LATVIJAS
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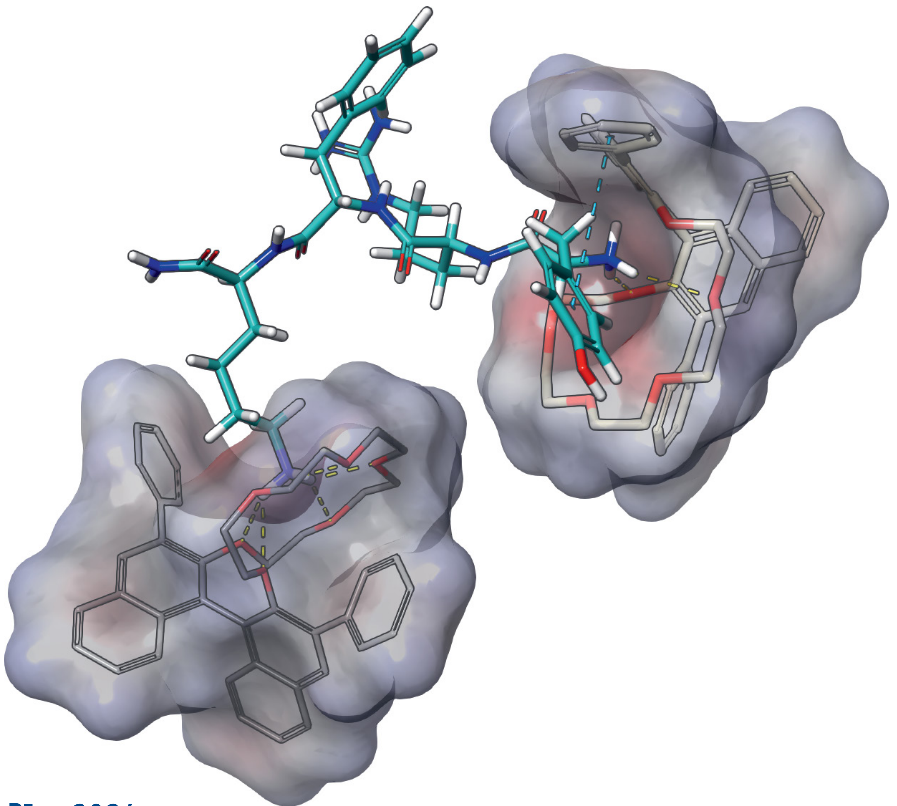
Toms Upmanis

HIRĀLĀS ATPAZĪŠANAS MEHĀNISMU PĒTĪJUMI ĪSO PEPTĪDU HROMATOGRĀFISKAJAI SADALEI UZ KRAUNA ĒTERU STACIONĀRAJĀM FĀZĒM

CHIRAL RECOGNITION MECHANISM STUDIES OF SHORT PEPTIDE CHROMATOGRAPHIC SEPARATION ON CROWN ETHER STATIONARY PHASES

Promocijas darbs / Doctoral Thesis



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LATVIJAS UNIVERSITĀTE

MEDICĪNAS UN DZĪVĪBAS ZINĀTŅU FAKULTĀTE

Toms Upmanis

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PROMOCIJAS DARBS

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ANOTĀCIJA

Hirālās atpazīšanas mehānismu pētījumi īso peptīdu hromatogrāfiskajai sadalei uz krauna ēteru stacionārajām fāzēm. Upmanis, T., zinātniskā vadītāja Dr. chem. Kažoka, H. Zinātnisko publikāciju kopas kopsavilkums analītiskās ķīmijas apakšnozarē, 76 lapaspuses, 20 attēli, 2 tabulas, 79 literatūras avoti. Latviešu un angļu valodās.

Neskatoties uz veiksmīgu krauna ēteru stacionāro fāžu izmantošanu dažādu hirālu pirmējo aminogrupu saturošu savienojumu enantiosadalē, trūkst pētījumu, kas apskata sarežģītākus analītus, kā piemēram, īsos peptīdus. Precīzs mehānisms, kas regulē krauna ētera saistīšanos ar pētāmā analīta stereoizomēriem, joprojām nav skaidrs. Izvēloties zināmu μ-opioīdu receptoru agonistu tetrapeptīdu Tyr-Arg-Phe-Lys-NH_2 kā modeļvielu, novērots, ka hirālās stacionārās fāzes, kam pamatā izmantoti (*R*) un (*S*)-(3,3'-difenil-1,1'-binaftil)-20-krauna-6 selektori nodrošina optimālus apstākļus šī tetrapeptīda hirālajai hromatogrāfiskai sadalei. Noskaidrots, ka uz šīm stacionārajām fāzēm iespējams izšķirt arī vairāku strukturāli līdzīgu tetrapeptīdu enantiomērus. Lai skaidrotu eksperimentāli novēroto hromatogrāfisko sadali, hirālās atpazīšanas mehānisms tika pētīts ar augstefektīvās šķidruma hromatogrāfijas, masspektrometrijas un kodolu magnētiskās rezonanses spektrometrijas metodēm.

KRAUNA ĒTERU HIRĀLĀS STACIONĀRĀS FĀZES; TETRAPEPTĪDI; HIRĀLĀ ATPAZĪŠANA; AMINOSKĀBES; ENANTIOSELEKTIVITĀTE

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APZĪMĒJUMU SARAKSTS

α	selektivitāte
ACN	acetonitrils
Arg	arginīns
COSY	korelācijas spektroskopija
CSP	hirālās stacionārās fāze
Cys	cistīns
ESI	elektroizsmidzināšanas jonizācija
Glu	glutamīnskābe
HILIC	hidrofilo mijiedarbību šķidruma hromatogrāfija
His	histidīns
HMBC	heteronukleārās daudzkāršo saišu korelācijas
HPLC	augstefektīvā šķidruma hromatogrāfija
HSQC	heteronukleārās viena kvanta korelācijas
HRMS	augstas izšķirtspējas masspektrometrija
k	aiztures faktors
Leu	leicīns
Lys	lizīns
MP	mobilā fāze
MS	masspektrometrija
NMR	kodolu magnētiskā rezonanse
Phe	fenilalanīns
RP	apgrieztā fāze
TOCSY	totālās korelācijas spektroskopija
TOF	nolidojuma laika analizators
t_R	aiztures laiks
Trp	triptofāns
Tyr	tirozīns

IEVADS

Īsie peptīdi ($n < 6$) ir iesaistīti dažādos bioloģiskos procesos un atbild par daudzām cilvēka veselībai svarīgām funkcijām. Savas augstās efektivitātes un selektīvās darbības dēļ, to izmantošana arvien vairāk piesaista zinātnieku interesi gan jaunu zāļvielu kandidātu izstrādei, gan kā jaunu transporta formu jau zināmu zāļvielu ievadīšanai. Peptīdi sastāv no noteiktā secībā virknē sakārtotām aminoskābēm, kuras visas, izņemot glicīnu, ir hirālas. Gan ražošanas, gan dažādu ārēju apstākļu ietekmē nereti iespējams veidoties vairākiem peptīda stereoizomēriem ar potenciāli atšķirīgu vai pat nevēlamu bioloģisko aktivitāti, tādēļ nepieciešamība pēc sarežģītu diastereomēru un enantiomēru maisījumu sadales metodēm ir būtiska gan bioloģijas, gan medicīnas zinātnes nozarēs, t.sk. farmācijā.

Mūsdienās, hirālu savienojumu enantiosadalei lieto dažādas analītiskās metodes, no kurām izplatītākā ir augstefektīvā šķidrumu hromatogrāfija (HPLC), izmantojot hirālas stacionārās fāzes (CSP). Ir zināms, ka CSP, kuru pamatā ir krauna ēteri, veiksmīgi izmantoti dažādu hirālu savienojumu enantiomēru atdalīšanai, kas satur pirmējās aminogrupas, tostarp dažādus neaizsargātus di- un tripeptīdus. Diemžēl trūkst pētījumu, kas apskata sarežģītākus analītus, piemēram tetrapeptīdus. Dalāmo savienojumu sarežģītās stereoķīmiskās struktūras dēļ ir praktiski neiespējami prognozēt kā pētāmā analīta stereoizomēri uzvedīsies hromatogrāfiskajā sistēmā. Tādēļ visbiežāk analītiskās sadales metodes tiek izstrādātas pēc mēģinājumu un kļūdu metodes (*trial and error*).

Kaut arī vispārīgi zināmi, precīzi peptīdu hirālās atpazīšanas mehānismi uz krauna ēteru CSP nav pilnībā izpētīti. No mehānistiskā viedokļa nav zināms, tieši kāds ir katras peptīda funkcionālās grupas ieguldījums mijiedarbībās ar hirālo selektoru. Lai labāk izprastu jau esošās pieejas kā arī radītu perspektīvas stratēģijas, izstrādājot analītiskās metodes hirālu īso peptīdu enantiosadalei, nepieciešams izveidot pamatu padziļinātai izpratnei par hirālās sadales mehānismiem uz krauna ētera fāzēm.

Promocijas darba mērķis ir izpētīt īso peptīdu hirālās atpazīšanas mehānismu uz krauna ētera stacionārajām fāzēm, apvienojot HPLC, augstas izšķirtspējas masas spektrometriju (HRMS) un kodolu magnētiskās rezonanses spektroskopijas (NMR) metodes, izmantojot zināmu μ-opioīdu agonistu Tyr-Arg-Phe-Lys-NH_2 kā tetrapeptīda modeļvielu.

Mērķa sasniegšanai tika izvirzīti sekojoši **uzdevumi**:

1. Veikt visu sešpadsmit Tyr-Arg-Phe-Lys-NH_2 stereoizomēru sintēzi un izpētīt komerciāli pieejamu krauna ēteru hirālo stacionāro fāžu pielietojamību Tyr-Arg-Phe-Lys-NH_2 stereoizomēru hromatogrāfiskajai sadalei.
2. Ievadot dažādas aminoskābes pie tetrapeptīda N-gala un Phe pozīcijā, sintezēt Tyr-Arg-Phe-Lys-NH_2 struktūras analogus un izvērtēt struktūrā ievadīto aminoskābju atlikumu ietekmi uz tetrapeptīdu aizturi un enantiosadali uz CROWNPAK **CR-I** fāzēm.
3. Sintezēt visus iespējamos Tyr-Arg-Phe-Lys-NH_2 struktūras analogus, kuros ar krauna ētera hirālajiem selektoriem saistīties spējīgās aminogrupas ir selektīvi aizstātas ar inertām hidroksilgrupām un noskaidrot, kura aminogrupa ir iesaistīta hirālajā atpazīšanā uz CROWNPAK **CR-I** fāzēm.
4. Sintezēt CROWNPAK **CR-I** stacionāro fāžu hirālos selektorus un izpētīt saistīšanos starp Tyr-Arg-Phe-Lys-NH_2 LLLL un DDDD-enantiomēriem un sintezētajiem selektoriem ar HRMS un NMR metodēm.

Promocijas darba zinātniskā novitāte:

1. Pirmo reizi ziņots par tetrapeptīda (Tyr-Arg-Phe-Lys-NH_2) hirālo sadali uz krauna ēteru hirālajām stacionārajām fāzēm.
2. Konstatēts, ka stacionārās fāzes, kas veidotas uz (*R*) un (*S*)-(3,3'-difenil-1,1-binaftil)-20-krauna-16 hirālo selektoru bāzes ir perspektīvākās Tyr-Arg-Phe-Lys-NH_2 un desmit tā struktūras analogu enantiosadalei.
3. Darbā pirmo reizi parādīts, ka, analizējot vienu analītu uz divām pretējas hiralitātes CROWNPAK **CR-I** (+) un **CR-I (-)** stacionārajām fāzēm, iespējams gan palielināt sadalīto tetrapeptīda stereoizomēru skaitu, gan izvērtēt CSP enantiosadales spēju attiecībā uz noteiktu enantiomēru pāri arī tad, ja pieejams tikai viens no enantiomēriem.
4. Apvienojot HPLC, HRMS un KMR metodes, izpētīts viena Tyr-Arg-Phe-Lys-NH_2 enantiomēru pāra hirālās atpazīšanas mehānisms uz (3,3'-difenil-1,1-binaftil)-20-kraunu-16 fāzēm.
5. Noskaidrots, ka vairāk kā viena aminogrupa Tyr-Arg-Phe-Lys-NH_2 struktūrā var vienlaikus saistīties ar vairākām selektora molekulām uz sorbenta virsmas. Šāda saistīšanās stehiometrija iepriekš literatūrā nav aprakstīta.

Pētījuma praktiskais pielietojums:

Darbā aprakstītā tetrapeptīdu hromatogrāfiskā uzvedība uz (3,3'-difenil-1,1-binaftil)-20-kraunu-16 stacionārajām fāzēm var būtiski palīdzēt turpmāk jaunu, nezināmu īso peptīdu enantiosadalē. Iegūtie dati ir viegli pielietojami praksē peptīda tipa zāļvielu kandidātu hirālās tīrības noteikšanas metožu izstrādē uz krauna ētera stacionārajām fāzēm. Ir pamats prognozēt, ka izstrādāto metožu izmantošana var tikt pielietota dažādu peptīdu attīrīšanā, kas ir svarīgi zinātniskajos pētījumos un farmācijas nozarē.

Promocijas darbā, izmantotā netradicionālā pieeja (divas hirālās kolonnas, ar pretējas hiralitātes selektoriem viena parauga analīzei), var kalpot sarežģītu stereoizomēru maisījumu sastāva noteikšanai. Turklāt, gadījumos, kad ir pieejams tikai viens savienojuma enantiomērs, šī pieeja ļauj salīdzinoši ātri un lēti novērtēt vai stacionārās fāzes hirālais selektors ir enantioselektivitīvs attiecībā pret noteiktas konfigurācijas enantiomēru pāri.

Darbā aprakstīto HPLC, HRMS un NMR metožu izmantošanu saistīšanās pētījumos var izmantot kā piemēru turpmākai sistemātiskai hirālās atpazīšanas mehānisma noskaidrošanai dažādām hirālo selektoru un analītu klasēm. Izpētīto starpmolekulāro mijiedarbību profils var kalpot par pamatu jaunu hirālo stacionāro fāžu izstrādei.

Publicētie zinātniskie raksti.

1. **Upmanis, T**.; Kažoka, H.; Arsenyan, P. A study of tetrapeptide enantiomeric separation on crown ether based chiral stationary phases. *J. Chromatogr. A* **2020**, *1622*, 461152. *Q1*, $IF_{2023} = 3.8$
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2. **Upmanis, T**.; Kažoka, H. Application of Commercially Available Crown Ether Chiral Stationary Phases for Separation of Tetrapeptide Stereoisomers, *Acta Pharm. Hung*. **2021**, *91*, 324–325.

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4. **Upmanis, T**.; Kažoka, H. Mechanistic insights in chiral recognition of μ-opioid receptor agonist tetrapeptide on crown ether chiral stationary phase. *J. Chromatogr. Open* **2021**, *1*, 100016.
   T. Upmanis izstrādāja 90% no eksperimentālā darba apjoma, izstrādāja plānu un uzrakstīja publikāciju, noformēja pētījuma rezultātus atbilstoši žurnāla prasībām, kā arī sagatavoja atbildes uz recenzentu jautājumiem un aizrādījumiem.

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   T. Upmanis izstrādāja 80% no eksperimentālā darba apjoma, izstrādāja plānu un uzrakstīja publikāciju, noformēja pētījuma rezultātus atbilstoši žurnāla prasībām, kā arī sagatavoja atbildes uz recenzentu jautājumiem un aizrādījumiem.

Zinātniskās konferences.

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1. PROMOCIJAS DARBA TEORĒTISKIE PAMATI UN PĒTĪTĀS SISTĒMAS

1.1. Peptīdu zāļvielas

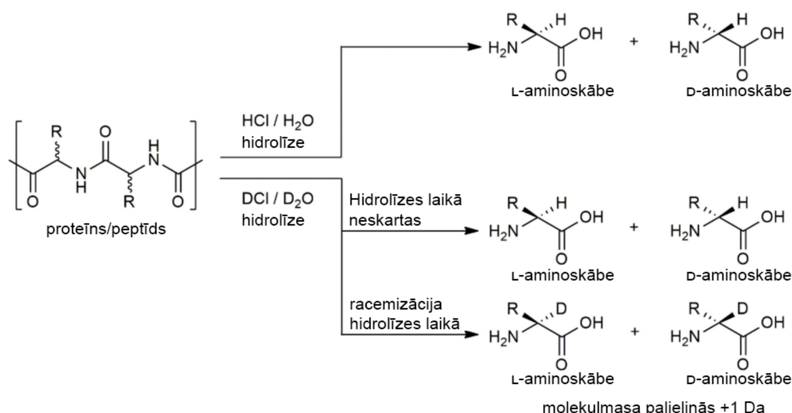
Zāļvielu atklāšanas nozarē pēdējo desmitgadu laikā ir novērojama arvien pieaugoša farmācijas industrijas interese peptīdu izmantošanai, šai savienojumu klasei ielaužoties tirgū visdažādāko slimību, kā piemēram, diabēta, vēža, osteoporozes, multiplās sklerozes, HIV, kā arī hronisku sāpju, ārstēšanai [1]. Strauji attīstoties bioloģijas un medicīnas zinātnēm, pētījumu skaits, saistībā ar dažādu peptīdu zāļvielu pielietojumu, arvien pieaug [2], veidojot nozīmīgu farmācijas tirgus daļu, 2019. gadā pārdošanas apjomam visā pasaulē pārsniedzot 70 miljardus ASV dolāru [1]. Uz šo brīdi, vairāki simti peptīdu zāļvielu kandidāti atrodas klīnisko pētījumu fāzē un vairāk kā 80 jau ir apstiprinātas klīniskai lietošanai visā pasaulē [3].

Īsie peptīdi pārstāv unikālu farmaceitisko preparātu klasi, kas sastāv no noteiktā secībā sakārtotu aminoskābju virknes un molekulārā izmēra ziņā tie atrodas starp mazmolekulāriem savienojumiem un olbaltumvielām [4], tomēr gan ķīmisko īpašību, gan darbības mehānismu ziņā peptīdi atšķiras no abiem iepriekš minētajiem [5]. Kā daudzu fizioloģisko funkciju regulējošu receptoru signālmolekulas, peptīdi (to dabiskajā formā vai modificēti) paver iespējas terapijām, izmantojot dabiskos regulācijas ceļus [6]. Citi nozīmīgi faktori, kas ir veicinājuši šo tendenci, ir augsta specifiskuma pakāpe un zems toksicitātes profils (ko nosaka to ārkārtīgi ciešā saistīšanās ar mērķiem), kas ļauj šai savienojumu klasei būt lieliskam papildinājumam vai pat vēlamajai alternatīvai mazmolekulāro savienojumu zāļvielām [7].

Peptīdu bioloģiskās funkcijas un to fizikālās īpašības ir atkarīgas no to stereoķīmiskās struktūras, ko pēc būtības nosaka virknē sakārtotu aminoskābju konfigurācija [8,9]. Visas dabas aminoskābes, izņemot glicīnu ir hirālas un pastāv D vai L formā, tādejādi iespējams veidoties vairākiem peptīda stereoizomēriem ar potenciāli atšķirīgu bioloģisko aktivitāti, [10,11]. Nereti peptīdu dabas savienojumos ir novērojama racemizācija (vai epimerizācija, atkarībā no iesaistīto stereocentru pozīcijas), kas var notikt gan sintēzes laikā, gan uzglabāšanas apstākļu ietekmē, kā arī metabolisku procesu rezultātā [12,13], veidojot sarežģītus enantiomēru / epimēru maisījumus, tādēļ precīzu un ātru hirālās tīrības kontroles metožu izstrāde peptīdiem ir ārkārtīgi būtiska gan farmācijas rūpniecībai, gan pētījumiem ķīmijas un bioloģijas zinātnes nozarēs.

1.2. HPLC pielietojums hirālu peptīdu analītu sadalei

Izplatītāka stratēģija dažādu peptīdu analītu optiskās tīrības noteikšanai ietver peptīda hidrolīzi par atsevišķām aminoskābēm [14]. Lai izvairītos no jebkādām ārēju faktoru izraisītām enantiomēru attiecības izmaiņām sākotnējā paraugā, peptīda hidrolīze tiek veikta deiterētos šķīdinātājos. Šādā gadījumā, jebkādu racemizāciju, kas notiek parauga sagatavošanas posmā, pavada deitērija apmaiņa (1.1. att.) pie α-oglekļa [15].



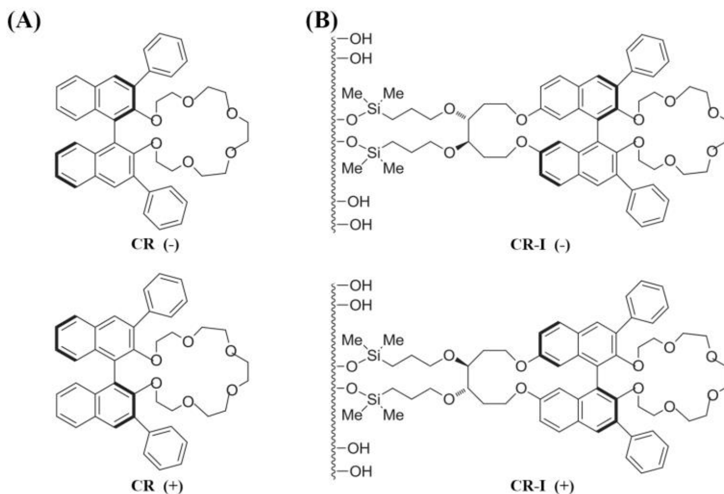
1.1. att. **DCl / D_2O peptīdu hidrolīzes shematisks attēlojums** [16].

Tālāk, hidrolīzes ceļā iegūtās aminoskābes tiek pakļautas hirālai derivatizācijai (piem., izmantojot *Mārfeja* reaģentu [17]) un ar šķidruma hromatogrāfijas-masspektrometrijas metodēm netieši (enantiomērus pārvēršot par diastereomēriem) analizētas ahirālos apstākļos, kur aminoskābju molekulas, kas racemizējušās hidrolīzes laikā un ir iezīmētas ar deitēriju (radot + 1 masas atšķirību) secīgi var tikt atdalītas masspektrometriski [17]. Aprakstītās stratēģijas trūkumi ietver sevī nepieciešamību pēc dārgiem deiterētiem šķīdinātājiem un masspektrometrijas aprīkojuma, piedevām, derivatizācijas solis ienes papildus sarežģītību paraugu sagatavošanas gaitā, paildzina analīzes laiku un samazina metodes robustumu, ievedot jaunus potenciālu kļūdu avotus [18].

Ideālā gadījumā, iepriekš aprakstītos metodes trūkumus varētu novērst, analizējot hirālus peptīdus tieši - tos iepriekš nemodificējot. Aminoskābju enantiomēru tiešās hromatogrāfiskās sadales metodēm izstrādāts plašs hirālo stacionāro fāžu klāsts, kas veidotas uz dažādu tipu hirālajiem selektoriem. Diemžēl, dalāmo analītu stereoķīmiskās sarežģītības dēļ (ar katru nākamo struktūrā ievadīto aminoskābi (n) peptīda stereoizomēru skaits palielinās eksponenciāli 2^n), panākumi dažādu tiešo sadales metožu izmantošanā, nemodificētu īso peptīdu hirālajā hromatogrāfiskajā sadalē, bijuši samērā ierobežoti, kas labi atspoguļojas relatīvi zemajā atrodamo publikāciju skaitā. Starp tām, piemēram, glicil-dipeptīdu enantiosadale panākta ar ligandu apmaiņas hromatogrāfiju [19]. Atsevišķas neaizsargātas neproteinogēnās aminoskābes un peptīdu dabas analītu enantiosadale veikta uz polisaharīdu atvasinājumu fāzēm [20,21]. Savukārt, dažādu neaizsargātu dipeptīdu un tripeptīdu hirālajā izšķiršanā, iepriekš ziņots par CSP, kas veidotas uz makrociklisko glikopeptīdu [22–24] un ciklodekstrīnu [25,26] bāzes, izmantošanu. Divu veidu hirālās stacionārās fāzes (uz hinīna alkaloīdu [27–29] un hirālu krauna ēteru bāzes [30]) ir radītas tieši aminoskābju un dažādu pirmējo aminogrupu saturošu savienojumu, kuriem pieskaitāmi arī peptīdi, sadalei.

1.3. Uz krauna ēteru bāzes veidotās hirālās stacionārās fāzes

Vienu no zināmākajiem hirālu krauna ēteru veidiem izstrādājusi Donalda Dž. Krama grupa (par to saņemot Nobela prēmiju ķīmijā 1987. gadā), eksperimentējot ar optiski aktīvu 1,1'-bi-2-naftil funkciju iekļaušanu krauna ētera struktūrā [31]. Grupas veiktajos pētījumos īpaši izcelta (*R*)- un (*S*)-(3,3'-difenil-1,1-binaftil)-20-krauna-16 (1.2. att., **A**) unikālā hirālās atpazīšanas spēja attiecībā uz dažādu α-aminoskābju un to metilesteru enantiomēriem [32]. Šīs īpašības atrada pielietojumu hirālajā hromatogrāfijā un, gandrīz 10 gadus vēlāk, dinamiski uzklājot hirālo selektoru uz silikagela, tika izveidotas pirmās komerciālās krauna ēteru CSP (no *Daicel Chemical Industries*) CROWNPAK **CR** (+) un **(-)** [33].



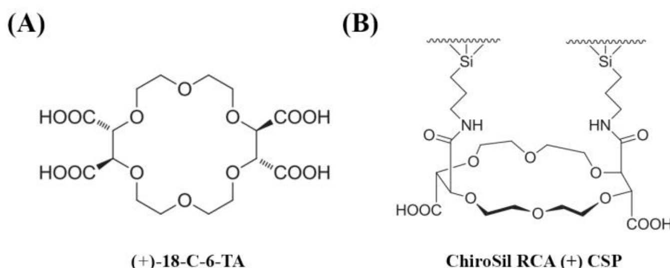
1.2. att. **Ķīmiskās struktūras:** (*R*)- un (*S*)-(3,3'-difenil-1,1-binaftil)-20-krauna-16 hirālie selektori CROWNPAK **CR (-)** un (+) CSP- (**A**); imobilizēta hirālā selektora versija CROWNPAK **CR-I (-)** un (+) CSP – (**B**).

Šīs hirālās stacionārās fāzes izrādījās ļoti efektīvas dažādu pirmējo α-aminoskābju [33,34], aril-α-aminoketonu [35], β-aminoskābju [36–38], γ-aminoskābju [39], kā arī dažādu citu hirālu savienojumu, kas satur pirmējo aminogrupu [40,41] tajā skaitā arī dipeptīdu [42,43] enantiosadalei. Diemžēl, CSP pagatavošanas specifikas dēļ bija jārēķinās ar būtiskiem trūkumiem kā rezultātā bija jāievēro stingri noteikumi attiecībā uz mobilās fāzes šķīdinātāju izvēli. Hirālā selektora dinamiskās uzklāšanas dēļ uz sorbenta virsmas, pat 15% MeOH satura pārsniegšana mobilajā fāzē varēja neatgriezeniski bojāt CSP veiktspēju, hirālo selektoru no kolonnas vienkārši "izmazgājot".

Šos trūkumus vēlāk izdevās novērst, hirālo selektoru kovalenti saistot ar brīvajām silanola grupām uz silikagela virsmas, kā rezultātā izstrādātas imobilizētās CROWNPAK **CR-I** (+) vai **(-)** CSP (1.2. att., **B**) [44]. Tika konstatēts, ka šāda stacionārās fāzes pagatavošanas metode ievērojami palielina tās stabilitāti, ļaujot izmantot plašāku šķīdinātāju klāstu (ACN, MeOH, EtOH, 2-PrOH, THF) mobilajā fāzē kā arī neierobežojot organiskā modifikatora koncentrāciju tajā, tādā veidā paplašinot iespējas variēt ar hromatogrāfisko apstākļu piemeklēšanu nepieciešamajai sadalei. Izmantojot **CR-I (+)** un **(-)** CSP izdevies sadalīt visu proteīnogēno aminoskābju enantiomērus (izņemot prolīnu) [45], tās ir veiksmīgi pielietotas plaša klāsta α- [46,47],

β- [38] un *γ*-aminosavienojumu [39] hirālajā izšķiršanā un vispārīgi nodrošina labāku sadales veiktspēju attiecībā pret tās priekštečiem – **CR** (+) un **(-)** CSP [39].

Otru zināmāko krauna ēteru CSP, kas veidots uz (+) vai (-)-(18-krauna-6)-2,3,11,12-tetrakarbonskābes pamata (**18-C-6-TA**; 1.3. att., **A**) izstrādājusi Žana-Marī Lēna grupa (par to arī saņemot Nobela prēmiju ķīmijā 1987. gadā), krauna ētera ciklā iekļaujot divus vīnskābes fragmentus [48]. Šis krauna ēteris plaši izmantots arī kā hirālās izšķiršanas reaģents kodolmagnētiskās rezonanses spektroskopijā [49], kapilārajā elektroforēzē [50] un masspektrometrijā [51].



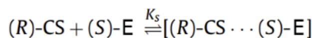
1.3. att. **Ķīmiskās struktūras:** (+)-(18-krauna-6)-2,3,11,12-tetrakarbonskābe – (**A**); imobilizēts (+)-**18-C-6-TA** hirālais selektors ChiroSil **RCA** (+) stacionārajā fāzē – (**B**).

Tā kā **18-C-6-TA** labi šķīst ūdenī, tikai tā imobilizētā versija, komerciāli pazīstama kā ChiroSil **RCA** (+) (1.3. att., **B**; vai **SCA (-)**, atkarībā no hirālā selektora konfigurācijas; izstrādājusi *RStech Corporation*) tiek izmantota hromatogrāfijā. CSP, kas veidotas uz **18-C-6-TA** bāzes veiksmīgi pielietotas dažādu dabas un sintētisko *α*- un *β*-aminoskābju [52–55], otrējo amīnu [56] kā arī īso peptīdu [57] enantiosadalēs.

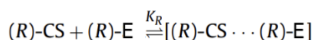
No apskatītās literatūras secināts, ka CSP, kas veidotas uz hirālu krauna ēteru bāzes ir vienas no daudzsološākajiem kandidātiem tiešajai īso peptīdu hirālajai sadalei. Tomēr, lai labāk izprastu to pielietojumu tik sarežģītu analītu izšķiršanai, vispirms nepieciešams apskatīt, kas ir zināms par šo abu CSP veidu hirālās atpazīšanas mehānismiem.

1.4. Hirālā atpazīšana

Parasti CSP tiek izstrādātas tā, lai tās veidotu tādu stēriski traucētu vidi, kurā viens no izomēriem spēj ar CSP saistīties spēcīgāk nekā otrs. Atkarībā no hirālā selektora struktūras, zināms, ka dažādas vispārzināmās starpmolekulārās mijiedarbības (piemēram, jonu, ūdeņraža saites, stēriski traucējumi, π-π, dipola-dipola, jonu-dipola, van der Vālsa) caur to pievilkšanās/atgrūšanās īpašībām vistiešākajā veidā ietekmē saistīšanās spēku starp hirālo analītu un selektoru. Līdzsvara reakcijas, kas uz stacionārās fāzes virsmas atgriezeniski veido diastereomēru pārus, ir parādītas 1.4. attēlā.



$$(R)\text{-CS} + (S)\text{-E} \overset{K_S}{\rightleftharpoons} [(R)\text{-CS} \cdots (S)\text{-E}]$$



$$(R)\text{-CS} + (R)\text{-E} \overset{K_R}{\rightleftharpoons} [(R)\text{-CS} \cdots (R)\text{-E}]$$

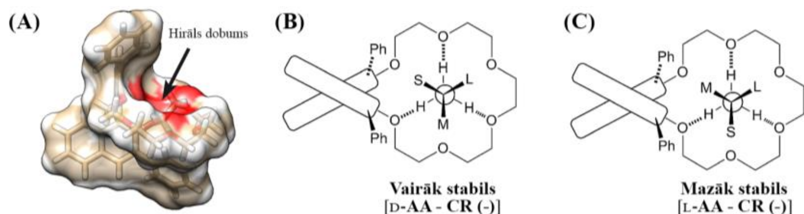
1.4. att. **Shematisks diastereomēru veidošanās attēlojums** [58]**.**

Dotajā piemērā hirālais selektors (CS) ar fiksētu konfigurāciju (*R*), mijiedarbojoties ar analīta *S*-enantiomēru (*S*)-E, veido diastereomēru kompleksu [(*R*)-CS···(*S*)-E], kura stabilitāti raksturo līdzsvara konstante K_S (1.4. att. – *augšā*). Tajā pat laikā notiek arī saistīšanās starp hirālo selektoru un analīta *R*-enantiomēru, veidojot [(*R*)-CS···(*R*)-E] kompleksu, kura stabilitāti raksturo līdzsvara konstante K_R

(1.4. att. – *apakšā*). Atšķirības abu kompleksu līdzsvara konstantēs atspoguļojas izomēru aizturē un kalpo par pamatu stereoselektīvai sadalei jebkurā hromatogrāfiskajā sistēmā. Izomērs, kas ar hirālo selektoru saistās vājāk, no kolonnas izdalās ātrāk, kamēr izomērs, kas ar hirālo selektoru veido stabilāku kompleksu, kolonnā aizturas ilgāk, tādā veidā, ļaujot maisījumu hromatogrāfiski sadalīt. Būtiska loma hirālajā atpazīšanā ir arī videi, kurā šīs mijiedarbības notiek, kas šķidruma hromatogrāfijā ir mobilā fāze (MP). Atkarībā no izmantoto šķīdinātāju dabas, mobilās fāzes molekulas ne tikai sacenšas ar analīta molekulām par piesaistes vietām uz hirālās stacionārās fāzes, bet arī var ietekmēt hirālā selektora stēriku, mainot hirālās atpazīšanas mehānismu.

1.4.1. Hirālā atpazīšana uz (3,3’-difenil-1,1-binaftil)-20-krauna-16 CSP

Apgrieztās fāzes (RP) apstākļos (ūdeni saturoša mobilā fāze), hirālā sadale tiek panākta, aizturot analītu kolonnā, tam selektīvi iekļaujoties selektora hirālajā dobumā (1.5. att., **A**). Saistīšanās pamatā ir trīs $^{+}N–H\cdots O$ ūdeņraža saišu veidošanās starp protonēto analīta amonija grupu un krauna ētera skābekļa atomiem, kam seko enantioselektīvas hidrofobās atgrūšanās mijiedarbības starp analītu un stacionārās fāzes selektora divām 3-fenilnaftil grupām. Saistīšanās shematiski parādīta *Ņūmena* projekcijās 1.5. attēla **B** un **C** piemēros.



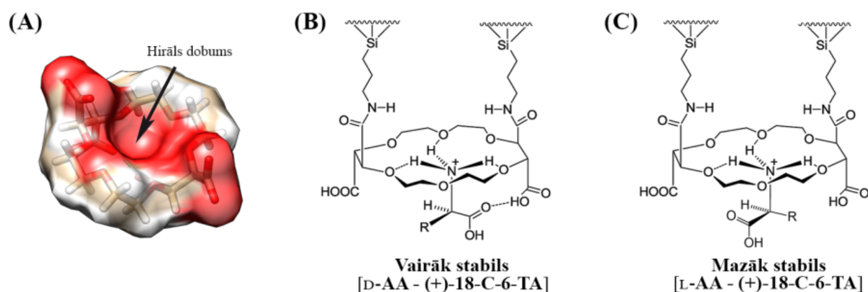
1.5. att. **Piedāvātais hirālās atpazīšanas mehānisms CR (-) selektoram: CR (-)** selektora elektrostatiskā potenciāla virsma, kas iegūta no kristāla struktūras [59], vizualizācija veidota ar *Chimera 1.16* programmatūru – (**A**); Saistīšanās rezultātā starp analīta pirmējo amonija jonu (R-NH_3^+) un **CR (-)** selektoru izveidojušies vairāk stabilie – (**B**); un mazāk stabilie kompleksi – (**C**) [32]. Aizvietotāji pie analīta hirālā oglekļa apzīmēti pēc to izmēra – liels (L), vidējs (M) un mazs (S).

Šajā gadījumā, lielākā funkcionālā grupa (L) pie analizējamā savienojuma hirālā centra aizņem telpu, kas atrodas vistālāk no selektora stēriski apjomīgās augšupvērstās 3-fenilnaftil grupas, kamēr atlikušie divi aizvietotāji, atkarībā no aminoskābes konfigurācijas, attiecīgi telpā novietojas automātiski. Stēriskie traucējumi, kas rodas starp hirālā selektora augšupvērsto 3-fenilnaftil fragmentu un L-aminoskābes vidējo funkcionālo grupu (M) ievērojami vairāk ierobežo tā piekļuvi selektora hirālajam dobumam (1.5. att., **A**), atšķirībā no tā attiecīgā D-enantiomēra, kur šo telpu aizņem mazākais (visbiežāk ūdeņradis) aizvietotājs (S). Rezultātā, ūdeņraža saites veidošanās starp L-enantiomēru un **CR (-)** selektoru ir stēriski traucēta, veidojot mazāk stabilu kompleksu (1.5. att., **C**), kas no kolonnas izdalās attiecīgi pirms D-enantiomēra [30]. Šādā veidā tiek skaidrota arī specifiskā enantiomēru izdalīšanās secība (L < D), kas uz **CR (-)** CSP ir spēkā visām hirālām aminoskābēm un kuru iespējams mainīt uz pretējo, izmantojot **CR** (+) CSP, kas satur pretējas konfigurācijas hirālo selektoru.

Atšķirīga enantioselektivitāte mobilajās fāzēs ar augstu organiskā modifikatora saturu ir atklāta tikai salīdzinoši nesen [45], jo vecākā tipa pārklātās CSP nepieļāva izmantot šādus hromatogrāfiskos apstākļus. Atšķirībā no RP apstākļiem, papildus stērisko faktoru virzītai iekļaušanas kompleksācijai caur ūdeņraža saišu veidošanos, iespējamas vēl papildus enantioselektīvas hidrofilās mijiedarbības (HILIC), tomēr precīzs šo mijiedarbību profila raksturs joprojām nav pilnībā izpētīts.

1.4.2. Hirālā atpazīšana uz (18-krauna-6)-2,3,11,12-tetrakarbonskābes CSP

Atkarībā no mobilās fāzes sastāva, arī (+) un (-)-**18-C-6-TA** CSP tiek apskatīti divi iespējamie mehānismi, lai izskaidrotu hirālo sadali [52,60]. RP apstākļos, papildus ūdeņraža saišu virzītai pirmējā amonija jona iekļaušanās kompleksācijai 18-krauna-6 cikla hirālajā dobumā (1.6. att., **A**), stēriski traucējumi starp **18-C-6-TA** divām karbonskābes grupām un stēriski apjomīgākajiem *α*-aminosavienojuma aizvietotājiem ierobežo analīta piekļuvi saistīšanās vietai. Rezultātā viens no enantiomēriem spēj saistīties ciešāk un veidot stabilāku diastereomēru kompleksu kā otrs, ļaujot tos hromatogrāfiski sadalīt. Līdzīgi kā **CR** (+) un (-) hirālajiem selektoriem, arī uz **18-C-6-TA** CSP ir raksturīga noteikta enantiomēru izdalīšanās secība. Aminoskābju D-enantiomēri veido stabilākus kompleksus ar (+)-**18-C-6-TA** hirālo selektoru, kamēr L-enantiomēri ar (-)-**18-C-6-TA**.



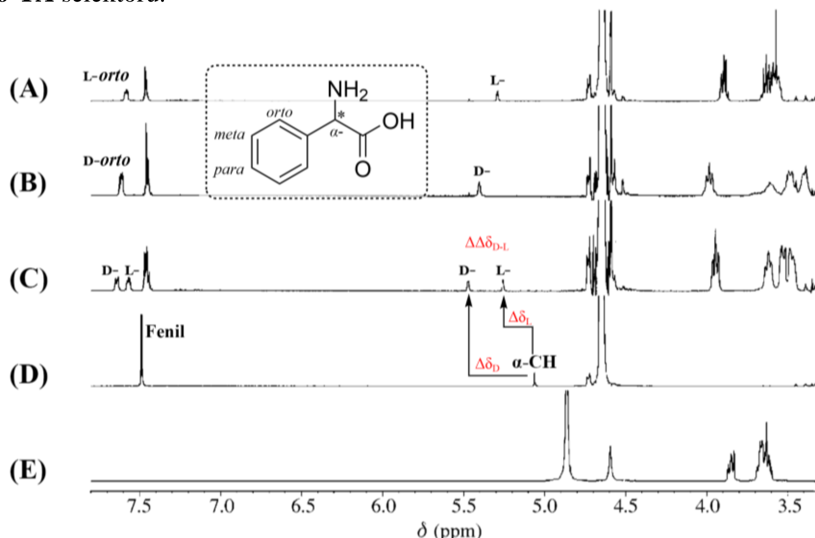
1.6. att. **Piedāvātais hirālās atpazīšanas mehānisms (+)-18-C-6-TA selektoram: (+)-18-C-6-TA** selektora elektrostatiskā potenciāla virsma, kas iegūta no kristāla struktūras [61], vizualizācija veidota ar *Chimera 1.16* programmatūru – (**A**); Iespējamie stabilākie (**B**) un mazāk stabilie (**C**) kompleksi kas izveidojušies, saistoties *α*-aminoskābēm ar CSP, kas veidotas uz (+)-**18-C-6-TA** selektora bāzes [62].

Ar organisko modifikatoru bagātās mobilajās fāzēs hirālā selektora divas sānu karbonskābes grupas var darboties kā papildu ūdeņraža saites donoru vai akceptoru grupas. Kā parādīts 1.6. attēla **B** un **C** piemēros, saistoties ar (+)-**18-C-6-TA**, *α*-aminoskābes D-enantiomēra karboksilgrupa veido papildu H-saiti ar vienu no selektora karboksilgrupām, radot stabilāku diastereomēru kompleksu (1.6. att., **B**). Savukārt, karboksilgrupa L-enantiomēra struktūrā ir telpā novirzīta prom no hirālā selektora karboksilgrupām un nevar piedalīties H-saites veidošanā, tādējādi veidojas mazāk stabils komplekss (1.6. att., **C**).

Diemžēl, literatūra aprakstītie pētījumi ir lielākoties teorētiski, tiem nav pietiekamas eksperimentālās bāzes un tie pārsvarā apskata vienkāršotus hirālās atpazīšanas modeļus, izmantojot aminoskābes. Jāņem vērā, ka peptīdu dabas analītu gadījumā ir jārēķinās ar vairāku hirālo centru klātbūtni, bez tam, peptīds var saturēt citus mijiedarbībās saistīties spējīgus fragmentus, tādēļ no mehānistiskā viedokļa nav zināms, tieši kāds ir katras peptīda funkcionālās grupas ieguldījums mijiedarbībās ar hirālo selektoru. Sīkāk par populārākajiem paņēmieniem hirālās atpazīšanas pētīšanai aprakstīts nākamajā nodaļā.

1.5. Dažādas pieejas hirālās atpazīšanas pētīšanai

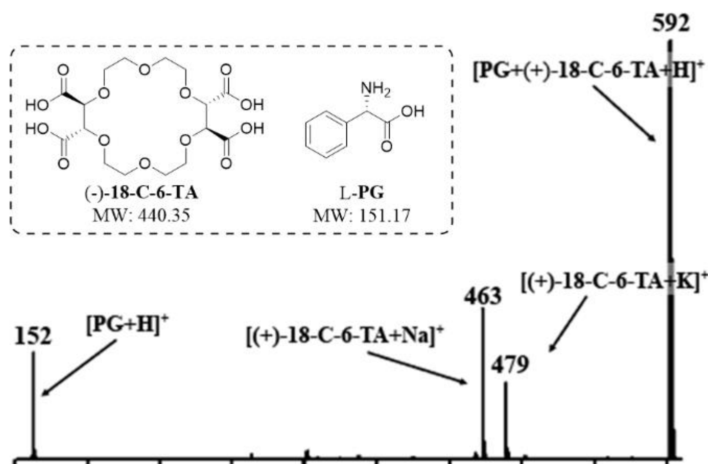
Hirālās atpazīšanas pētījumiem lietotas dažādas zināmas organisko savienojumu pētīšanas metodes. Starp spektroskopiskajām metodēm [63–65], viena no plašāk pielietotajām ir kodolu magnētiskā rezonanse, ar kuras palīdzību hirālā atpazīšana skaidrota dažādiem šķidruma hromatogrāfijā [66–68] un kapilārajā elektroforēzē [69,70] izmantotajiem hirālajiem selektoriem. Kā parādīts 1.7. attēla piemērā, ^{1}H-NMR lietota hirālās kompleksēšanās pētījumiem starp fenilglicīna enantiomēriem un (+)-**18-C-6-TA** selektoru.



1.7. att. **^{1}H-NMR fenilglicīna un ekvimolāru fenilglicīna/(+)-18-C-6-TA kompleksu (2 mM šķīdumi MeOH-*d$_4$*) spektri:** L-fenilglicīns ar (+)-**18-C-6-TA** - (**A**); D-fenilglicīns ar (+)-**18-C-6-TA** - (**B**); fenilglicīna racemāts ar (+)-**18-C-6-TA** - (**C**), fenilglicīna racemāts - (**D**), brīvs (+)-**18-C-6-TA** - (**E**) [68].

Saistoties divām molekulām, elektrisko un magnētisko lauku pārklāšanās ietekmē iesaistīto atomu kodolu rezonansi, ko novēro kā ķīmisko nobīžu (δ) izmaiņas (Δδ) NMR spektrā. Dotajā piemērā (1.7. att., **C**), redzams, ka, pievienojot ekvimolāru daudzumu (+)-**18-C-6-TA** selektora fenilglicīna racemātam, fenilglicīna *α*-protona un *orto*-fenilprotonu signāli sašķeļas divās signālu kopās, norādot uz atšķirīgām diastereomēru kompleksu ķīmiskajām struktūrām, kas veidojušās saistoties L-fenilglicīnam ar (+)-**18-C-6-TA** un D-fenilglicīnam ar (+)-**18-C-6-TA**. Turklāt, pēc ķīmisko nobīžu izmaiņu (Δδ) lieluma NMR spektrā var secināt, kuri atomi ir tieši iesaistīti starpmolekulārās mijiedarbībās.

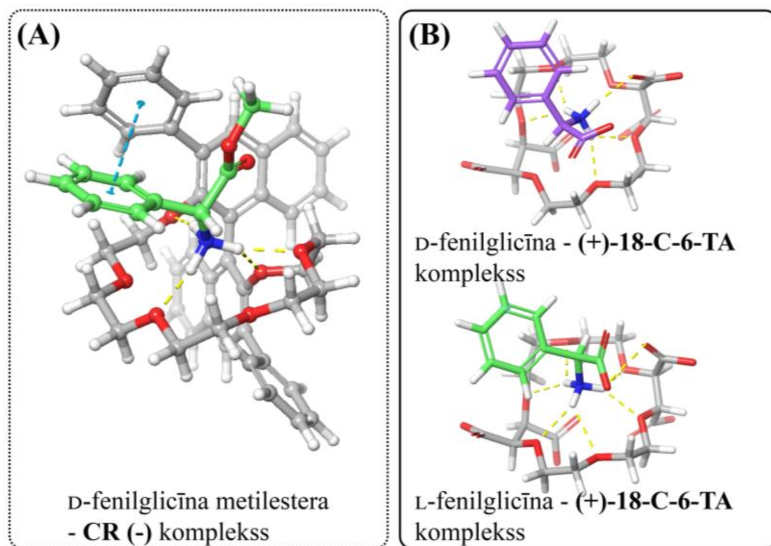
Kā cita parocīga metode, lai pētītu divu molekulu saistīšanos kompleksā [51,71], kā arī apstiprinātu kompleksēšanās stehiometriju [72], tiek izmantota arī masspektrometrija. Šādā veidā, mākslīgi sajaucot pētāmo analītu un hirālo selektoru šķīdumā un to tieši ievadot masspektrometrā, ar elektroizsmidzināšanas jonizācijas (ESI) nolidojuma laika (TOF) masspektrometrijas metodi pētīta kompleksēšanās starp L-fenilglicīnu un (-)-**18-C-6-TA** selektoru (1.8. att.).



1.8. att. **Daļējs ESI-TOF masspektrs ekvimolāram (10^{-5} M šķīdums MeOH) L-fenilglicīna (PG) un (-)-18-C-6-TA maisījumam** [51].

Kā redzams attēlotajos masspektros, izveidojušais komplekss spēj izturēt pāreju no šķidruma fāzes sagatavotajā parauga šķīdumā uz gāzes fāzi masspektrometrā un tā molekulārais jons redzams pie *m/z* vērtības 592. Papildus, novēro arī (-)-**18-C-6-TA** nātrija (*m/z* 463) un kālija (*m/z* 479) aduktus, kā arī nesaistītu L-fenilglicīnu (*m/z* 152). Lai arī šādi MS eksperimenti apstiprina apskatīto divu molekulu saistīšanos kompleksā, vieni paši tie nesniedz padziļinātu informāciju par hirālo atpazīšanu un vairāk kalpo kā papildinājums citām organisko savienojumu pētīšanas metodēm.

Kā vēl vienu svarīgu struktūras analīzes metodi, ieskata gūšanai par dažādu molekulu savstarpēju saistīšanos, jāmin rentgenstaru difrakciju, kas sniedz visaptverošu informāciju par izveidotā kompleksa strukturālajām īpašībām cietā stāvoklī [73,74]. Rentgenstaru difrakcija izmantota arī lai skaidrotu krauna ēteru spēju hirāli atpazīt dažādu aminoskābju enantiomērus. Šādā veidā, kokristalizējot D-fenilglicīna metilestera perhlorātu ar **CR (-)** hirālo selektoru metanolā [59], skaidrots saistīšanās mehānisms starp abām molekulām. Šajā piemērā, kokristāla struktūras analīze (1.9. att., **A**) norāda uz trīskāršu $^{+}$N–H···O ūdeņraža saites veidošanos starp D-fenilglicīna metilestera amonija jonu un krauna ētera cikla skābekļiem, kā arī C-H···π mijiedarbības starp abu molekulu aromātiskajiem fragmentiem. Citā darbā [74], lai demonstrētu atšķirīgus saistīšanās mehānismus starp fenilglicīna enantiomēriem un (+)-**18-C-6-TA** hirālo selektoru, iegūti attiecīgie kokristāli no 10 mM $HClO_4$ ūdens šķīduma (1.9. att., **B**). Diemžēl labas kvalitātes kristālu, it īpaši noteiktu molekulu kokristālu iegūšana, bieži ir problemātiska.

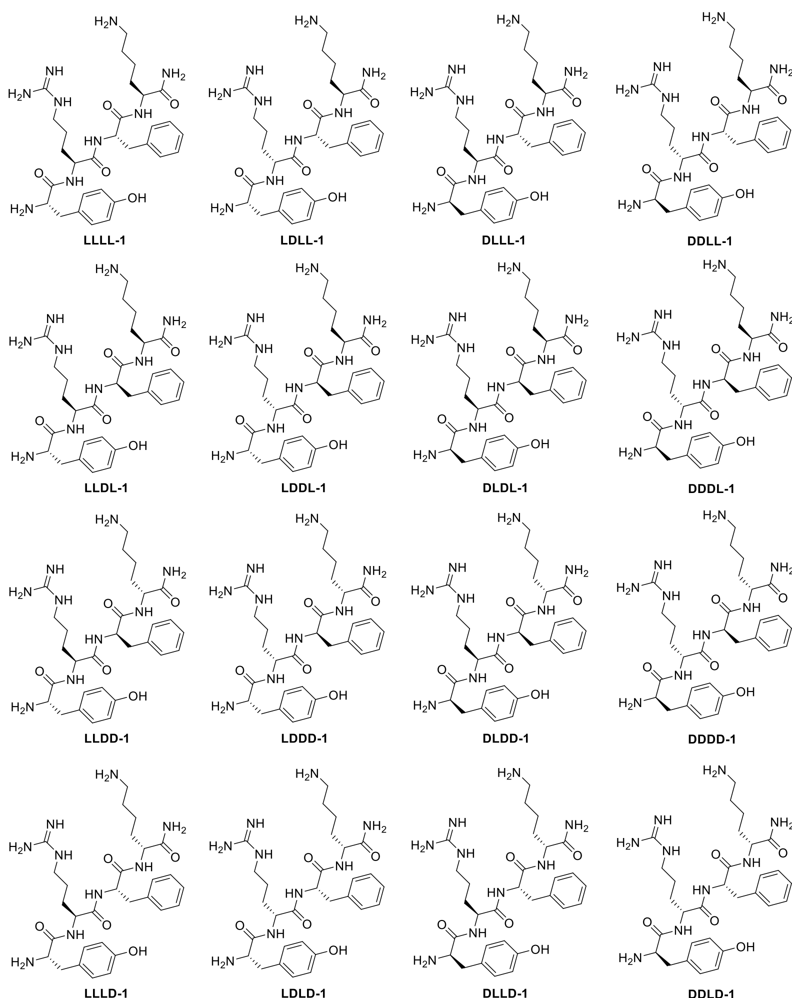


1.9. att. **Starpmolekulāro mijiedarbību stereoskati kompleksos, kas iegūti ar rentgenstaru difrakcijas metodi:** D-fenilglicīna metilestera koplekss ar **CR (-)** selektoru [59] – (**A**); fenilglicīna enantiomēru kompleksi ar (+)**-18-C-6-TCA** hirlo selektoru [74] – (**B**). Mijiedarbības vizualizētas ar *Maestro 13.4* programmatūru.

Pēdējos gados hromatogrāfijas zinātne tieši hirālās atpazīšanas pētījumu virzienā kļuvusi arvien pieņemošāka attiecībā pret dažādām skaitļošanas metodēm, kombinējot dažādas molekulārās modelēšanas metodes ar eksperimentālām tehnikām, lai raksturotu molekulu kompleksēšanos [75,76]. Tomēr svarīgi ir ņemt vērā, ka lielākā daļa skaitļošanas metožu ir izstrādātas proteīnu izpētei un šo metožu pielāgošana hromatogrāfijas problēmu pētīšanai var izrādīties problemātiska, kaut vai ticamu hirālo selektoru struktūru iegūšanai.

1.6. Pētāmie objekti

Lai pētītu krauna ēteru spēju hirāli atpazīt īso peptīdu izomērus, promocijas darbā kā modeļviela izvēlēts Tyr-Arg-Phe-Lys-NH_2 tetrapeptīds (**1**; 1.10. att.), kura LDLL-stereoizomērs zināms kā selektīvs μ-opioīdu receptoru agonists *DALDA* [77]. Tetrapeptīda **1** struktūra satur 4 hirālos centrus, kā rezultāta iespējami 16 stereoizomēri, kas, uzsākot darbu, tika sintezēti.



1.10. att. **Tyr-Arg-Phe-Lys-NH_2 stereoizomēru struktūras.**

Šis tetrapeptīds tika izvēlēts, jo tas sastāv no četrām atšķirīgām aminoskābēm, satur dažādus ūdeņraža saites donorus – brīvu α-aminogrupu pie N-gala tirozīna, ε-aminogrupu C-gala lizīna atlikumā, kā arī guanidīna fragmentu arginīnā, kuri teorētiski visi var piedalīties mijiedarbībās ar hirālo selektoru. Tādejādi no mehānisma skata punkta nav zināms katras šīs grupas ieguldījums kompleksa veidošanā. Papildus, šis tetrapeptīds arī satur atšķirīgus aromātiskos aizvietotājus, kas ne tikai nodrošina UV absorbciju, bet var arī iesaistīties papildus mijiedarbībās ar hirālo selektoru.

2. REZULTĀTI UN TO IZVĒRTĒJUMS

2.1. Krauna ēteru CSP pielietojums Tyr-Arg-Phe-Lys-NH_2 enantiomēru un stereoizomēru hromatogrāfiskajai sadalei[1]

Lai noskaidrotu uz krauna ēteru bāzes veidoto CSP perspektīvas īso peptīdu hirālajā sadalē, uz divām komerciāli pieejamām krauna ēteru CSP: CROWNPAK **CR-I** (+) (1.2. att., **B**) un ChiroSil **RCA** (+) (1.3. att., **B**), pētīta visu sešpadsmit Tyr-Arg-Phe-Lys-NH_2 (**1**; 1.10. att.) steroizomēru hromatogrāfiskā uzvedība. Detalizētāks eksperimentālā darba apraksts atrodams I Pielikuma 2.2. – 2.5. nodaļās.

Lai nodrošinātu pilnīgu tetrapeptīda **1** aminogrupu protonēšanos, tika secināts, ka tetrapeptīda **1** enantiomēru sadalei uz **CR-I** (+) un **RCA** (+) CSP optimālus apstākļus var sasniegt mobilajai fāzei pievienojot $HClO_4$ (visās pētāmajās MP ar nemainīgu koncentrāciju – 50 mM; ūdens šķīdumā atbilst pH 1.5).

Sākot eksperimentālo darbu, par MP organisko modifikatoru izvēlēts acetonitrils (ACN). Vispirms, tika izpētīti Tyr-Arg-Phe-Lys-NH_2 stereoizomēru aiztures profili uz abām krauna ēteru CSP izokrātiskā režīmā, mainot ACN daudzumu ACN/ūdens MP no 15 līdz 95% uz **CR-I** (+) un no 10 līdz 80% uz **RCA** (+) CSP. Grafiska aiztures laika (t_R) atkarība no ACN koncentrācijas mobilajā fāzē astoņiem pētītajiem tetrapeptīda **1** enantiomēru pāriem redzama 2.1. attēlā. Visiem tetrapeptīda **1** stereoizomēriem novērotas paraboliskas līknes ar minimumiem 50-75% ACN diapazonā uz **CR-I** (+) un 50% ACN uz **RCA** (+) CSP. Redzams, ka stereoizomēru aizture palielinās mobilajās fāzēs ar zemāku (<25% uz **CR-I** (+); <35% uz **RCA** (+) CSP) un augstāku (>90% uz **CR-I** (+); >75% uz **RCA** (+) CSP) acetonitrila saturu. Līdzīga paraboliska aiztures atkarība no mobilās fāzes organiskā modifikatora daudzuma iepriekš literatūrā aprakstīta metilaizvietotiem anilīniem [78] un dažādām proteīnogēnām aminoskābēm [45], un skaidrota ar atšķirīgiem saistīšanās mehānismiem uz krauna ēteru CSP: apgrieztās fāzes vai RP mehānismu (ūdens bagātās mobilajās fāzēs) un HILIC-veida mehānismu (augsta ACN satura MP).

Iepriekš nav veikti pētījumi, kas apraksta izdalīšanās secību savienojumiem, kas satur vairākas potenciāli saistīties spējīgas funkcionālās grupas kā tas ir īsajos peptīdos. Ir zināms (1.4. nodaļa), ka uz **CR-I** (+) CSP raksturīgā aminoskābju izdalīšanās secība ir D < L, savukārt uz **RCA** (+) CSP - L < D. Darbā noskaidrots, ka arī tetrapeptīda **1** LXXX-konfigurācijas (L-Tyr pie N-gala; 1.10. att.) stereoizomēri **CR-I** (+) kolonnā aizturas spēcīgāk kā to attiecīgie D-enantiomēri (DXXX; 2.1. att., **A**), savukārt uz **RCA** (+) - enantiomēru izdalīšanās secība ir pretēja (DXXX > LXXX; 2.1. att., **B**). Novērotās izdalīšanās secības norāda uz iespējamu N-gala α-aminoskābes – Tyr iesaisti kompleksu veidošanā starp krauna ētera ciklu CSP un analīta pirmējo amonija jonu (R-NH_3^+). Lai šo hipotēzi apstiprinātu, tālākajā darbā veikta padziļināta izpēte.

Kā redzams no stereoizomēru aiztures profiliem (2.1. att., **A**), izmantojot MP ar ACN daudzumu virs 90%, strauji palielinās LXXX-izomēru aizture, kamēr to attiecīgo DXXX-konfigurācijas enantiomēru aizture palielinās ievērojami lēnāk. Šāda hromatogrāfiskā uzvedība norāda ne tikai uz atšķirībām enantiomēru saistīšanā ar hirālo selektoru, bet kalpo arī par pamatu enantiosadales pieaugumam (2.1. att., **C**). Vispārīgi pieņemts, ka $\alpha > 1.2$ uzskatāma par pietiekamu divu savienojumu (šajā gadījumā – enantiomēru) hromatogrāfiskajai sadalei. Lai gan MP ar ACN saturu <85% starp DLDD/LDLL-enantiomēriem tika novērota vāja sadale ($\alpha < 1.05$; 2.1. att., **C**; DLDD/LDLL

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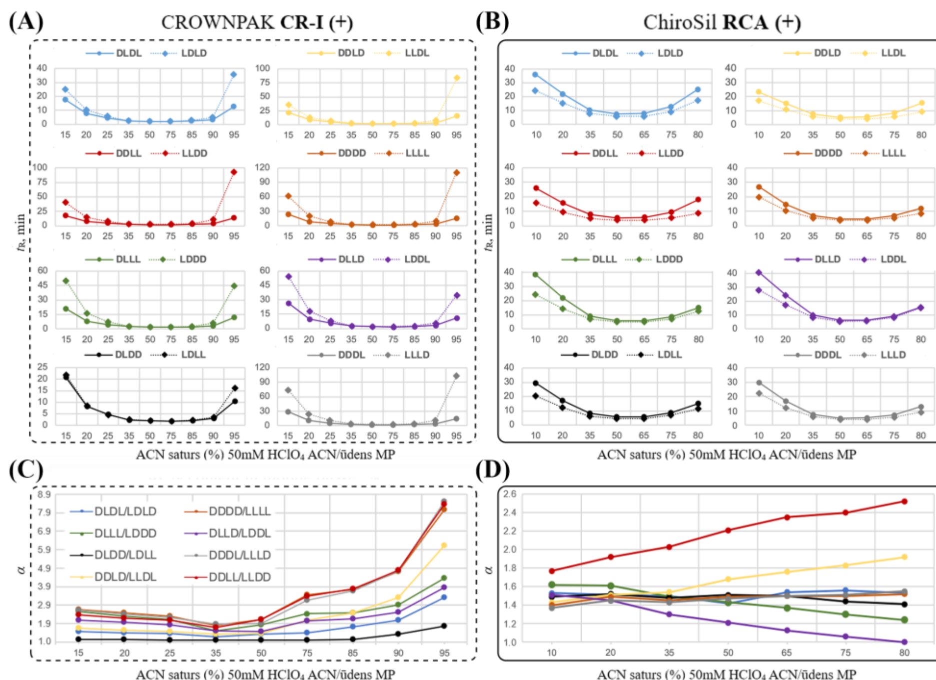
[1] **Upmanis, T.**; Kažoka, H.; Arsenyan, P. A study of tetrapeptide enantiomeric separation on crown ether based chiral stationary phases. *J. Chromatogr. A* **2020**, *1622*, 461152 (I Pielikums).

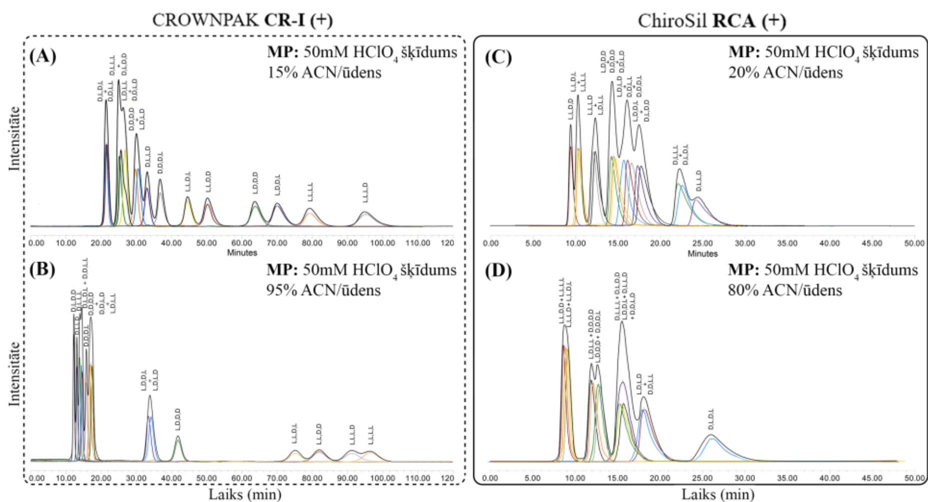
- *attēlots melnā krāsā*), palielinot ACN saturu MP virs 90%, uz **CR-I** (+) CSP izdevies sadalīt visus astoņus tetrapeptīda **1** enantiomēru pārus ar $\alpha > 1.2$.



2.1. att. **Tyr-Arg-Phe-Lys-NH₂ stereoizomēru aiztures laiku (t_R) un enantiomēru sadales (α) atkarība no ACN daudzuma mobilajā fāzē:** uz CROWNPAK **CR-I** (+) – **(A)** un **(C)**; uz ChiroSil **RCA** (+) – **(B)** un **(D).**

Interesanti, ka uz **RCA** (+) CSP, bāzes līnijas sadali ($\alpha > 1.2$; 2.1. att., **D**) visiem astoņiem tetrapeptīda **1** enantiomēru pāriem iespējams iegūt, tikai strādājot RP apstākļos. Palielinot ACN daudzumu MP, sadales faktora α vērtības starp DDLX/LLDX-enantiomēru pāriem pieaug, kamēr starp DLLX/LDDX-enantiomēru pāriem α samazinās. Tajā pat laikā, ACN izmaiņas mobilajā fāzē praktiski neietekmē atlikušo 4 pētāmo DDDX/LLLX un DLDX/LDLX-enantiomēru pāru sadali, norādot, ka pat vienas molekulas ietvaros, atkarībā no aizvietotāju stēriskā izvietojuma tetrapeptīda **1** struktūrā, hirālās atpazīšanas mehānismi atšķiras, tādēļ, strādājot ar nezināmu pētāmo objektu, svarīgi izpētīt abu eluēšanas režīmu pielietojumu.

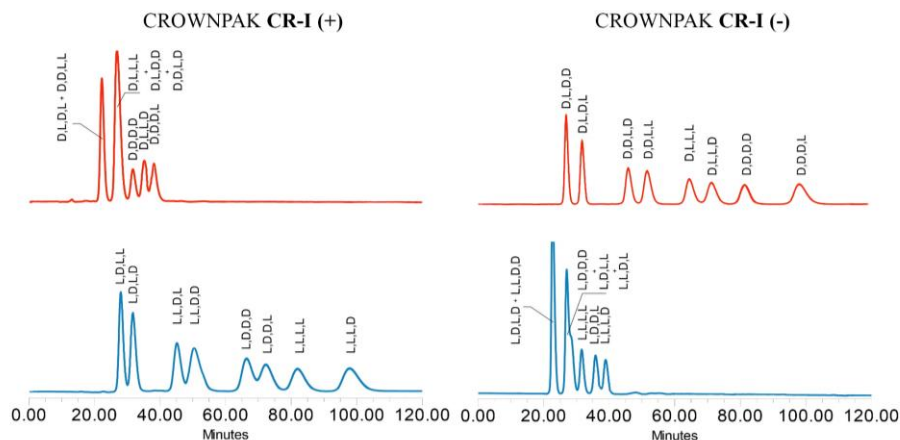
Tā, veicot vienlaicīgu sešpadsmit tetrapeptīda **1** stereoizomēru hromatogrāfisko analīzi (2.2. att. - *attēloti melnā krāsā*) uz **CR-I** (+) un **RCA** (+) CSP, tika konstatēts, ka perspektīvākā ir **CR-I** (+) CSP, uz kuras, lietojot MP ar zemu acetonitrila saturu (15% ACN), izdevies izšķirt septiņus, galvenokārt, LXXX-konfigurācijas stereoizomērus (2.2. att., **A**). Izmantojot ar ACN bagātu mobilo fāzi (2.2. att., **B**) no maisījuma pilnībā atdalīts tikai LDDD-stereoizomērs. Savukārt, neatkarīgi no mobilās fāzes sastāva, krietni vājāka stereoselektivitāte attiecībā pret tetrapeptīda **1** izomēriem, novērota uz **RCA** (+) CSP (2.2. att., **C** un **D**), tādēļ turpmāk promocijas darbā pētītas tikai **CR-I** fāzes.



2.2. att. **Tyr-Arg-Phe-Lys-NH_2 stereoizomēru (*attēloti melnā krāsā*) un enantiomēru (*attēloti krāsaini*) sadale uz CR-I** (+) **CSP - (A) un (B); RCA** (+) **CSP – (C) un (D)**. Kolonnas: CROWNPAK **CR-I** (+) (3.0 ×150 mm, 5µm); ChiroSil **RCA** (+) (4.6 ×150 mm, 5µm); Plūsmas ātrums: F = 0.4 mL/min uz **CR-I** (+); F = 1 mL/min uz **RCA** (+); Injekcijas tilpums: 10 µL; UV detektēšana veikta pie $\lambda = 220$ nm.

2.2. Krauna ēteru stacionāro fāžu pretējās hiralitātes izmantošana Tyr-Arg-Phe-Lys-NH_2 stereoizomēru sadalē[2]

Lai risinātu problēmu ar N-gala D-tirozīna saturošo Tyr-Arg-Phe-Lys-NH_2 DXXX-konfigurācijas stereoziomēru vājo sadali (2.2. att., **A** un **B**) uz **CR-I** (+) CSP, tika izmēģināta uz *R*-(3,3’-difenil-1,1’-binaftil)-20-krauna-6 hirālā selektora veidotā **CR-I (-)** CSP (1.2. att., **B**). Zinot, ka enantiomēru izdalīšanās secību uz **CR-I** CSP iespējams mainīt uz pretējo, veicot hromatogrāfisko analīzi uz stacionārās fāzes, kas satur pretējas hiralitātes selektoru, šajā darbā piedāvāta netradicionāla pieeja, lai palielinātu izšķirto tetrapeptīda **1** stereoizomēru skaitu. Kā redzams no 2.3. attēla, maisījumu, kas satur visus astoņus tetrapeptīda **1** DXXX-konfigurācijas stereoizomērus, iespējams sadalīt līdz bāzes līnijai uz **CR-I (-)** CSP, savukārt, identiskos apstākļos uz **CR-I** (+) fāzes, DXXX-stereoizomēru sadale ir vāja. Šādā veidā, veicot visu Tyr-Arg-Phe-Lys-NH_2 stereoizomēru maisījuma analīzi uz abām **CR-I** kolonnām, teorētiski iespējams identificēt un izšķirt 12 no 16 tetrapeptīda **1** stereoizomēru.



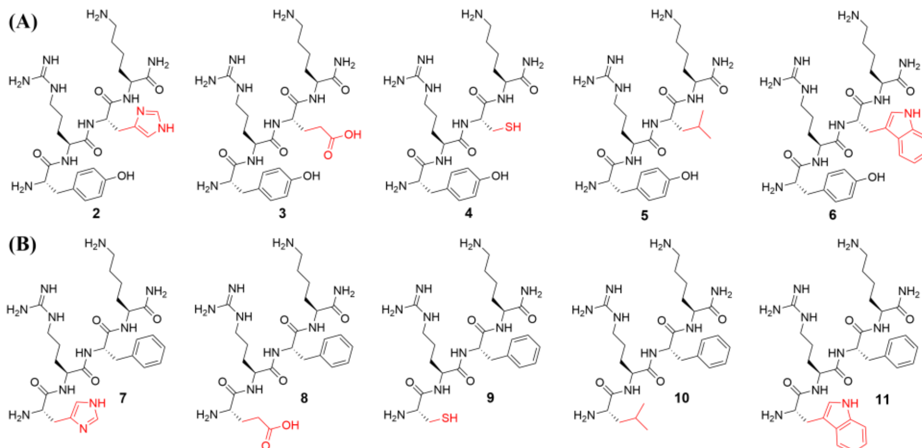
2.3. att. **Tetrapeptīda 1 DXXX (*attēlots sarkanā krāsā*) un LXXX-stereoizomēru (*attēlots zilā krāsā*) sadale uz CR-I (+) un CR-I (-) CSP.** Mobilā fāze: 50 mM $HClO_4$ šķīdums 15% ACN/ūdens.

Šāda pieeja aprakstīta pirmo reizi un būtu jāapsver reālu paraugu analīzē, kur pretējas hiralitātes selektoru **CR-I** CSP unikālā selektivitāte var sniegt plašāku ieskatu par hirālo piemaisījumu (stereoizomēru) sastāvu sarežģītos izomēru maisījumos, kā piemēram, peptīdos.

[2] **Upmanis, T**.; Kažoka, H. Application of Commercially Available Crown Ether Chiral Stationary Phases for Separation of Tetrapeptide Stereoisomers, *Acta Pharm Hung*. **2021**, *91*, 324–325 (II pielikums).

2.3. Ievadītās aminoskābes dabas ietekme uz tetrapeptīda 1 struktūras analogu hromatogrāfisko uzvedību uz CR-I CSP[3]

Ir labi zināms, ka pat nelielas izmaiņas hirāla analīta struktūrā var pilnībā izmainīt tā hromatogrāfisko uzvedību. Tādēļ, lai paplašinātu zināšanas šajā jomā, tika sintezēti desmit tetrapeptīdi **2** – **11** (2.4. att.; LLLL-enantiomēri).



2.4. att. **Modificēto tetrapeptīda 1 analogu struktūras:** tetrapeptīdi **2** – **6** modificēti Phe pozīcijā – (**A**); tetrapeptīdi **7** – **11** modificēti pie N-gala – (**B**).

Lai aptvertu iespējami atšķirīgāku aminoskābju klāstu, izvēlētas: histidīns (His, polāra, bāziska), glutamīnskābe (Glu; polāra, skāba); cistīns (Cys; polāra, neitrāla, sēru saturoša), leicīns (Leu; nepolāra, alifātiska) un triptofāns (Trp; nepolāra, aromātiska). Aminoskābes ievadītas tetrapeptīda **1** Phe pozīcijā (2.4. att., **A**) kā arī N-gala aminoskābes pozīcijā (2.4. att., **B**), ar mērķi izpētīt kā aminoskābes daba un pozīcija tetrapeptīdu **2** - **11** struktūrās ietekmē to hromatogrāfisko uzvedību uz **CR-I** (+) un **(-)** CSP (eksperimenti sīkāk aprakstīti III Pielikuma 2.2. – 2.4. nodaļās).

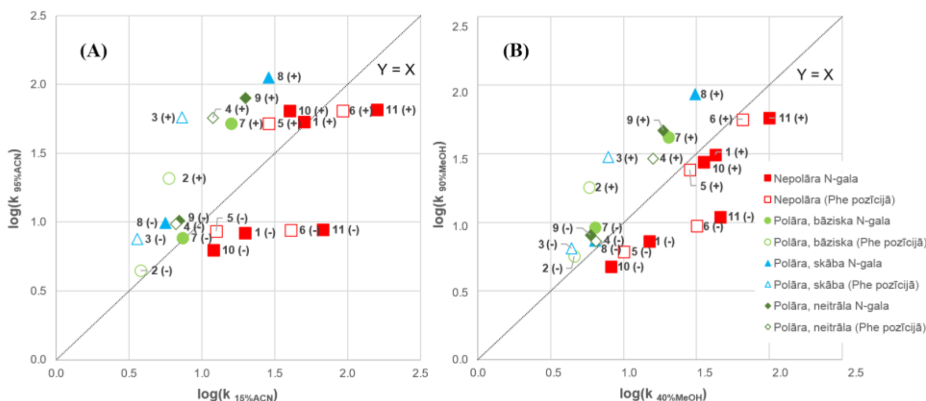
2.3.1. Ievadītās aminoskābes ietekme uz tetrapeptīda aizturi

Vispirms, lai pārliecinātos, ka jaunsintezēto tetrapeptīdu **2** – **11** aizture seko uz tetrapeptīda **1** novērotajām tendencēm, tika izpētīta to hromatogrāfiskā uzvedība uz **CR-I** (+) un **(-)** CSP ar 50 mM $HClO_4$ ACN/ūdens mobilajām fāzēm. Pēc tam tika veikti pētījumi arī ar MeOH saturošām MP. Lai arī pastāv uzskats, ka MeOH kā protisks šķīdinātājs un H-saites donors var traucēt saistīšanos starp analītu un hirālo selektoru, tam atspoguļojoties kā aiztures kritumam, pētāmo savienojumu hromatogrāfiskajā uzvedībā tika novērots pretējais.

Visiem desmit tetrapeptīdiem **2** – **11**, līdzīgi kā tetrapeptīdam **1** uz **CR-I** (+) un **(-)** CSP novērota paraboliska aiztures atkarība attiecībā pret MP izmantotā organiskā modifikatora daudzumu: ar minimumiem 50-75% ACN (60-80% MeOH) diapazonā un paaugstinātu savienojuma aizturi zema (<25% ACN vai <40% MeOH) un augsta (>90% ACN vai >80% MeOH) organiskā modifikatora satura MP, norādot uz atšķirīgiem saistīšanās mehānismiem.

[3] **Upmanis, T**.; Kažoka, H. Influence of amino acid residue on chromatographic behaviour of μ–opioid receptor agonist tetrapeptide analogue on crown ether based chiral stationary phase. *J. Chromatogr. A* **2022**, *1673*, 463059 (III Pielikums)

Lai uzskatāmāk parādītu ievadīto aminoskābju dabas ietekmi uz tetrapeptīdu aizturi uz **CR-I** CSP, izveidots grafiskais attēlojums (2.5. att.), kur uz X ass atliktas eksperimentāli noteikto savienojumu aiztures faktoru (k) logaritmu vērtības pie zema organiskā modifikatora satura MP, bet uz Y ass atliktas vērtības augsta organiskā šķīdinātāja satura MP. Grafikā attēlotie punkti, kas atrodas zem Y=X taisnes norāda, ka, no mehānistiska skatupunkta, šo savienojumu aizturē dominē hidrofobās mijiedarbības, savukārt, savienojumus, kuru aizturē noteicošās ir hidrofilās (polāras) mijiedarbības, grafikā apzīmē punkti, kuri atrodas virs Y=X taisnes.



2.5. att. **Struktūrā ievadītās aminoskābes ietekmes uz tetrapeptīdu 1 – 11 aizturi, atkarībā no mobilās fāzes sastāva:** log ($k_{15\%ACN}$) *vs.* log ($k_{95\%ACN}$) – 50 mM $HClO_4$ ACN/ ūdens MP – (**A**); log ($k_{40\%MeOH}$) *vs.* log ($k_{90\%MeOH}$) –50 mM $HClO_4$ MeOH/ūdens MP – (**B**); Datu punkti, kas apzīmēti ar (+) iegūti uz **CR-I** (+) CSP; ar (-) apzīmētie punkti iegūti uz **CR-I (-)** CSP; savienojumi **1** – **11** sagrupēti pēc krāsām, atkarībā no ievadīto aizvietotāju ķīmiskās dabas (nepolārs/polārs - skābs, neitrāls, bāzisks).

Balstoties uz 2.5. attēla datiem var secināt, ka galvenokārt, tetrapeptīdi, kuros ievadītas nepolāras aminoskābes (grafikā attēloti kā sarkani punkti) RP apstākļos tiek spēcīgāk aizturēti, kamēr analīti, kuri modificēti ar polārām aminoskābēm (grafikā attēloti kā zilas un zaļas krāsas punkti) spēcīgāk aizturas ar organisko modifikatoru bagātās mobilajās fāzēs, pie tam, šāds dalījums ir vēl izteiktāk redzams ar MeOH saturošām mobilajām fāzēm.

2.3.2. Krauna ēteru stacionāro fāžu pretējās hiralitātes izmantošana enantiosadales izvērtēšanai

Selektivitāte vai sadales faktors (α) apraksta hromatogrāfiskās sistēmas spēju atdalīt vielas maisījumā un to aprēķina kā divu secīgu pīķu aiztures faktoru (k) attiecību (2.1. vienādojums).

$$\alpha = \frac{k_2}{k_1}, \qquad (2.1.)$$

Kur α – selektivitāte;

k_2 – aiztures faktors spēcīgāk aizturētajam enantiomēram;

k_1 – aiztures faktors vājāk aizturētajam enantiomēram.

Tā kā parasti savienojums racēmiskā formā ir vieglāk pieejams par tā enantiomēriski tīro formu, tad, lai noskaidrotu vai CSP ir enantioselektīva attiecībā uz noteiktu hirālo savienojumu, parasti izmanto šo savienojumu racēmisko formu un CSP – enantiomēriski tīrā formā. Tomēr, īpaši aminoskābēm un to atvasinājumiem (tostarp peptīdiem), enantiomēriski tīrās formas bieži vien ir vieglāk pieejamas kā to racemāti.

Tādēļ šajā darbā izmēģināta literatūrā iepriekš neaprakstīta pretēja koncepcija, sadali pētot ar enantiomēriski tīru savienojumu uz CSP “racēmiskā formā” kā tas ir **CR-I** (+) un (**-**) fāzēs (1.2. att., **B**) un α vietā kā enantioselektivitāti raksturojošu parametru šajā gadījumā var izmantot α^* (2.2. vienādojums).

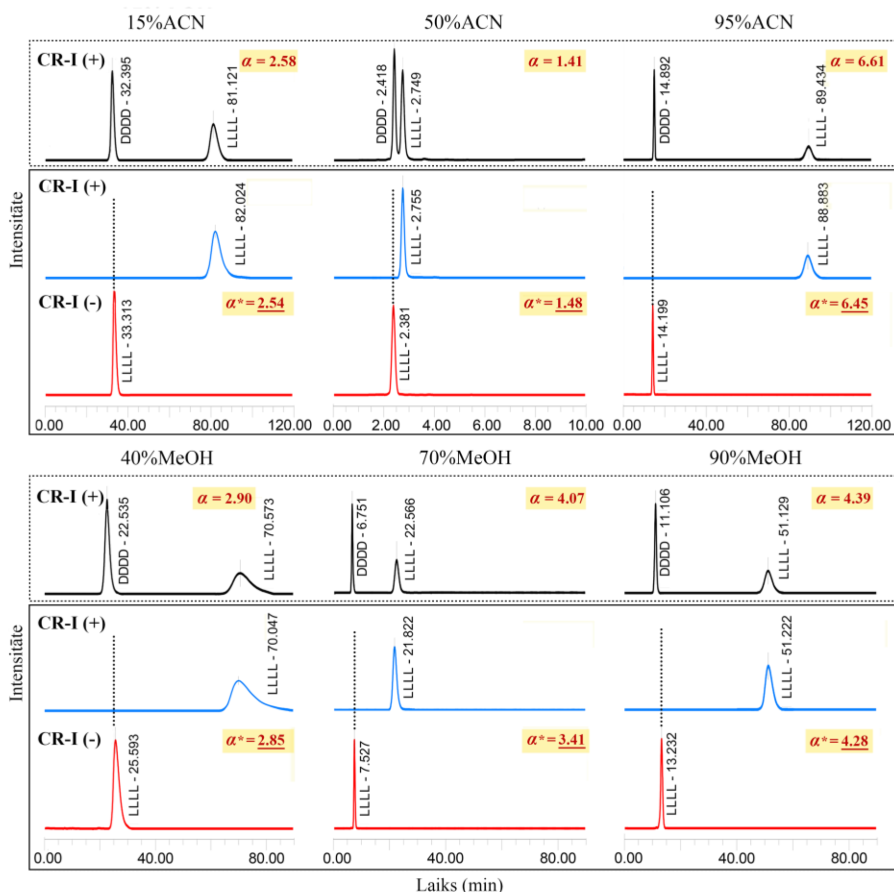
$$\alpha \sim \alpha^* = \frac{k_{(+)}}{k_{(-)}}, \tag{2.2.}$$

Kur α^* - enantioselektivitāti raksturojošs parametrs;

$k_{(+)}$ – aiztures faktors noteiktas konfigurācijas enantiomēram uz **CR-I** (+) CSP;

$k_{(-)}$ – aiztures faktors tam pašam enantiomēram uz pretējās hiralitātes **CR-I** (**-**) CSP.

Lai apstiprinātu šo koncepciju, tika veikta virkne eksperimentu ar tetrapeptīda **1** LLLL-enantiomēru. Kā redzams no 2.6. attēla, α vērtības, kas raksturo tetrapeptīda **1** LLLL/DDDD enantiomēru sadali uz **CR-I** (+) CSP (piem., 15% ACN - $\alpha = 2.58$; 95% ACN – $\alpha = 6.61$), būtiski neatšķiras no aprēķinātajām α^* vērtībām, (15% ACN - $\alpha^* = 2.54$; 95% ACN - $\alpha^* = 6.45$) kas iegūtas no LLLL-enantiomēra aiztures datiem uz **CR-I** (+) un (**-**) CSP.



2.6. att. **Tetrapeptīda 1 LLLL un DDDD enantiomēru hromatogrāfisko uzvedību raksturojošās hromatogrammas uz CROWNPAK CR-I CSP:** tetrapeptīda **1** LLLL/DDDD enantiomēru pāra sadale uz **CR-I** (+) (*attēlota melnā krāsā*); LLLL enantiomēra aizture uz **CR-I** (+) (*zilā krāsā*); LLLL enantiomēra aizture uz **CR-I** (**-**) (*sarkanā krāsā*). Mobilās fāzes: 50 mM $HClO_4$ ACN (MeOH)/ūdens.

Iegūtie rezultāti apstiprina, ka α^* parametru var droši izmantot, lai turpmāk raksturotu tetrapeptīdu **2 - 11** LLLL/DDDD enantiomēru pāru sadali, neatkarīgi no MP izmantotā organiskā modifikatora un sastāva.

2.3.3. Struktūrā ievadītās aminoskābes dabas ietekme uz tetrapeptīdu enantiosadali

Iepriekš aprakstītā koncepcija izmantota, lai noskaidrotu vai hirālie selektori **CR-I** CSP ir enantioselektīvi attiecībā arī uz modificētajiem tetrapeptīda **1** analogiem **2** – **11** (2.4. att.). Lai novērtētu mobilās fāzes organiskā modifikatora dabas un sastāva ietekmi uz pētāmo tetrapeptīdu LLLL/DDDD pāru enantiosadali, tika aprēķinātas α^*, jeb enantioselektivitāti raksturojošā parametra vērtības (2.2. vienādojums). Zinot, ka $\alpha >$ 1.2 uzskatāma par pietiekamu divu enantiomēru hromatogrāfiskajai sadalei, šie paši kritēriji tika piemēroti attiecībā uz α^* vērtībām.

Ņemot vērā atšķirīgo enantiomēru aizturi, atkarībā no MP dabas (kā piemēram, 2.6. att.), eksperimenti veikti gan ar zema (15% ACN vai 40% MeOH), gan augsta (95% ACN vai 90% MeOH) organiskā modifikatora satura MP. Dati, kas raksturo tetrapeptīdu **1** – **11** enantioselektivitāti uz **CR-I** CSP apkopoti 2.1. tabulā.

2.1. Tabula

Eksperimentāli iegūto tetrapeptīdu 1 - 11 (LLLL-enantiomēru) aiztures faktoru *k* un aprēķinātās α^* vērtības uz CR-I (+) un (-) CSP

Savienojums (2.4. att.)		ACN (%) daudzums 50 mM $HClO_4$ ACN/ūdens MP				MeOH (%) daudzums 50 mM $HClO_4$ MeOH/ūdens MP			
	CR-I	15		95		40		90	
		k	α^*	*k*	α^*	*k*	α^*	*k*	α^*
1	(+)	50.27	2.54	53.74	6.45	42.78	2.85	31.01	4.27
	(-)	19.82		8.33		15.00		7.27	
2	(+)	5.95	1.94	20.88	4.68	5.80	1.26	17.95	3.16
	(-)	3.07		4.46		4.61		5.69	
3	(+)	7.31	2.53	57.55	7.62	7.87	1.79	29.99	4.64
	(-)	2.89		7.55		4.41		6.47	
4	(+)	11.91	2.19	57.16	5.91	15.93	2.46	29.08	3.98
	(-)	5.45		9.67		6.48		7.31	
5	(+)	28.71	2.75	52.14	6.10	28.38	2.84	24.08	3.96
	(-)	10.46		8.54		10.00		6.08	
6	(+)	93.09	2.70	64.33	7.34	65.30	2.05	56.25	6.97
	(-)	34.43		8.77		31.90		9.42	
7	(+)	15.95	2.62	52.27	6.82	20.38	3.19	41.63	4.53
	(-)	6.08		7.67		6.39		9.21	
8	(+)	28.74	6.22	111.61	11.30	28.14	4.43	85.39	22.63
	(-)	4.62		9.88		6.35		7.35	
9	(+)	20.00	3.46	79.70	7.81	18.77	3.15	46.72	5.78
	(-)	5.78		10.21		5.96		8.08	
10	(+)	40.01	4.00	64.55	10.26	35.60	4.36	27.51	5.75
	(-)	10.01		6.29		8.17		4.78	
11	(+)	159.21	2.78	65.30	7.43	100.00	2.18	57.60	5.25
	(-)	57.28		8.79		45.88		10.97	

Balstoties uz 2.1. tabulas datiem, redzams, ka, neatkarīgi no izmantotās mobilās fāzes, visiem apskatītajiem tetrapeptīdiem aprēķinātās enantiosadali raksturojošā faktora α^* vērtības pārsniedz 1.2. Sevišķi augsta enantioselektivitāte novērota, strādājot ar augsta organiskā modifikatora satura MP.

Lai raksturotu dažādu ievadīto aminoskābju dabas (kā arī pozīcijas tetrapeptīda struktūrā) ietekmi uz enantiosadali, tika ieviests parametrs $\frac{\alpha^{*}(\mathbf{1})}{\alpha^{*}(\mathbf{2-11})}$, kurš salīdzina

tetrapeptīda **1** enantiosadali raksturojošo parametru ($\alpha^*_{(1)}$) ar α^* vērtību, kas vienādos hromatogrāfiskajos apstākļos iegūta katram struktūras analogam **2** – **11** un rezultāti apkopoti 2.2. tabulā. Aprēķinātās parametra vērtības, kas zemākas par 1 norāda uz enantioselektivitātes palielināšanos, kamēr vērtības, kas augstākas par 1 – uz samazinājumu, kas izraisīts tetrapeptīdu **2** – **11** struktūrā ievadīto aminoskābju dabas dēļ.

2.2. Tabula

Aprēķinātās parametra $\frac{\alpha^*_{(1)}}{\alpha^*_{(2-11)}}$ vērtības

Ievadītā aminoskābe (2.4. att.)	Ievadītās aminoskābes pozīcija (tetrapeptīda **1** struktūrā)	ACN (%) daudzums 50 mM $HClO_4$ ACN/ūdens MP		MeOH (%) daudzums 50 mM $HClO_4$ MeOH/ūdens MP	
		15%	95%	40%	90%
His (**2**)	Phe	1.31	1.38	2.26	1.35
Glu (**3**)		1.00	0.85	1.59	0.92
Cys (**4**)		1.16	1.09	1.16	1.07
Leu (**5**)		0.92	1.06	1.00	1.08
Trp (**6**)		0.94	0.88	1.39	0.72
His (**7**)	N-gala	0.97	0.95	0.89	0.94
Glu (**8**)		0.41	0.57	0.64	0.37
Cys (**9**)		0.73	0.83	0.90	0.74
Leu (**10**)		0.64	0.63	0.65	0.74
Trp (**11**)		0.91	0.87	1.31	0.81

Balstoties uz 2.2. tabulas datiem, redzams, ka ietekme uz enantiosadali, ko izraisa atšķirīgu aminoskābju ievadīšana tetrapeptīda **1** Phe pozīcijā, ir mazāk nozīmīga kā modifikācija pie tetrapeptīda N-gala. Saskaņā ar pieņēmumu, ka tetrapeptīda N-gala aminoskābes aminogrupa varētu būt atbildīga par saistīšanos ar krauna ētera selektoru (2.1. nodaļa), stēriskie efekti, ko izraisa α-oglekļa sānu ķēdes izmērs varētu būt svarīgs faktors analizējamās vielas hirālajā atpazīšanā. Stēriski pieclocekļu imidazola gredzens His (**7**) telpā aizņem līdzīgu daudzumu vietas kā sešlocekļu cikls Tyr fenilgredzenā (**1**), tādēļ abu šo savienojumu enantiosadale sevišķi neatšķiras, savukārt, mazāki, fleksiblāki aizvietotaji tetrapeptīdos **8**, **9** un **10**, šķietami uzlabo savienojumu enantiosdali.

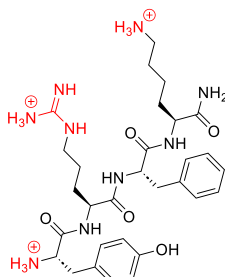
Iegūtie dati ļauj secināt, ka tetrapeptīdu **1** – **11** enantiosadali uz **CR-I** (+) un **(-)** CSP nosaka galvenokārt stēriski faktori, ko būtiski ietekmē aizvietotāja sānu ķēdes garums pie tetrapeptīda N-gala α-oglekļa, nevis pašas ievadītās aminoskābes polaritāte vai daba (lādēta/ nelādēta).

2.4. Tetrapeptīda 1 hirālās atpazīšanas pētījumi uz CR-I CSP[4,5]

Tetrapeptīda **1** LLLL un DDDD enantiomēri tika izvēlēti kā modeļvielas turpmākajos hirālās atpazīšanas mehānisma pētījumos. Lai labāk saprastu starpmolekulāros procesus, kas ir pamatā tetrapeptīda **1** enantiomēru sadalei uz **CR-I** hirālajām stacionārajām fāzēm, ar HPLC, HRMS un NMR metodēm tika pētīta krauna ēteru selektoru saistīšanās ar LLLL-**1** un DDDD-**1**. Līdzīgu apstākļu nodrošināšanai gan NMR ampulā, gan hromatogrāfiskajā sistēmā, visi eksperimenti veikti 50 mM $HClO_4$ metanolā šķīdumos.

2.4.1. Struktūras – hromatogrāfiskās uzvedības sakarības pētījumi saistīšanās vietas identifikācijai

Teorētiski tetrapeptīda **1** saistīšanās ar krauna ētera selektoru var piedalīties trīs funkcionālās grupas - α-aminogrupa Tyr; ε-aminogrupa Lys, kā arī guanidīna fragments Arg (2.7. att.).



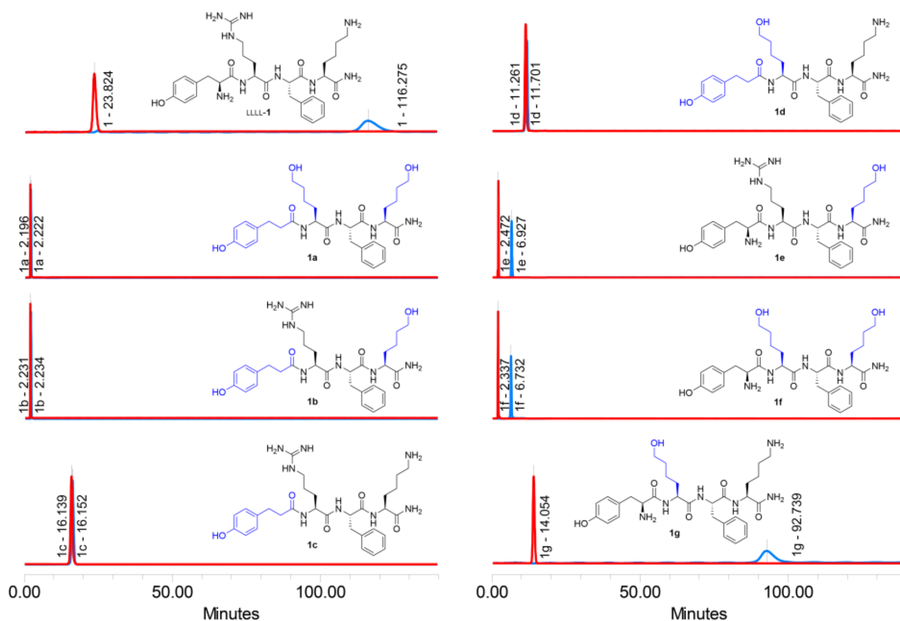
2.7. att. **Mijiedarbībās stāties spējīgās tetrapeptīda 1 funkcionālās grupas.** Potenciālie H-saišu donori apzīmēti sarkanā krāsā.

Savienojumi **1a** – **1g** sintezēti (2.8. att.) ar mērķi sistemātiski izslēgt konkrētas mijiedarboties spējīgās aminogrupas tetrapeptīda **1** sekvencē, aizvietojot tās ar OH grupām, vai izslēdzot tās pavisam, tajā pat laikā, saglabājot jaunizveidoto molekulu stereoķīmiski pēc iespējas līdzīgāku tetrapeptīda **1** struktūrai. Lai izslēgtu N-gala aminogrupu Tyr, tetrapeptīda **1** struktūrā Tyr pozīcijā ievadīta 3-(4-hidroksifenil)-propānskābe (savienojumos **1a** – **1d**). Lai izvairītos no iespējamās saistīšanās Lys (savienojumos **1a**; **1b**; **1e** un **1f**) un Arg (savienojumos **1a**; **1d**; **1f** un **1g**) atlikumos, šīs aminoskābes tika aizvietotas ar 6-hidroksinorleicīnu. Pieņemot, ka enantioselektīva saistīšanās ar krauna ētera selektoru vienādos hromatogrāfiskos apstākļos ir iespējama tikai gadījumos, kad analīta viena un tā paša enantiomēra aizture uz **CR-I** (+) un **(-)** CSP atšķirsies (2.3.2. nodaļa), darbā salīdzināta tetrapeptīda **1** un tā struktūras analogu **1a – 1g** hromatogrāfiskā uzvedība.

Balstoties uz 2.8. attēla datiem, redzams, ka izslēdzot visas trīs saistīties spējīgās aminogrupas tetrapeptīda **1** struktūrā, savienojumu **1a** nav iespējams aizturēt ($t_R \sim t_0$) uz **CR-I** CSP. Līdzīga hromatogrāfiskā uzvedība novērota arī savienojumam **1b**, no kā secināms, ka guanidīna fragments Arg nesaistās ar krauna ētera selektoriem. Atšķirībā no **1a** un **1b**, savienojumi **1c** un **1d** aizturas, pateicoties pirmējai ε-aminogrupai Lys atlikumā, kas spēj piedalīties $^{+}$N–H···O ūdeņraža saišu veidošanā ar krauna ētera selektoru. Tomēr, savienojumu līdzīgā aizture (piem., **1c**: $k_{(-)} \sim k_{(+)} = 8.9$), kas novērota

[4] **Upmanis, T.**; Kažoka, H. Mechanistic insights in chiral recognition of μ-opioid receptor agonist tetrapeptide on crown ether chiral stationary phase. *J. Chromatogr. Open* **2021**, *1*, 100016. (IV Pielikums)
[5] **Upmanis, T.**; Sevostjanovs, E.; Kažoka, H. Chiral recognition mechanism studies of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide on crown ether based chiral stationary phase. *Chirality* **2024**, *36(1)*, e23619 (V Pielikums)

uz **CR-I** (+) un **CR-I** (-) CSP, norāda uz to, ka šī saistīšanās nav enantioselektīva. Visticamāk, 4 C atomu attālums starp ε-aminogrupu lizīnā un hirālo centru ir pārāk liels, lai krauna ētera selektora stēriski apjomīgās bifenil- un fenilgrupas (1.5. att.), spētu izveidot stereoselektīvu vidi.



2.8. att. **LLLL-1 un struktūras analogu 1a – 1g aizture uz CR-I** (+) (***attēlots zilā krāsā***) **un CR-I (-) CSP (*sarkanā krāsā*).** Mobilā fāze: 50mM $HClO_4$ MeOH.

Iespējama enantioselektīva saistīšanās, norādot uz dažādu mijiedarbošanos starp analītu un krauna selektoriem, novērota struktūras analogiem **1e** - **1g**, kuru aizture, līdzīgi kā LLLL-**1**, atšķiras uz **CR-I** (+) un **(-) CSP.** Salīdzinot **1e** – **1g** struktūras (2.8. att.), redzams, ka šie savienojumi satur N-gala α-aminogrupu Tyr atlikumā, tādejādi norādot uz šīs aminogrupas nozīmīgumu hirālajā atpazīšanā.

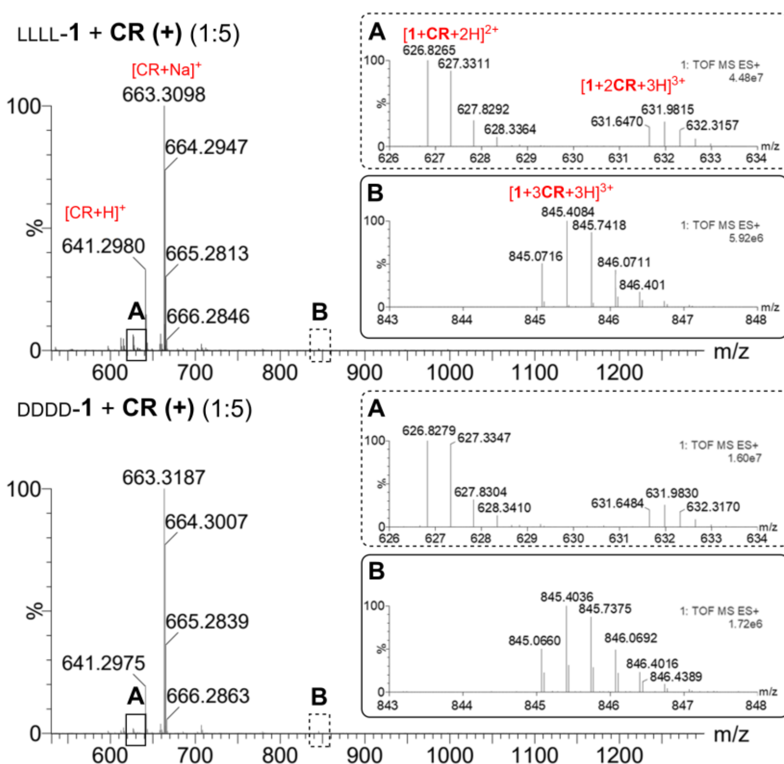
Interesanti, ka struktūras analogu **1e** ($k_{(-)} = 0.53$; $k_{(+)} = 3.28$) un **1f** ($k_{(-)} = 0.44$; $k_{(+)} = 3.15$) aizture būtiski atšķiras no **1g** ($k_{(-)} = 7.67$; $k_{(+)} = 56.2$) un LLLL-**1** ($k_{(-)} = 13.71$; $k_{(+)} = 70.8$). Var pieņemt, ka tieši brīvās ε-aminogrupas klātbūtne savienojuma struktūrā, ievērojami palielina **1g** un LLLL-**1** aizturi. Šis novērojums var liecināt par to, ka tetrapeptīda **1** hirālajā atpazīšanā varētu piedalīties gan N-gala α-amino grupa Tyr, gan ε-amino grupa Lys. Lai šo pieņēmumu apstiprinātu, veikti tālākie HRMS un NMR eksperimenti.

2.4.2. Augstas izšķiršanas MS pielietojums tetrapeptīda 1 – krauna selektora kompleksu pierādīšanā

Lai pierādītu tetrapeptīda **1** spēju saistīties kompleksos ar **CR** selektoriem ārpus hromatogrāfiskās kolonnas, veikti augstas izšķiršanas masspektrometrijas (HRMS) pētījumi, izmantojot elektroizsmidzināšanas jonizāciju (ESI), kas iestatīta pozitīvā jonizācijas režīmā. Eksperimentālās daļas apraksts atrodams V Pielikuma 2.6. nodaļā.

Tika sintezēti optiski tīri (*S*)- and (*R*)-(3,3'-difenil-1,1'-binaftil)-20-krauna-6 enantiomēri (attiecīgi **CR** (+) un **(-)** selektori; 1.2. att.), kas tika sajaukti pieckāršā pārākumā pret attiecīgo tetrapeptīda **1** LLLL vai DDDD-enantiomēru (5:1; 50 mM $HClO_4$

metanolā) un pagatavotie šķīdumi tieši ievadīti nolidojuma laika (TOF) masspektrometrā. Nesaistītais krauna ētera selektora atlikums masspektrā redzams (2.9. att.) kā intensīvs signāls ar masas lādiņa attiecību *m/z* 641 un 663 (atbilst Na^{+} aduktam). Tuvāka iegūtā masspektra apskate parāda vairākus zemākas intensitātes signālus, no kuriem svarīgākie atrodami pie *m/z* 626 un 631, un atbilst 1:1 un 1:2 stehiometrijas kompleksiem starp tetrapeptīda **1** enantiomēriem un krauna selektoru (2.9. att., **A**). Piedevām, novērots arī 1:3 kompleksēšanās stehiometriju raksturojošais signāls pie *m/z* 845 (2.9. att., **B**), norādot uz krauna ētera selektora spēju gāzes fāzē saistīties ar visām trīs pieejamajām tetrapeptīda **1** struktūras aminogrupām (2.7. att.). Līdzīgas intensitātes visu trīs iepriekšminēto kompleksu veidošanās vērojama arī DDDD-**1** enantiomēram, saistoties ar **CR** (+) selektoru. Tas varētu liecināt par citādāku (ne stereospecifisku) saistīšanās mehānismu gāzes fāzē, ierobežojot šīs metodes priekšrocības hirālās atpazīšanas mehānismu pētījumos. Neskatoties uz to, lietojot ESI-HRMS demonstrēta nekovalentu kompleksu veidošanās starp tetrapeptīda **1** enantiomēriem un krauna selektoriem un iegūts vērtīgs ieskats kompleksēšanās stehiometrijā.

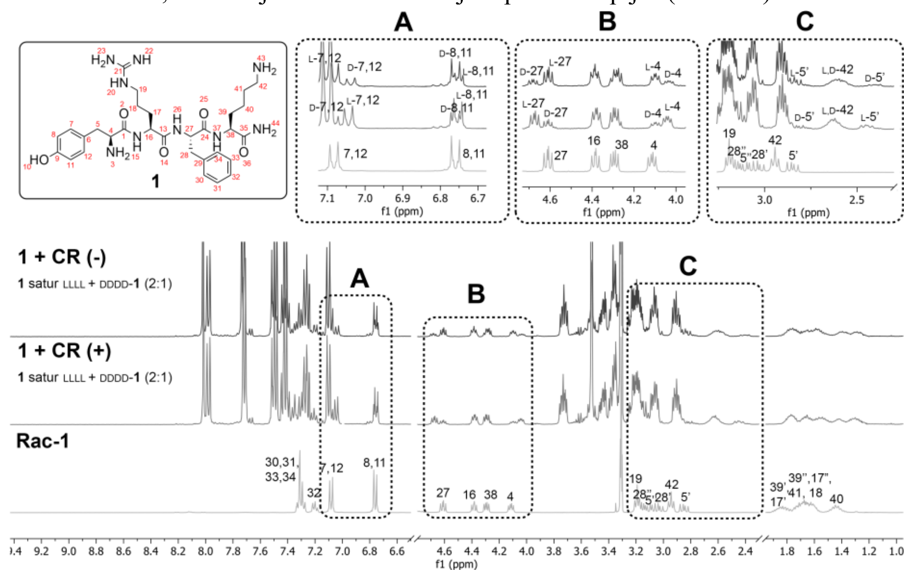


2.9. att. **Daļējs tetrapeptīda 1 kompleksēšanās ESI-TOF masspektrs:** LLLL-**1** saistoties ar **CR** (+) hirālo selektoru (pieckāršā pārākumā; *attēlots augšā*); DDDD-**1** saistoties ar **CR** (+) hirālo selektoru (pieckāršā pārākumā; *attēlots apakšā*). Signāli, kas atbilst 1:1 un 1:2 attiecības kompleksiem attēloti izgriezumos (**A**); Masas signāli kas atbilst 1:3 attiecības kompleksiem attēloti izgriezumos (**B**).

2.4.3. Kodolu magnētiskās rezonanses spektroskopijas pielietojums tetrapeptīda 1 – krauna selektora kompleksu pierādīšanā

Lai labāk saprastu krauna ēteru selektoru saistīšanos ar tetrapeptīda **1** LLLL un DDDD-enantiomēriem, veikti virkne pētījumu ar 1D un 2D NMR metodēm (eksperimentos izmantotie instrumenti un paraugu pagatavošana aprakstīta V Pielikuma 2.7. nodaļā). Lai nodrošinātu līdzīgu vidi kāda tā ir hromatogrāfiskajā kolonnā (2.4.1. nodaļa) ampulā tika sajaukts tetrapeptīda **1** pseidoracemāts tā brīvās bāzes formā (identifikācijas nolūkos LLLL:DDDD = 2:1) ar katru no krauna ētera selektoriem **CR** (+) un **(-)** divkāršā pārākumā MeOH-d_4, kas satur $HClO_4$ 50 mM daudzumā (2.10. att.). Eksperimentāli tika noskaidrots, ka krauna selektora pievienošana lielākā pārākuma, neatspoguļojās būtiskās novēroto ķīmisko nobīžu izmaiņās.

Tetrapeptīda **1** ^{1}H-NMR signāli (apzīmēti saskaņā ar 2.10. attēlā redzamo numerācijas shēmu) tika identificēti, kombinējot ^{1}H-^{1}H korelāciju spektroskopijas (COSY), ^{1}H-^{13}C heteronukleārās viena kvanta korelācijas (HSQC) un heteronukleārās daudzkāršo saišu korelācijas (HMBC) metodes. Diastereomēru kompleksu veidošanos apstiprina tetrapeptīda **1** pseidoracemāta protonu signālu šķelšanās krauna ētera selektoru klātbūtnē, kas parādīta ^{1}H-NMR spektrā 2.10. att. Spektrā pārklājušies signāli tika identificēti, izmantojot totālās korelācijas spektroskopijas (TOCSY) metodi.



2.10. att. **^{1}H-NMR (400 MHz) spektri:** nesaistītam 5 mM tetrapeptīda **1** pseidoracemātam (Rac-**1**; LLLL:DDDD = 2:1; *attēlots apakšā*); 5 mM tetrapeptīda **1** pseidoracemātam 10 mM **CR** (+) (*pa vidu*) un 10mM **CR (-)** selektoru (*augšā*) klātbūtnē. Visi spektri uzņemti metānolā-d_4, kas satur 50 mM $HClO_4$. Signālu šķelšanās Tyr aromātiskajos protonos izcelta izgriezumā (**A**); Signālu šķelšanās Phe un Tyr atlikumu α-protonos izcelta izgriezumā (**B**); Signālu ķīmiskās nobīdes Tyr atlikuma β-protonos izcelta izgriezumā (**C**).

Novērots, ka saistoties ar **CR** (+) selektoru, LLLL-**1** protoni NMR spektrā nobīdās izteiktāk, attiecībā pret nesaistītu tetrapeptīdu **1**, salīdzinot ar tā DDDD-enantiomēru. Savukārt, pretēja uzvedība novērota pseidoracemātam, saistoties ar **CR (-)** selektoru, kur, attiecīgi spēcīgāk nobīdās DDDD-**1** signāli. Piedevām no abiem uzņemtajiem spektriem redzams (2.10. att.), ka tie savā starpā ir spoguļattēli, apstiprinot, ka LLLL-**1** saistoties ar **CR** (+), un DDDD-**1** ar **CR (-)** selektoru (attiecīgi arī LLLL-**1** saistoties ar **CR (-)** un DDDD-**1** ar **CR** (+) selektoru) pakļaujas identiskiem

kompleksēšanās mehānismiem. Tādēļ tālākajā hirālās atpazīšanas mehānisma diskusijā apskatīta tikai **CR** (+) selektora kompleksēšanās ar tetrapeptīda **1** LLLL un DDDD-enantiomēriem.

Izteikta nobīdīšanās stiprākos laukos NMR spektros tika novērota tetrapeptīda **1** Lys atlikuma H42 protona signālam. Šāda veida uzvedība norāda uz starpmolekulāru mijiedarbību, visticamāk H-saišu veidošanos starp tetrapeptīdu **1** un krauna ētera selektoru. Šo pieņēmumu apstiprina iepriekš veikti pētījumi [79], kur, saistoties aminoskābēm kompleksā ar **CR** (+) selektoru caur H-saitēm, novērots līdzīgs ekranēšanas efekts. Tomēr, ņemot vērā gandrīz identiskās ķīmisko nobīžu izmaiņu vērtības starp kompleksiem, ko ar **CR** (+) selektoru veido LLLL-**1** un DDDD-**1**, var pieņemt, ka abi enantiomēri ir pakļauti līdzīgam (ne-enantioselektīvam) kompleksēšanās mehānismam, Lys ε-NH_3^+ grupai saistoties ar skābekļa atomiem krauna ētera ciklā.

Būtiskas atšķirības (2.10. att., **C**) protonu ķīmiskajās nobīdēs tika novērotas LLLL-**1** Tyr atlikuma β-protonos H5'' un H5', kuri, līdzīgi kā Lys atlikuma protoni, nobīdījās stiprākā laukā, kā arī Tyr α-protonam H4 (2.10. att., **B**) un aromātiskajiem protoniem H7 un H12 (2.10. att., **A**). Tajā pat laikā šie Tyr atlikuma protonu signāli praktiski nenobīdās, ar **CR** (+) selektoru saistoties DDDD-**1**. Balstoties uz šiem novērojumiem, var secināt, ka papildus ne-enantioselektīvai ūdeņraža saites veidošanai starp tetrapeptīda **1** Lys NH_3^+ grupu un **CR** (+) selektoru, notiek arī sekundāra saistīšanās ar starp LLLL-**1** tirozīna atlikuma α-NH_3^+ grupu un vēl vienu **CR** (+) selektora molekulu (attiecīgi DDDD-**1** ar vēl vienu **CR (-)** molekulu), kas varētu atbildēt par tetrapeptīda **1** hirālo atpazīšanu. Šāds pieņēmums būtu saskaņā gan ar tetrapeptīda **1** hirālās sadales (2.1. nodaļas) rezultātiem, gan struktūras analogu **1a** – **1g** hromatogrāfiskās uzvedības analīzi 2.4.1. nodaļā.

Atšķirībā no iepriekš apskatītajām ūdeņraža saistīšanās izraisītajām protonu ķīmiskajām nobīdēm stiprākos laukos, **CR** (+) klātbūtnē tetrapeptīda **1** LLLL-enantiomēra Phe α-protonam H27 (2.10. att., **B**), β-protoniem H28'', H28' un aromātiskajiem protoniem H30, H31, H33, H34 novērota vāja dezekranizācija (signālu nobīdīšanās vājākos laukos). Balstoties uz ķīmisko nobīžu izmaiņu atšķirīgo raksturu, var pieņemt, ka šajā gadījumā tiek novērotas cita veida starpmolekulārās mijiedarbības, iespējams, starp Phe atlikuma π-sistēmu un **CR** (+) selektora aromātiskajiem aizvietotājiem.

Kopumā, tetrapeptīda **1** un tā struktūras analogu **1a** – **1g** hromatogrāfiskās uzvedības analīze uz **CR-I** (+) un **(-)** CSP norāda uz to, ka LLLL/DDDD-**1** enantiosadale varētu būt iespējama, pateicoties ievērojamām atšķirībām enantiomēru saistīšanās mehānismos. No hromatogrāfijas skata punkta, spēcīgāk aizturētajam tetrapeptīda **1** enantiomēram [LLLL-**1 - CR-I** (+)] un [DDDD-**1** - **CR-I (-)**], NH_3^+ grupas Tyr un Lys atlikumos vienlaicīgi var saistīties ar divām krauna ētera selektora molekulām uz CSP virsmas, kamēr vājāk aizturētajam tetrapeptīda **1** enantiomēram [DDDD-**1 - CR-I** (+)] un [LLLL-**1** - **CR-I (-)**], par saistīšanos ar krauna ētera selektoru (un līdz ar to arī aizturi) atbildīga ir tikai NH_3^+ grupa Lys atlikumā.

SECINĀJUMI

1. Izpētīts komerciāli pieejamu krauna ēteru stacionāru fāžu pielietojums īso peptīdu enantiosadalē par modeļvielu izmantojot Tyr-Arg-Phe-Lys-NH_2 (sintezēti visi 16 stereoizomēri). Labāka enantioselektivitāte ir novērota uz CROWNPAK **CR-I** (+) fāzes: visi astoņi tetrapeptīda enantiomēru pāri tika sadalīti ar ACN bagātu mobilo fāzi.
2. Desmit jaunsintezētiem Tyr-Arg-Phe-Lys-NH_2 struktūras analogiem (pie N-gala vai Phe pozīcijā ievadītas dažādas aminoskābes) izpētīta hromatogrāfiskā uzvedība uz **CR-I** fāzēm. Noskaidrots, ka modificēšanai Phe pozīcijā ir mazāka ietekme uz enantioselektivitāti kā pie N-gala un enantiosadale lielā mērā ir atkarīga no stēriskiem efektiem, ko izraisa α-oglekļa sānķēžu lielums pie N-gala, nevis ievadītās aminoskābes daba.
3. Sintezēti 7 savienojumi ar mērķi sistemātiski izslēgt ar hirālo sorbentu mijiedarboties spējīgās tetrapeptīda aminogrupas, tās aizvietojot ar OH grupām (vai izslēdzot tās pavisam) un pētīta to hromatogrāfiskā uzvedība uz **CR-I** (+) un **(-)** fāzēm, kas satur pretējas hiralitātes selektorus. Secināts, ka:
   a. Guanidīna fragments Arg nesaistās ar krauna ētera selektoriem;
   b. Pirmējā ε-aminogrupai Lys atlikumā spēj piedalīties $^{+}N{-}H\cdots O$ ūdeņraža saišu veidošanā ar krauna ētera selektoru, bet šī saistīšanās nav enantioselektīva;
   c. Iespējams, ka tieši α-aminogrupa Tyr fragmentā ir nozīmīga hirālajā atpazīšanā.
4. Ar HRMS metodi noskaidrots, ka Tyr-Arg-Phe-Lys-NH_2 LLLL un DDDD-enantiomēri ar krauna ētera selektoriem vienlaicīgi spēj saistīties gan 1:1, 1:2, gan 1:3 stehiometrijas kompleksos.
5. Ar NMR metodēm pētot LLLL un DDDD-enantiomēru kompleksēšanos ar krauna ēteru selektoriem secināts, ka vienā no tetrapeptīda enantiomēram, NH_3^+ grupas Tyr un Lys atlikumos vienlaicīgi var saistīties ar divām krauna ētera selektora molekulām uz sorbenta virsmas, kamēr otram enantiomēram par saistīšanos ar krauna ētera selektoru atbildīga ir tikai NH_3^+ grupa Lys atlikumā kas nav pretrunā ar HPLC datiem.

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UNIVERSITY
OF LATVIA

FACULTY OF MEDICINE AND LIFE SCIENCES

Toms Upmanis

CHIRAL RECOGNITION MECHANISM STUDIES OF SHORT PEPTIDE CHROMATOGRAPHIC SEPARATION ON CROWN ETHER STATIONARY PHASES

DOCTORAL THESIS

Submitted for the Degree of Doctor of Science (Ph. D.)
in Natural Sciences (in the field of Chemistry)
Subfield of Analytical Chemistry

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3) Prof. Bezhan Chankvetadze, PhD (Ivane Javakhishvili Tbilisi State University)

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The thesis is available at the Library of the University of Latvia (Riga, Raiņa Blvd. 19).

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ABSTRACT

Chiral recognition mechanism studies of short peptide chromatographic separation on crown ether stationary phases. Upmanis, T., supervisor Dr. chem., Kažoka, H. Summary of the collection of scientific articles in the subfield of analytical chemistry, 76 pages, 20 figures, 2 tables, 79 literature references. In Latvian and English.

Despite the successful use of the stationary phases based on crown ethers for the enantioseparations of variety of chiral compounds containing primary amino groups, the lack of studies investigating more structurally complex analytes such as short peptides are evident. Exact mechanisms governing the intermolecular binding between crown ether selector and analyte stereoisomers remain unclear. By choosing a known μ-opioid receptor agonist tetrapeptide Tyr-Arg-Phe-Lys-NH_2 we have observed that chiral stationary phases, based on *R* and *S*-(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 chiral selectors provide optimal conditions for tetrapeptide chiral chromatographic separations. It was later found out that these stationary phases also resolve other structurally similar tetrapeptide enantiomers. In order to rationalize the experimentally observed chromatographic separations, the mechanism of chiral recognition was investigated by high-performance liquid chromatography, mass spectrometry and nuclear magnetic resonance spectrometry.

CROWN ETHER CHIRAL STATIONARY PHASES; TETRAPEPTIDE; CHIRAL RECOGNITION; AMINO ACIDS; ENANTIOSELECTIVITY

ABBREVIATIONS

α	selectivity
ACN	acetonitrile
Arg	arginine
COSY	correlation spectroscopy
CSP	chiral stationary phase
Cys	cysteine
ESI	electrospray ionization
Glu	glutamic acid
HILIC	hydrophilic interaction liquid chromatography
His	histidine
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum correlation
HRMS	high resolution mass spectrometry
k	retention factor
Leu	leucine
Lys	lysine
MP	mobile phase
MS	mass spectrometry
NMR	nuclear magnetic resonance
Phe	phenylalanine
RP	reversed phase
TOCSY	total correlation spectroscopy
TOF	time of flight
t_R	retention time
Trp	tryptophan
Tyr	tyrosine

INTRODUCTION

Short peptides ($n < 6$) are involved in a variety of biological processes and are responsible for many functions crucial for human health. Due to their high efficiency and selective action, the use of short peptides has attracted increasing interest from scientists, both for the development of new drug candidates and as new delivery vehicles for existing drugs. Peptides consist of amino acids arranged in a sequence, all of which are chiral apart from glycine. During manufacturing process and other external factors, multiple peptide stereoisomers with potentially different or even undesired biological activity can often be formed. Therefore, the demand for separation techniques of complex diastereomeric and enantiomeric mixture is of paramount importance for the biological and medical sciences and is necessary for the pharmaceutical industry.

Several analytical methods are used today for the enantioseparation of chiral compounds, the most common being high performance liquid chromatography (HPLC) using chiral stationary phases (CSPs). Among them, CSPs based on crown ethers are known to have been successfully used for the separation of enantiomers of various chiral compounds containing primary amino groups, including various unprotected di- and tripeptides. Due to the complex stereochemical structure of the compounds, it is practically impossible to predict how the stereoisomers of a specific analyte will behave in the chromatographic system, thus, most often analytical separation methods are developed by *trial and error* approach.

Although generally known, the exact mechanisms of chiral recognition for the resolution of short peptides have not been fully investigated. In order to better understand the existing approaches as well as to design promising strategies for the development of analytical methods to monitor the enantiomeric purity of chiral short peptides, it is necessary to establish a basis of an in-depth understanding of the chiral separation mechanisms on the crown ether phases.

Thus, **the aim of this work** is to investigate the chiral recognition mechanism underlying short peptide separations on crown ether chiral stationary phases, by combining HPLC, high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy by employing known μ-opioid agonist Tyr-Arg-Phe-Lys-NH_2 as a tetrapeptide model compound.

The **tasks** set to achieve the aim were as follows:

1. To synthesize all sixteen stereoisomers of Tyr-Arg-Phe-Lys-NH_2 and clarify the prospects of using commercially available crown ether based chiral stationary phases for chromatographic separations of Tyr-Arg-Phe-Lys-NH_2 stereoisomers.
2. To synthesize modified analogues of the Tyr-Arg-Phe-Lys-NH_2 by introducing different amino acids at the N-terminus and Phe position and to evaluate the effect of introduced residual amino acid on tetrapeptide retention and enantioseparation on CROWNPAK **CR-I** phases.
3. To synthesize all possible Tyr-Arg-Phe-Lys-NH_2 structure analogues in which the amino groups capable of binding to the crown ether selectors are selectively substituted with inert hydroxyl groups and to determine which of the three amino groups are involved in chiral recognition on CROWNPAK **CR-I** phases.
4. To synthesize the chiral selectors found in CROWNPAK **CR-I** stationary phases and to investigate the intermolecular binding between LLLL and DDDD-enantiomers of Tyr-Arg-Phe-Lys-NH_2 and synthesized selectors by HRMS and NMR.

Scientific novelty of the work:

1. Chiral separations of tetrapeptide (Tyr-Arg-Phe-Lys-NH_2) performed on crown ether chiral stationary phases are reported for the first time.
2. It has been established that stationary phases based on (*R*) and (*S*)-(3,3'-diphenyl-1,1-binaphthyl)-20-crown-16 chiral selectors are the most perspective for enantioseparation of Tyr-Arg-Phe-Lys-NH_2 and its ten structural analogues.
3. This work is the first to demonstrate an unconventional approach. By analysing the same analyte on two CROWNPAK **CR-I** (+) and **CR-I (-)** stationary phases of opposite chirality allowed us to increase the number of separated tetrapeptide stereoisomers as well as to assess the enantioselectivity towards a particular enantiomeric pair even in cases, where only one of the enantiomers is available.
4. By combining HPLC, HRMS and NMR techniques the chiral recognition mechanism for one enantiomer pair of Tyr-Arg-Phe-Lys-NH_2 on (3,3'-diphenyl-1,1-binaphthyl)-20-crown-16 phases has been fully investigated.
5. The obtained results indicate that more than one amino moiety in tetrapeptide structure can simultaneously bind to multiple chiral selector molecules on sorbent surface, which as a concept has not been discussed in the chromatography community.

Practical application of the work:

The chromatographic behaviour of tetrapeptides on (3,3'-diphenyl-1,1-binaphthyl)-20-crown-16 stationary phases described in this work may contribute significantly to enantioseparations of various new, unknown short peptide type analytes. The obtained results are easily applicable to the development of analytical methods for chiral purity determination of peptide type drug candidates on stationary phases based on crown ethers. It is expected that the developed methods can be applied for the purification of peptides, which is important in scientific research and pharmaceutical industry

The unconventional approach used in this thesis (two chiral columns with selectors of opposite chirality for the analysis of one sample) can be used to determine the composition of complex stereoisomeric mixtures. In addition, this approach allows a relatively quick and cost efficient assessment of whether a stationary phase chiral selector is enantioselective towards a particular enantiomeric pair in circumstances where only one enantiomer of a compound is available.

The use of HPLC, HRMS and NMR techniques in binding studies discussed in this work can serve as guidelines for further systematic investigation of the chiral recognition mechanisms for different classes of chiral selectors and analytes. The investigated intermolecular interaction profile can be of great help in the design of new chiral stationary phases.

List of publications

1. **Upmanis, T**.; Kažoka, H.; Arsenyan, P. A study of tetrapeptide enantiomeric separation on crown ether based chiral stationary phases. *J. Chromatogr. A* **2020**, *1622*, 461152. *Q1*, IF_{2022} = 4.1
   T.Upmanis carried out 90% of the experimental work, developed the concept (80%) and wrote the article, prepared the experimental results according to the journal guidelines, as well as prepared the answers to the questions and remarks given by the reviewers.

2. **Upmanis, T**.; Kažoka, H. Application of Commercially Available Crown Ether Chiral Stationary Phases for Separation of Tetrapeptide Stereoisomers, *Acta Pharm. Hung*. **2021**, *91*, 324–325.
   T.Upmanis carried out 90% of the experimental work, developed the concept and wrote the article, prepared the experimental results according to the journal guidelines, as well as prepared the answers to the questions and remarks given by the reviewers.

3. **Upmanis, T**.; Kažoka, H. Influence of amino acid residue on chromatographic behaviour of μ–opioid receptor agonist tetrapeptide analogue on crown ether based chiral stationary phase. *J. Chromatogr. A* **2022**, *1673*, 463059. *Q1,* IF_{2022} = 4.1
   T.Upmanis carried out 90% of the experimental work, developed the concept and wrote the article, prepared the experimental results according to the journal guidelines, as well as prepared the answers to the questions and remarks given by the reviewers.

4. **Upmanis, T**.; Kažoka, H. Mechanistic insights in chiral recognition of μ-opioid receptor agonist tetrapeptide on crown ether chiral stationary phase. *J. Chromatogr. Open* **2021**, *1*, 100016.
   T.Upmanis carried out 90% of the experimental work, developed the concept and wrote the article, prepared the experimental results according to the journal guidelines, as well as prepared the answers to the questions and remarks given by the reviewers.

5. **Upmanis, T**.; Sevostjanovs, E.; Kažoka, H. Chiral recognition mechanism studies of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide on crown ether based chiral stationary phase. *Chirality* **2024**, *36(1)*, e23619. *Q3*, IF_{2022} = 2.0

   T.Upmanis carried out 80% of the experimental work, developed the concept and wrote the article, prepared the experimental results according to the journal guidelines, as well as prepared the answers to the questions and remarks given by the reviewers.

Attended conferences

1. **Upmanis T**.; Kažoka H.; Arsenyan P. Chiral resolution of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide stereoisomers on crown ether chiral stationary phases. *12th Balaton Symposium on High-Performance Separation Methods*, **2019**, Siofok, Hungary (poster presentation/ in book of abstracts).
2. **Upmanis T.**; Kažoka H.; Arsenyan P. HPLC study of tetrapeptide enantiomeric separation on crown ether based chiral stationary phase. *78th International Scientific Conference of the University of Latvia*, **2020**, Riga, Latvia (oral presentation/ in book of abstracts).
3. **Upmanis T.**; Kažoka H. Application of commercially available crown ether chiral stationary phases for separation of tetrapeptide stereoisomers. *International Conference on Advances in Pharmaceutical Drug Development, Quality Control and Regulatory Sciences* (*DDRS 2021)*, **2021**, Budapest, Hungary (poster presentation/ in book of abstracts).
4. **Upmanis T.**; Kažoka H. Approach of using the opposite chirality of crown ether stationary phases in chiral recognition of tetrapeptide enantiomers. *80th International Scientific Conference of the University of Latvia*, **2022**, Riga, Latvia (oral presentation/ in book of abstracts).
5. **Upmanis T.**; Kažoka H. Chiral Recognition Mechanism Studies of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide on crown ether based chiral stationary phases. *33rd*

International Symposium on Chromatography (ISC 2022), **2022**, Budapest, Hungary (poster presentation/ in book of abstracts).

6. **Upmanis T**. Chiral recognition mechanism studies of short peptide chromatographic separation on crown ether stationary phase. *3rd LIOS conference*, **2023**, Riga, Latvia (oral presentation).

1. RESEARCH BACKGROUND

1.1. Peptide therapeutics

The growing interest in peptide drug discovery field within the pharmaceutical industry has become evident over the past decades, reaching the market for a wide range of diseases, including diabetes, cancer, osteoporosis, multiple sclerosis, HIV and chronic pain [1]. With the rapid development of biological and medical sciences, the use and research of peptide therapeutics is continuously evolving [2], accounting for a significant proportion of the pharmaceutical market, with worldwide sales of more than $70 billion in 2019 [1]. Currently there are several hundred peptide drugs undergoing clinical developments, with over 80 already approved for clinical use worldwide [3].

Short peptides represent a unique class of pharmaceutical agents composed of a series of well-ordered amino acids, which in terms of their molecular size are between small molecular compounds and proteins [4], however, both in terms of their chemical properties and their mechanisms of action, peptides differ from the two mentioned above [5]. As intrinsic signalling molecules for a multitude of physiological functions, peptide drugs (either in their native or modified forms) present an opportunity for therapeutic intervention that closely mimics the natural pathways [6]. Other notable factors that have contributed to this trend are the high specificity and low toxicity profile (deriving from their extremely tight binding to their targets), allowing this class of compounds to be an excellent complement or even a preferable alternative to small molecule drugs [7].

The essential biological functions and physical properties of peptides depend on their stereochemistry which is inherently controlled by the configuration of the amino acid components [8,9]. Amino acids exist in D and L forms (apart from glycine) and as a result the peptide can exist as several stereoisomers with different biological properties [10,11]. During synthesis, storage or metabolic processes stereoisomers may experience racemization (or epimerization depending on the position of the stereogenic centres involved) [12,13], resulting in complex enantiomeric / epimeric compositions, therefore, enantiomeric purity control of peptide analytes is an important challenge in the biological and medical sciences and is necessary for the pharmaceutical industry therefore, development of accurate and rapid analytical techniques to determine chiral purity in peptides is of paramount importance for pharmaceutical industry as well as chemical and biological research.

1.2. Application of HPLC for separation of chiral peptide analytes

The most common strategy for optical purity testing of peptide analytes has been to hydrolyse the peptide into individual amino acid constituents [14]. To avoid any artificial enhancement of the enantiomeric composition in the initial peptide sample, the hydrolysis is performed in deuterated solvents so that any racemization occurring during this step of sample preparation is accompanied by deuterium exchange (Fig. 1.1.) at the α-C position [15].

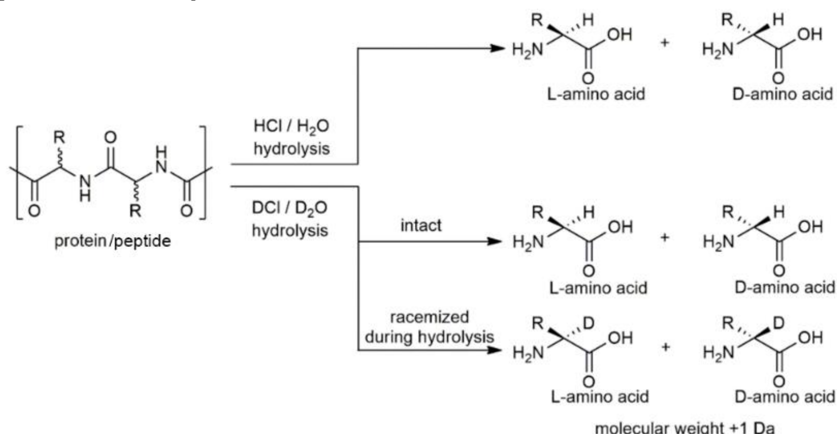


Fig. 1.1. **Schematic representation of DCl / D_2O peptide hydrolysis** [16].

The resulting hydrolysed amino acids are then subjugated to chiral derivatization (e.g., by *Marfey's* reagent [17]) and analysed indirectly by achiral liquid chromatography-mass spectrometry. Amino acid molecules that racemize during hydrolysis are labelled with deuterium (resulting in a + 1 mass difference) and can be subsequently excluded by MS techniques [17]. The downside of this approach includes the necessity for costly deuterated solvents and mass spectrometry equipment. Furthermore, sample derivatization step adds extra complexity, requires time, and decrease the robustness of the method by introducing additional potential sources of error [18].

Ideally, these shortcomings could be avoided by directly analysing chiral peptides in their intact form. For direct separation methods, various chiral stationary phases based on different types of chiral selectors have been developed for separation of amino acid enantiomers using LC. Unfortunately, due to the complexity of the systems (number of stereoisomers increase exponentially 2^n for every newly introduced chiral amino acid moiety (n) in peptide structure) success of direct LC chiral separations of intact short peptides have been rather limited, which is well reflected in the relatively small number existing publications. Among them, enantioseparation of glycyl dipeptides has been achieved by ligand exchange chromatography [19]. Some unprotected nonproteinogenic amino acids and peptide like analyte enantioseparation has been performed on polysaccharide derivatives [20,21]. Unprotected dipeptide and tripeptide chiral resolution has been previously reported on CSPs based on macrocyclic glycopeptides [22–24] and cyclodextrins [25,26]. Two types of chiral stationary phases, based on cinchona alkaloids [27–29] and chiral crown ethers [30] have been developed specifically for the separation of amino acids and various compounds containing primary amino groups, among them peptides. The later will be further discussed in more detail.

1.3. Chiral stationary phases based on crown ethers

The first type of chiral crown ethers was developed by Donald J. Cram and co-workers (for that being awarded the Nobel Prize in chemistry in 1987) by introducing optically active 1,1'-bi-2-naphthyl units into crown ether system [31]. Further research pointed towards the unique chiral recognition abilities of (*R*)- or (*S*)-(3,3'-diphenyl-1,1-binaphthyl)-20-crown-16 (Fig. 1.2., **A**) towards various *α*-amino acid enantiomers and their methyl esters [32], which nearly a decade later were commercialized into CROWNPAK **CR** (+) or **(-)** CSPs (by *Daicel Chemical Industries*) by dynamically coating the chiral selector onto silica gel [33].

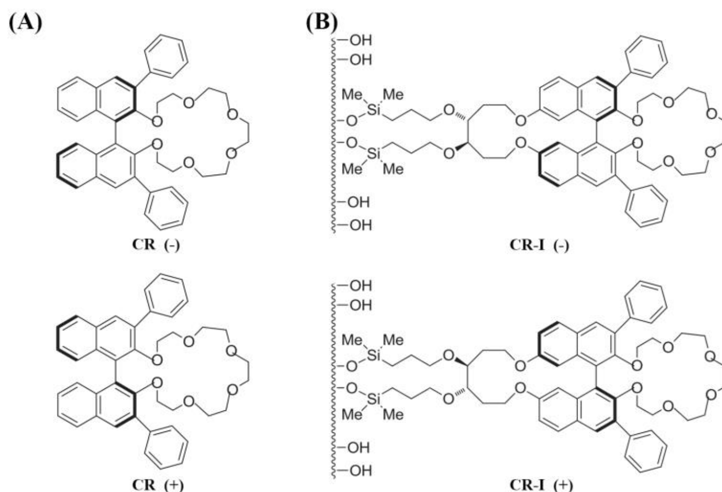


Fig. 1.2. **Chemical structures of:** (*R*)- and (*S*)-(3,3'-diphenyl-1,1-binaphthyl)-20-crown-16 chiral selectors found in CRONWPAK **CR (-)** and (+) CSPs - (**A**); immobilized chiral selectors found in CROWNPAK **CR-I** (-) and (+) CSPs – **(B).**

These chiral stationary phases were found to be very useful for the resolution of primary *α*-amino acids [33,34], aryl *α*-amino ketones [35], some *β*-amino acids [36–38], *γ*-amino acids [39] as well as various other chiral drugs, containing a primary amino group [40,41], including dipeptides [42,43]. However, these CSPs came with a major drawback and strict rules had to be followed regarding the mobile phase solvents. Due to the dynamic coating of chiral selector on to sorbent surface, exceeding 15% MeOH content in mobile phase was not recommended as it would result in *column bleeding* and deterioration of CSP performance.

This issue was solved by covalently binding the chiral selector to the silica support resulting in CROWNPAK **CR-I** (+) or **(-)** CSPs (Fig. 1.2., **B**) [44]. This immobilized phase was found to be very stable, showing the compatibility with broader variety of mobile phase solvents (ACN, MeOH, EtOH, 2-PrOH, THF), containing any percentage of organic modifier and presented more opportunities in finding the optimal chromatographic conditions for the necessary separation. **CR-I** (+) and **(-)** CSPs have been able to separate the enantiomers of all proteinogenic amino acids (with the exception of proline) [45] and have been successfully used for the resolution of even wider range of various primary *α*- [46,47], *β*- [38] and *γ*-amino compounds [39], and overall have presented better separation performance than the previously made **CR (+)** and **(-)** CSPs [39].

The second type of chiral crown ether, which is used nowadays, is based on (+) or (-)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (**18-C-6-TA**; Fig. 1.3., **A**) and was developed by Jean-Marie Lehn's group (also awarded Nobel Prize in chemistry in 1987) by incorporating two tartaric acid units into the crown ether ring system [48]. This crown ether has been widely used as a chiral resolution agent by nuclear magnetic resonance spectroscopy [49], capillary electrophoresis [50] and mass spectrometry [51].

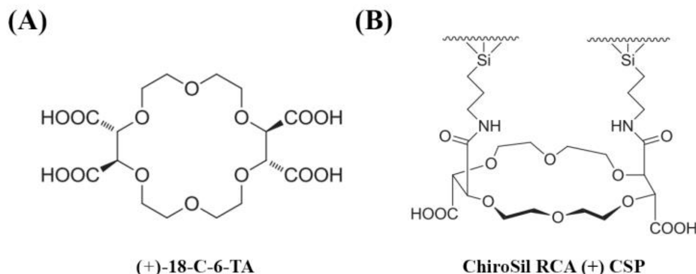


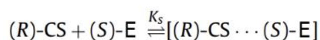
Fig. 1.3. **Chemical structures of:** (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid – **(A)**; immobilized **(+)-18-C-6-TA** chiral selector found in ChiroSil **RCA** (+) CSP – **(B)**.

Since **18-C-6-TA** is water soluble, only it's immobilized version, commercially known as ChiroSil **RCA** (+) (Fig. 1.3., **B;** or **SCA (-)**, depending on the chirality of the selector; developed by *RStech Corporation*) is used for chromatography. CSPs, based on **18-C-6-TA** unit have been successfully used for the enantioseparations of primary natural and unnatural α- and β-amino acids [52–55], secondary amines [56] and even short peptides [57].

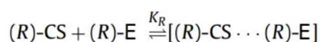
It is clear that CSPs based on crown ethers are one of the most promising candidates for direct short peptide chiral separations. However, to better understand the application of crown ether CSPs for more complex analyte separations, it is first necessary to look at what is known about chiral recognition mechanisms of both types of CSPs.

1.4. Chiral recognition

Usually, CSPs are designed to create a steric environment, where one of the isomers binds more favourably than the other. Depending on the chiral selector structure, a variety of intermolecular interactions (e.g., coulomb, hydrogen bonding, steric hindrance, π-π, dipole-dipole, ion-dipole, van der Waals forces) via their attractive or repulsive properties are known to directly affect the bonding strength between the chiral analytes and the chiral selector. The reactions that reversibly form diastereomeric pairs on the surface of the stationary phase are illustrated in Figure 1.4.



$$(R)\text{-CS} + (S)\text{-E} \overset{K_S}{\rightleftharpoons} [(R)\text{-CS} \cdots (S)\text{-E}]$$



$$(R)\text{-CS} + (R)\text{-E} \overset{K_R}{\rightleftharpoons} [(R)\text{-CS} \cdots (R)\text{-E}]$$

Fig. 1.4. **Schematic representation of diastereomer formation** [58]**.**

In the given example chiral selector (CS) with a fixed configuration (*R*) interacts with the *S*-enantiomer of an analyte (*S*)-E to form a transient diastereomeric complex [(*R*)-CS···(*S*)-E] with equilibrium constant K_S (Fig. 1.4. – *top*). At the same time, chiral selector also interacts with the *R*-enantiomer to form [(*R*)-CS···(*R*)-E] complex with equilibrium constant K_R (Fig. 1.4. – *bottom*). The differences between the equilibrium constants of two diastereomeric complexes reflects in isomer retention and

are the fundamental basis for stereoselective separations in any chromatographic system. The weaker bound isomer elutes first, while the stronger bound isomer is retained longer, thus allowing us to separate the mixture. An important variable in chiral recognition is the interaction medium - mobile phase (MP), where, based on the solvent nature, mobile phase modifiers not only compete for chiral binding sites with the chiral solutes, but also may alter the steric environment of the chiral selector, thus altering chiral recognition mechanism.

1.4.1. Chiral recognition on (3,3'-diphenyl-1,1-binaphthyl)-20-crown-16 CSPs

Under reversed phase (RP) conditions (water containing mobile phase), chiral separations are achieved primarily by retaining the analyte through inclusion complexation (Fig. 1.5., **A**), driven via triple $^{+}$N–H···O hydrogen bond formation between the ammonium ion in the protonated analyte and oxygens of the crown ether selector followed by enantioselective hydrophobic interactions between the solute and the binaphthyl and two phenyl groups of the stationary phase which is schematically represented by *Newman* projections in Figures 1.5., **B** and 1.5., **C**.

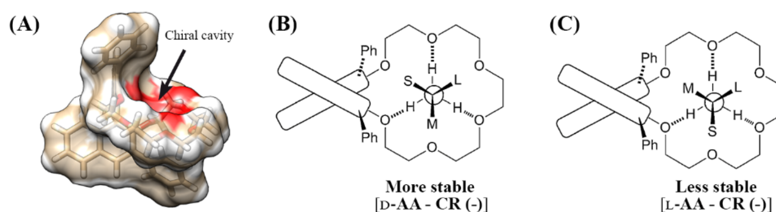


Fig. 1.5. **The proposed chiral recognition mode CR (-) selector:** Electrostatic potential surface of **CR** (-) chiral selector obtained from crystal structure [59], visualized by *Chimera 1.16* software – (**A**); The proposed chiral recognition mode showing the more – (**B**); and less stable complexes – (**C**) formed between the primary ammonium ion (R-NH_3^+) containing three different substituents of large (L), medium (M) and small (S) functional groups at the chiral centre and CR (-) selector [32].

In this instance, the larger functional group (L) at the chiral centre of analytes occupies the space furthest from the sterically most demanding upward facing 3-phenylnaphthyl group. The other two groups at the chiral centre are positioned automatically, based on the configuration of the amino acid. The steric hindrance between chiral selectors upward facing 3-phenylnaphthyl unit and the medium sized substituent (M) of L-amino acid is greater than for its corresponding enantiomer, where the same space is occupied by the smaller (usually hydrogen) substituent (S), limiting its ability to fully access the chiral cavity of the selector (Fig. 1.5., **A**). As a result, formation of the hydrogen bond formation is sterically hindered for the complex between the L-enantiomer and the **CR (-)** selector (creating the less stable complex; Fig. 1.5., **C**) and can be eluted prior to its corresponding D-enantiomer [30]. This specific enantiomer elution order (L < D) on **CR (-)** CSPs is valid for all chiral amino acids and can be inverted by using **CR** (+) CSP, which contains chiral selector of opposite chirality.

Different enantioselectivity in mobile phases with high organic content have been discovered relatively recently [45], since the older coated versions of the CSP did not allow such chromatographic conditions. Contrary to RP conditions, in addition to sterically driven inclusion complexation through H-bond formation, additional enantioselective polar interactions may occur, however exact characteristics of the interaction profile is not yet clear.

1.4.2. Chiral recognition on (18-crown-6)-2,3,11,12-tetracarboxylic acid CSPs

Two possible separation mechanisms have also previously been suggested for (+) and (-)-**18-C-6-TA** CSPs [52,60]. In lower organic modifier content mobile phases in addition to the complexation of primary ammonium ion inside the cavity of the 18-crown-6 ring (Fig. 1.6., **A**) via hydrogen bonding, steric hindrance between the two carboxylic acid groups of the **18-C-6-TA** selector and the sterically bulky substituents in α-amino compounds controls the analyte's ability to approach the active site and form H-bonds. As a result, one of the enantiomers would create more stable diastereomeric complex, than the other and could be chromatographically separated. Similarly, to **CR** (+) and **(-)** chiral selectors, a distinct enantiomer elution order is also characteristic for **18-C-6-TA** CSPs. Amino acid D-enantiomers form stronger complexes with the (+)-**18-C-6-TA** chiral selector, while L-enantiomers form stronger complexes with (-)-**18-C-6-TA**.

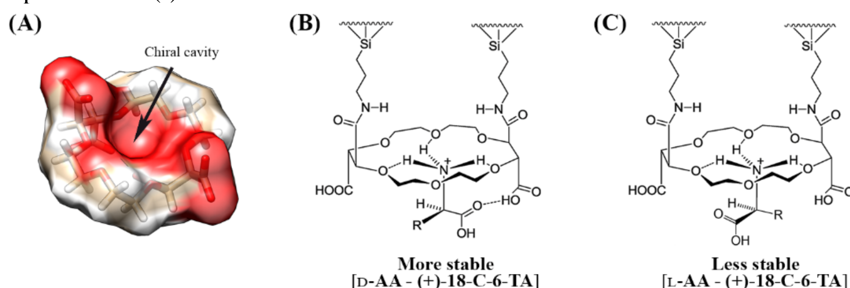


Fig. 1.6. **The proposed chiral recognition mode of (+)-18-C-6-TA selector:** Electrostatic potential surface of **(+)-18-C-6-TA** selector, obtained from crystal structure [61], visualized by *Chimera 1.16* software – **(A)**; The proposed chiral recognition mode showing the more stable **(B)** and the less stable **(C)** complexes formed between generalized α-amino acids and CSP, based on (+)-**18-C-6-TA** selector [62].

In organic modifier rich mobile phases, the side two carboxylic acid groups in chiral selector may act as additional hydrogen bonding donor or acceptor groups. As shown in example in Fig. 1.6, upon binding with (+)-**18-C-6-TA**, carboxyl group of α-amino acid D-enantiomer forms additional H-bond with one of the carboxylic acid units in the selector, creating more stable diastereomeric complex (Fig. 1.6., **B**). The carboxyl group in L-enantiomer structure is directed away from the carboxylic acid groups of the chiral selector and cannot participate in additional H-bonding, thus forming less stable complex (Fig. 1.6., **C**).

Unfortunately, the existing studies are mostly theoretic, have insufficient experimental support and covers only simplified chiral recognition models using amino acids. Peptides often contain multiple amino moieties, any of which can theoretically bind to the crown ether selector, so from a mechanistical point of view, the contribution of each interacting moiety must be studied. The most popular approaches on studying chiral recognition are briefly covered in the following paragraph.

1.5. Approaches on studying chiral recognition

Many characterization methods have been employed to rationalize chiral recognition. Among numerous spectroscopic techniques [63–65], nuclear magnetic resonance has been extensively used to study chiral recognition of various classes of chiral selectors applied in liquid chromatography [66–68] and capillary electrophoresis [69,70]. As shown in an example in Fig. 1.7. ^{1}H-NMR has been used to study the chiral complexation between racemic phenylglycine and (+)-**18-C-6-TA** selector.

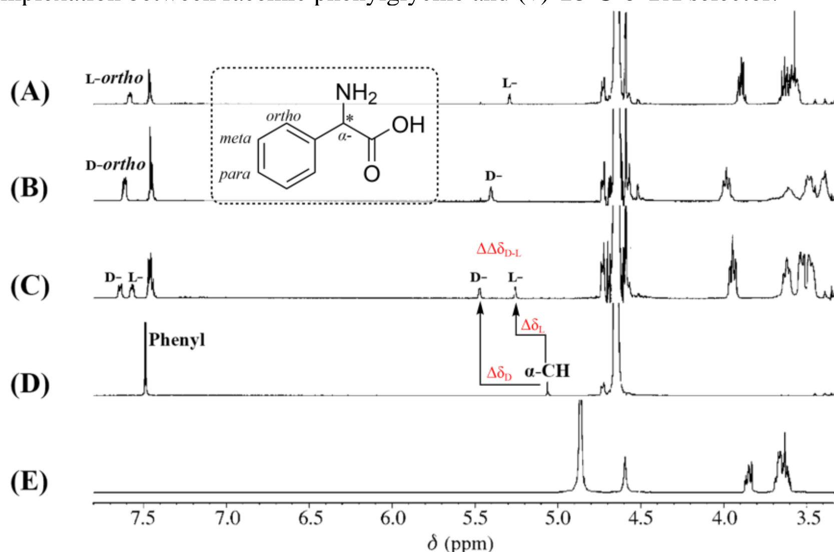


Fig. 1.7. **^{1}H-NMR spectra of phenylglycine and phenylglycine/(+)-18-C-6-TA complex with equimolar mixtures (2 mM each in MeOH-*d₄*):** L-phenylglycine with (+)-**18-C-6-TA** - **(A)**; D-phenylglycine with (+)-**18-C-6-TA** - **(B)**; racemic phenylglycine with (+)-**18-C-6-TA** - **(C)**, racemic phenylglycine - **(D)**, free (+)-**18-C-6-TA** - **(E)** [68].

Upon binding, the proximity of electric and magnetic fields between the neighboring molecules affects the resonance of the involved nuclei, which can be observed as a chemical shift (δ) changes (Δδ) in the NMR spectrum. In the given example (Fig. 1.7., **C**), when an equimolecular amount of (+)-**18-C-6-TA** was added to racemic phenylglycine, both *α*-proton and *ortho* phenyl protons in phenylglycine were split into two sets of signals, indicating a different chemical structures of the formed diastereomeric complexes between L-phenylglycine and (+)-**18-C-6-TA** and D-phenylglycine - (+)-**18-C-6-TA**. Furthermore, the most significant chemical shift changes (Δδ) in the NMR spectra often indicate on which atoms are directly involved in intermolecular binding.

Mass spectrometry can also be useful for the formed host-guest complex detection [51,71], as well as proving stoichiometry of the complexation between molecules [72]. This way, by mixing host and guest compounds in a solution and directly injecting the solution into instrument, complexation between L-phenylglycine and (-)-**18-C-6-TA** selector has been previously studied by electrospray ionization (ESI) time-of-flight (TOF) mass spectrometry (Fig. 1.8.).

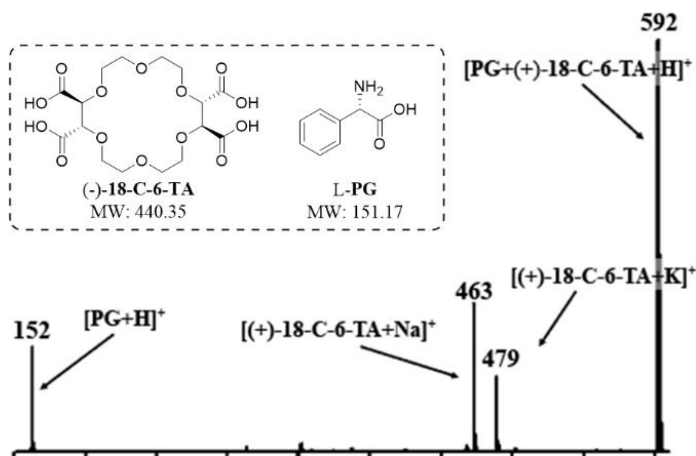


Fig. 1.8. **Partial ESI-TOF mass spectra of an equimolar solution (10^{-5} M in MeOH) of L-phenylglycine (PG) with (-)-18-C-6-TA** [51]**.**

As shown in the mass spectrum, the formed complex transfers from the solution phase into gas phase of the mass spectrometer and can be observed at *m/z* 592. Sodium (*m/z* 463) and potassium (*m/z* 479) adducts of (-)-**18-C-6-TA** can also be found in visible amounts, as well as unbound L-phenylglycine (*m/z* 152). The MS experiments confirms the binding between both molecules, however it does not provide more specific insights about chiral recognition and can be rather utilized as a complimentary tool to other techniques.

Another useful tool for gaining insight into different host-guest binding modes is X-ray diffraction by providing comprehensive information on the structural features of the formed complex structure in the solid state [73,74]. X-ray diffraction has been used to rationalize chiral recognition of various amino acids on chiral crown ethers. This way, by co crystalizing D-phenylglycine methyl ester perchlorate with **CR (-)** selector from methanol, binding mechanism between both molecules has studied [59]. The analysis of the crystal structure revealed triple $^{+}$N–H···O hydrogen bond forming between the protonated D-phenylglycine methyl ester ammonium ion and crown ether oxygens as well as C-H···π interaction between the aromatic units of host and guest (Fig. 1.9., **A**). In another work [74], different binding mechanisms have been demonstrated by co-crystallizing phenylglycine enantiomers with (+)-**18-C-6-TA** chiral selector from 10 mM $HClO_4$ (Fig. 1.9., **B**). Unfortunately, obtaining good quality crystals, especially co-crystals of specific host-guest complexes, can often be problematic.

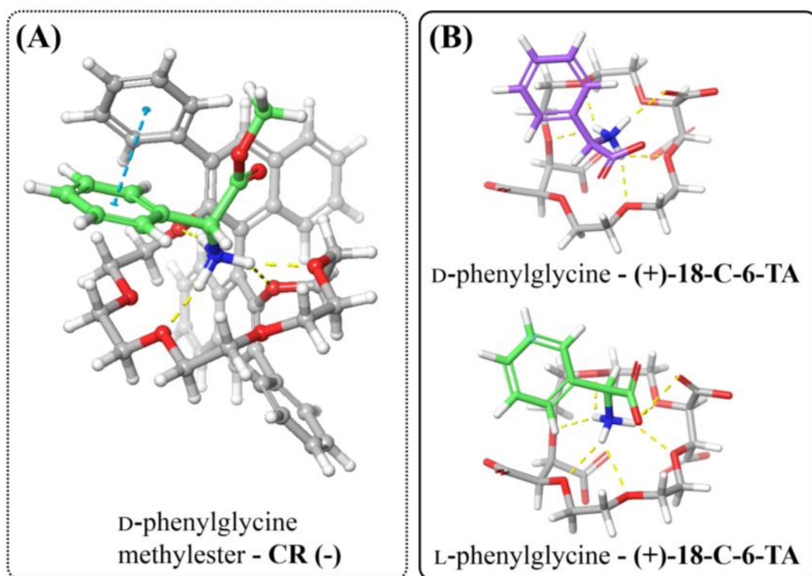


Fig. 1.9. **Stereoview of the intermolecular interactions in complex crystals obtained by X-ray diffraction:** D-phenylglycine methylester and **CR (-)** selector [59] – **(A)**; between phenylglycine enantiomers and **(+)-18-C-6-TCA** selector [74] – **(B)**. Interactions were visualized with *Maestro 13.4* software.

Lately, computational methods have started gaining more acceptance in the community and various molecular modelling methods, together with other experimental techniques have been employed to characterize different selector-selectand complexes [75,76]. However, most of the computational approaches have been designed for studying proteins and adjusting these methods for chromatography can be challenging, especially attaining credible structures of chiral selectors.

1.6. Objects of investigation

To study the chiral recognition ability of crown ethers towards isomers of short peptides, tetrapeptide Tyr-Arg-Phe-Lys-NH_2 (**1**; Fig. 1.10.), whose LDLL-stereoisomer is known as a selective μ-opioid receptor agonist *DALDA* [77] was chosen as a model compound. The structure of tetrapeptide **1** contains 4 chiral centres, resulting in 16 possible stereoisomers, which were synthesised at the initial phase of the work.

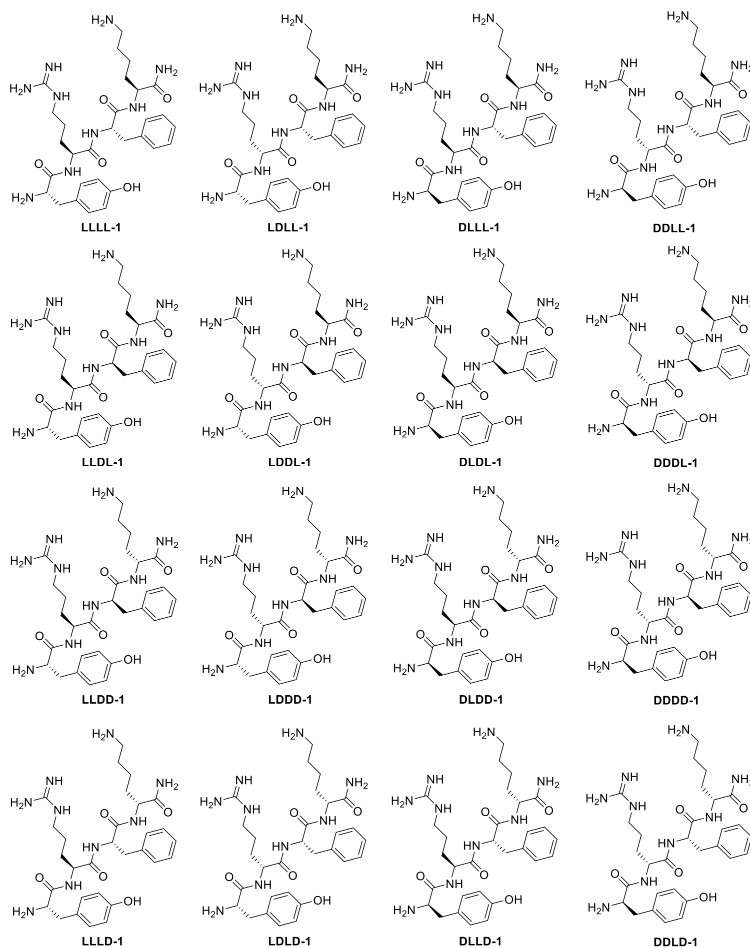


Fig. 1.10. **Structures of Tyr-Arg-Phe-Lys-NH_2 stereoisomers.**

This tetrapeptide was chosen because its sequence consists of four different amino acids and therefore contains several different hydrogen bond donors - a free *α*-amino group on the N-terminal tyrosine, an ε-amino group on the C-terminal lysine and a guanidine fragment in arginine - all of which can theoretically be involved in interactions with the chiral selector, so that from a mechanistic point of view the contribution of each of these groups to complexation is unknown. In addition, this tetrapeptide also contains various aromatic substituents which, in addition to providing UV absorption, may also be involved in additional interactions with the chiral selector.

2. RESULTS AND DISCUSSION

2.1. The use of crown ether CSPs for chromatographic separation of Tyr-Arg-Phe-Lys-NH_2 enantiomers and stereoisomers[1]

To clarify the prospects of using crown ether-based CSPs for chiral resolution in short peptides, the chromatographic behaviour of all sixteen Tyr-Arg-Phe-Lys-NH_2 (**1**; Fig. 1.10) stereoisomers was studied on two commercially available crown ether CSPs: CROWNPAK **CR-I (+)** (Fig. 1.2., **B**) and ChiroSil **RCA (+)** (Fig. 1.3., **B**). A more detailed description of the experimental work can be found in Appendix I, Sections 2.2. - 2.5.

To ensure full protonation of the amino groups in tetrapeptide **1**, the addition of $HClO_4$ (50 mM of total MP volume; corresponds to pH 1.5 in aqueous solutions) was found to provide the optimal conditions for resolution of tetrapeptide **1** enantiomers on both **CR-I (+)** and **RCA (+)** CSPs.

Acetonitrile (ACN) was chosen as the MP organic modifier for the initial experiments and the retention profiles of Tyr-Arg-Phe-Lys-NH_2 stereoisomers were studied on both crown ether CSPs under isocratic conditions by varying the ACN content in ACN/water MP from 15 to 95% on **CR-I (+)** and from 10 to 80% on **RCA (+)** CSPs. Plots of the retention times (t_R) of eight pairs of tetrapeptide **1** enantiomers against the ACN content are represented in Figure 2.1. All tetrapeptide **1** stereoisomers showed U-shaped curves with minima within 50–75% ACN range for **CR-I (+)** and 50% ACN for **RCA (+)** CSPs, where increased stereoisomer retention was observed at lower (<25% for **CR-I (+)** and <35% for **RCA (+)** CSPs) and at higher (90% for **CR-I (+)** and >75% for **RCA (+)** CSPs) acetonitrile content in the MP. Similar U-shaped retention dependence against the eluent composition has been previously reported for methyl-substituted anilines [78] and various proteinogenic amino acids [45] and is commonly explained by different binding mechanisms occurring for crown ether based CSPs: reversed-phase mechanism in aqueous and HILIC-like mechanism in organic solvent rich mobile phases.

There are no previous studies characterising the elution order in compounds consisting of multiple binding sites, e.g., multiple amino groups in short peptides. It is known (Section 1.4.) that for amino acids typical elution order on **CR-I (+)** CSP is D < L, whereas on **RCA (+)** it is L < D. Tetrapeptide **1** stereoisomers possessing LXXX-configuration (L-Tyr at the N-terminus; Fig. 1.10., **A**) retained more strongly than their D-antipodes (DXXX; Fig. 2.1., **A**) on **CR-I (+)** column, while on **RCA (+)** the elution order was opposite (DXXX > LXXX; Fig. 2.1., **B**). The observed elution order suggests that the α-amino acid at the N-terminus – Tyr could be involved in complexation between the crown ether ring of the CSP and the primary ammonium ions (R-NH_3^+) of the analyte. In order to confirm this hypothesis, further in-depth studies have been carried out.

As shown from the retention profiles illustrated (Fig. 2.1., **A**), upon the increase of ACN in mobile phase (> 90% ACN), rapid increase in retention can be observed for tetrapeptide enantiomers possessing LXXX-configuration. Such chromatographic behaviour not only indicates on different enantiomer binding mechanisms in mobile phases with high organic modifier content, but also serves as a base for the improved chiral resolution observed in these conditions (Fig. 2.1., **C**). Generally, α > 1.2 is considered sufficient for chromatographic separation between two compounds

[1] **Upmanis, T**.; Kažoka, H.; Arsenyan, P. A study of tetrapeptide enantiomeric separation on crown ether based chiral stationary phases. *J. Chromatogr. A* **2020**, *1622*, 461152 (Appendix I).

(enantiomers in this case). Even though such criteria were not met for the separation of LDLL/DLDD-enantiomers ($\alpha < 1.05$; Fig. 2.1., **C**; DLDD/LDLL - *represented in black*) with MP containing <85% ACN, increasing the ACN content in MP above 90% allowed the successful separation ($\alpha > 1.2$) of all eight tetrapeptide **1** enantiomeric pairs on **CR-I** (+) CSP.

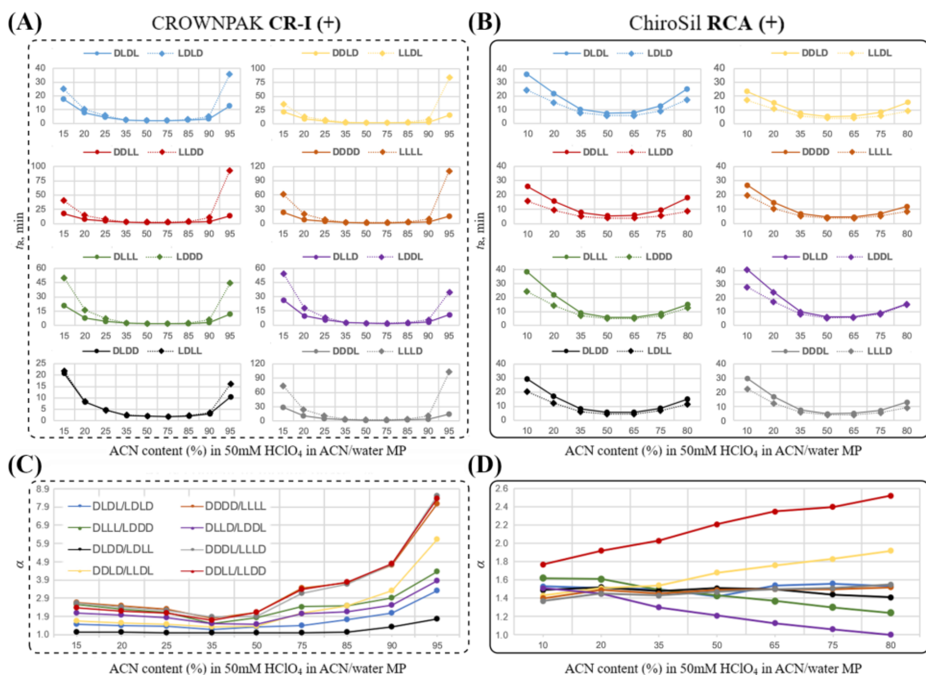


Fig. 2.1. **Effect of ACN content in mobile phase on Tyr-Arg-Phe-Lys-NH$_2$ stereoisomer retention times (t_R) and enantiomer separation (α):** on CROWNPAK **CR-I** (+) – (**A**) and (**C**); on ChiroSil **RCA** (+) – (**B**) and (**D**).

Interestingly, all eight enantiomeric pairs were baseline separated on **RCA** (+) column with low ACN content mobile phases (Fig. 2.1., **B** and **D**). Separation factor α for DDLX/LLDX-enantiomers increased, when ACN content in mobile phase was increased. In contrast, α value for DLLX/LDDX-enantiomers decreased with the increase in ACN content, whereas no significant changes in selectivity were observed for the remaining 4 DDDX/LLLX and DLDX/LDLX-enantiomeric pairs in the studied ACN range. The obtained results indicate that multiple chiral recognition mechanisms are possible within a single molecule and steric arrangement of the substituents in tetrapeptide **1** structure is crucial for enantiomer separation, thus both elution modes are worth investigating.

After attempting simultaneous chromatographic analysis of all tetrapeptide **1** stereoisomers (Fig. 2.2. - *represented in black*) on **CR-I** (+) and **RCA** (+) CSPs, it was established, that **CR-I** (+) CSP shows more potential for the separation of all Tyr-Arg-Phe-Lys-NH$_2$ stereoisomers. As a result, seven (mostly possessing LXXX-configuration) tetrapeptide **1** stereoisomers were separated with MP containing lower acetonitrile (15%) content (Fig. 2.2., **A**). Selectivity towards tetrapeptide **1** stereoisomer separation decreased with the increase of mobile phase organic modifier increase and only LDDD-stereoisomer was fully separated from the mixture in ACN rich (95%) mobile phase (Fig. 2.2., **B**). Much weaker stereoselectivity towards tetrapeptide **1** stereoisomers was observed on **RCA** (+) CSP (Fig. 2.2., **C** and **D**) independent of

ACN concentration in the mobile phase. Therefore, the following discussion in the thesis will only focus on studies done regarding **CR-I** phases.

Fig. 2.2. **Overlay of chromatograms of all 16 Tyr-Arg-Phe-Lys-NH$_2$ stereoisomer (*in black*) and enantiomer (*in colour*) separations on CR-I (+) CSP - (A) and (B); RCA (+) CSP – (C) and (D).** Columns: CROWNPAK **CR-I** (+) (3.0 ×150 mm, 5μm); ChiroSil **RCA** (+) (4.6 ×150 mm, 5μm); Flow rate: F = 0.4 mL/min on **CR-I** (+); F = 1 mL/min on **RCA** (+); Injection volume: 10 μL; UV detection: λ = 220 nm.

2.2. Employing the opposite chirality of the crown ether chiral stationary phase for the separation of tetrapeptide 1 stereoisomers[2]

To address the weak resolution observed for tetrapeptide **1** stereoisomers possessing DXXX-configuration on **CR-I** (+) CSP (Fig. 2.2., **A**), a CSP, based on *R*-(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 chiral selector (**CR-I (-)**; Fig 1.2., **B**) was introduced. Knowing that enantiomer elution order on **CR-I** CSPs can be inverted by performing the chromatographic analysis on stationary phase containing selector of opposite chirality, an unconventional approach was suggested in this work to increase the number of separated of tetrapeptide **1** stereoisomers. As shown in an example illustrated in Figure 2.3., a mixture containing all eight tetrapeptide **1** DXXX-stereoisomers could be separated on **CR-I (-)** CSP, whereas, under identical chromatographic conditions on **CR-I** (+) phase, weak separation was observed.

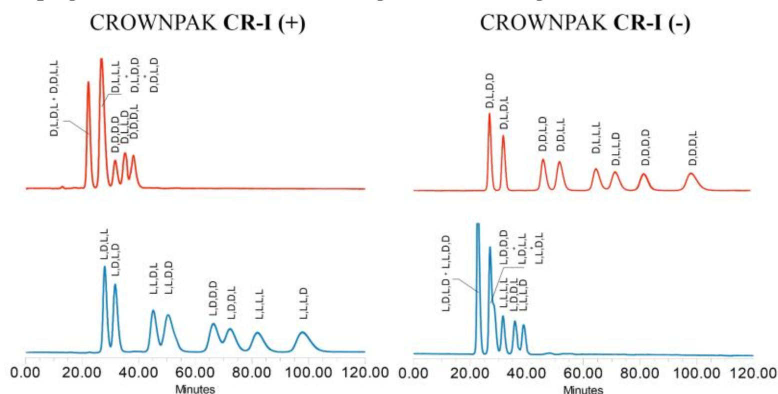


Fig. 2.3. **Separation chromatograms of mixed stereoisomer standard solutions:** of eight DXXX (***in red***) and LXXX (***in blue***) Tyr-Arg-Phe-Lys-NH_2 stereoisomers on CROWNPAK **CR-I** (+) and CROWNPAK **CR-I (-)** columns. Mobile phase: 50 mM $HClO_4$ in ACN/water 15/85 (v/v).

This way, by performing analysis of the same sample on both **CR-I** columns, in summary, 12 out of 16 tetrapeptide **1** stereoisomers could be separated and identified. The discussed approach has been reported here for the first time and should be considered in *real life* sample analysis as the complimentary use of opposite chirality selectors in **CR-I** CSPs may provide broader insight of the chiral impurity (stereoisomer) composition in complex isomeric mixtures (e.g., peptides).

[2] **Upmanis, T**.; Kažoka, H. Application of Commercially Available Crown Ether Chiral Stationary Phases for Separation of Tetrapeptide Stereoisomers, *Acta Pharm Hung*. **2021**, *91*, 324–325 (Appendix II).

2.3. Influence of amino acid residue on chromatographic behaviour of tetrapeptide 1 analogues on CR-I CSPs[3]

It is well known, that even slight changes in chiral analyte structure can completely change its chromatographic behaviour. In order to extend our knowledge in peptide chiral chromatographic analysis on **CR-I** CSPs, ten tetrapeptides **2** – **11** (Fig. 2.4.; LLLL-isomers) were synthesized.

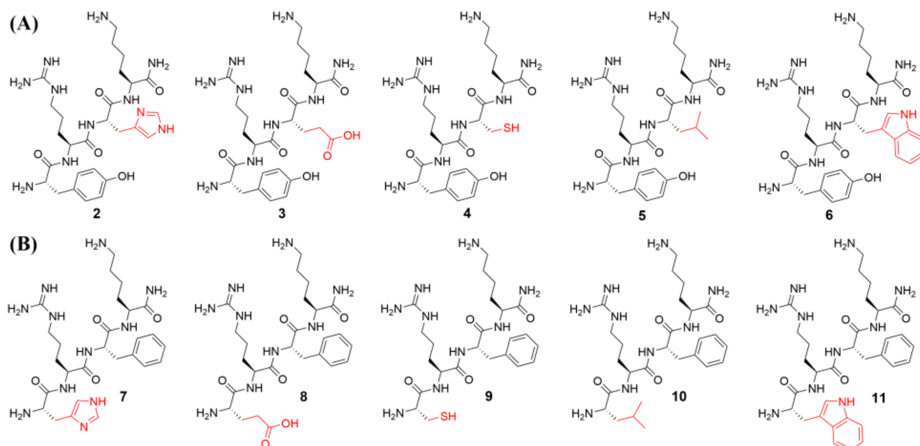


Fig. 2.4. **Structures of tetrapeptide 1 modified analogues:** tetrapeptides **2** – **6** modified at Phe position – (**A**); tetrapeptides **7** – **11** modified at N-terminus – (**B**).

To cover a wider range of different amino acid classes, histidine (His – polar basic); glutamic acid (Glu – polar acidic); cysteine (Cys – polar neutral; S-containing); leucine (Leu - nonpolar; aliphatic) and tryptophan (Trp – nonpolar; aromatic) were introduced at the Phe position (Fig. 2.4., **A**) or at the N-terminus (Fig. 2.4., **B**) of tetrapeptide **1** structure. The effects of the amino acid residue, as well as the influence of position of the amino acid residue in tetrapeptide sequence, on tetrapeptide **2** – **11** chromatographic behaviour on **CR-I** (+) and (**-**) phases is further discussed (additional information on experimental work can be found in Appendix III, Sections 2.2. – 2.4.).

2.3.1. Influence of the amino acid residue on tetrapeptide retention

First, to verify that newly synthesized tetrapeptide **2** - **11** retention follows the trends observed previously on tetrapeptide **1**, their chromatographic behaviour on **CR-I** (+) and (**-**) CSPs with 50 mM $HClO_4$ in ACN/water mobile phases was investigated. Subsequently, studies were also carried out with MeOH-containing MPs. Even though a common belief is that MeOH, as a protic solvent and H-bond donor, may impair binding between the chiral solute and selector on the CSP, which would reflect as a decrease of analyte retention, the opposite chromatographic behaviour was observed.

All ten studied tetrapeptides **2** - **11**, similarly to tetrapeptide **1** showed U-shaped curves with minima within the range of 50–75% ACN (60–80% MeOH) with an increased retention at low (<25% ACN or <40% MeOH) and high (>90% ACN or

[3] **Upmanis, T.**; Kažoka, H. Influence of amino acid residue on chromatographic behaviour of μ–opioid receptor agonist tetrapeptide analogue on crown ether based chiral stationary phase. *J. Chromatogr. A* **2022**, *1673*, 463059 (Appendix III)

>80% MeOH) mobile phase organic modifier contents indicating on possible different binding mechanisms.

To further rationalize the relationship between analyte retention (k) and the nature of amino acid residue on **CR-I** CSPs, log k values experimentally obtained at 15% ACN (40% MeOH – representing increased retention under RP conditions; X-axis) content were plotted against the log k values obtained at 95% ACN (90% MeOH – representing increased retention under high mobile phase organic modifier conditions; Y-axis) content and are summarized in Figure 2.5. The area beneath X = Y trend line represents hydrophobic interactions as significant contributor to the analyte retention, whereas hydrophilic (polar) interactions are considered dominant in the area above the trend line.

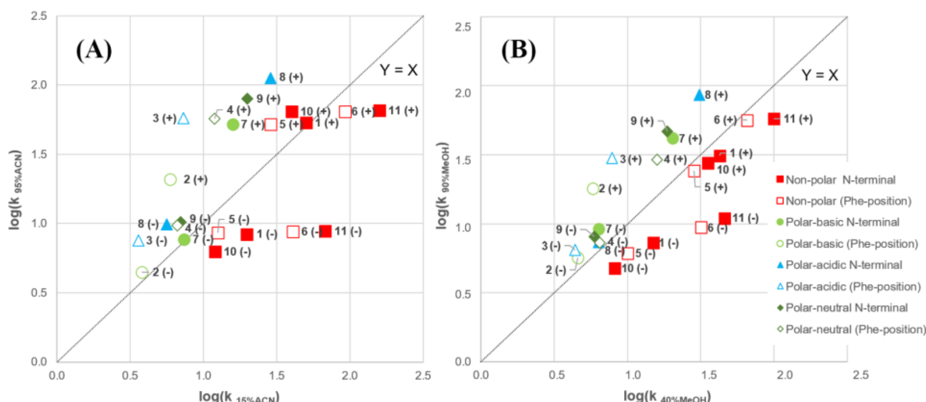


Fig. 2.5. **The effect of introduced amino acid residue on tetrapeptide 1 – 11 retention depending on the mobile phase composition:** plots of log ($k_{15\%ACN}$) *vs.* log ($k_{95\%ACN}$) – for 50 mM $HClO_4$ in ACN/water containing MPs – **(A)**; log ($k_{40\%MeOH}$) *vs.* log ($k_{90\%MeOH}$) – for 50 mM $HClO_4$ in MeOH/water containing MPs – **(B)**; data points marked with (+) were obtained on **CR-I** (+) CSP; data points marked with (-) were obtained on **CR-I (-)** CSP; compounds **1** – **11** were grouped by colour, depending on the nature of substituents (nonpolar/polar - acidic, neutral, basic).

According to data represented in Figure 2.5., as a general trend, tetrapeptides modified with nonpolar residues (represented as data points in red) are retained stronger under RP mode, while, analytes modified with polar residues (blue and green data points), are retained stronger in organic solvent rich mobile phases. This distribution was found to be even more pronounced, when using MeOH containing mobile phases.

2.3.2. Employing the opposite chirality of crown ether CSPs for evaluation of enantioseparation

Selectivity, also known as separation factor (α) describes the ability of the chromatographic system to distinguish between sample components. It is usually measured as a ratio of the retention factors (k) of the two peaks in question (Equation 2.1.).

$$\alpha = \frac{k_2}{k_1}, \qquad (2.1.)$$

Where α - selectivity

k_2 – retention factor of stronger retained enantiomer;

k_1 – retention factor of weaker retained enantiomer.

Usually, the racemic form of the analyte is easier available compared to its enantiomerically pure form, therefore, common way of studying, whether the CSP is

enantioselective towards the chiral analyte, employs the analyte in a racemic form and a CSP in a "single enantiomeric form". However, especially for amino acids and their derivatives (amongst them peptides), enantiomerically pure forms are often easier available than the racemates. Therefore, opposite concept, specifically, using a single enantiomer of a chiral compound and a "racemic form" of the CSPs (with opposite stereochemical configuration as it is with chiral selectors in **CR-I** (+) and **(-)** phases; Fig. 1.2., **B**) was introduced (Equation 2.2.).

$$\alpha \sim \alpha^* = \frac{k_{(+)}}{k_{(-)}}, \qquad (2.2.)$$

Where α^* - "apparent" enantioselectivity;

$k_{(+)}$ – retention observed for specific enantiomer on **CR-I** (+) CSP;

$k_{(-)}$ – retention for same enantiomer on the opposite chirality **CR-I (-)** CSP.

In order to test the validity of this concept, a series of experiments was carried out with the LLLL-enantiomer of tetrapeptide **1**. As shown in the example represented in Figure 2.6., the obtained values of selectivity α, characterizing the separation of tetrapeptide **1** LLLL/DDDD enantiomers on **CR-I** (+) (e.g., 15% ACN – α = 2.58; 95% ACN – α = 6.61), does not differ significantly from the calculated α^* values (15% ACN - α^* = 2.54; 95% ACN - α^* = 6.45), obtained from separate injections of LLLL-**1** on both **CR-I** CSPs of opposite configuration.

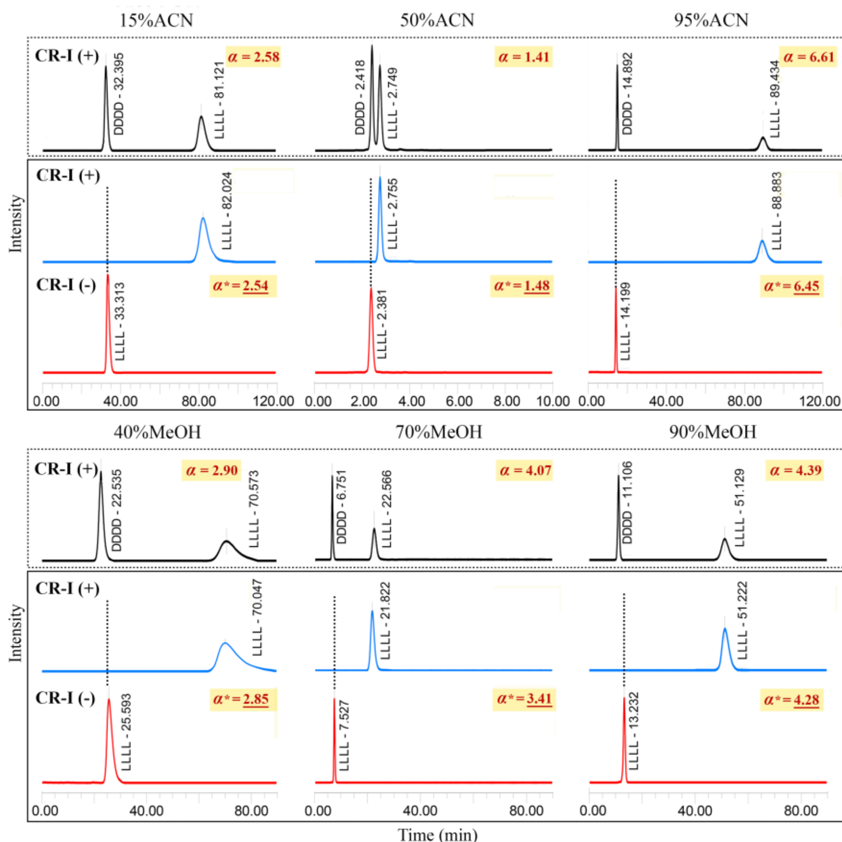


Fig. 2.6. **Chromatograms characterizing tetrapeptide 1 LLLL and DDDD enantiomer chromatographic behaviour on CROWNPAK CR-I CSPs:** tetrapeptide **1** LLLL/DDDD enantiomer separation on **CR-I (+)** (*represented in black*); retention of LLLL enantiomer on **CR-I (+)** (*in blue*); retention of LLLL enantiomer on **CR-I (-)** (*in red*). Mobile phases: 50 mM $HClO_4$ in ACN (MeOH)/water.

The obtained results confirm that the newly introduced "apparent" selectivity α^* parameter can be used to further characterize the separation of tetrapeptide **2 - 11** LLLL/DDDD enantiomeric pairs, regardless of the organic modifier type and composition of MP.

2.3.3. Influence of the modified *amino* acid residue on tetrapeptide enantioseparation

The concept described above was used to investigate whether the chiral selectors in **CR-I** CSPs are also enantioselective towards modified tetrapeptide **1** analogues **2 – 11** (Fig. 2.4.). In order to assess the effect of the nature and composition of the mobile phase organic modifier on the enantioselectivity of the studied tetrapeptide LLLL/DDDD pairs, the values of α^*, or the "apparent" enantioselectivity, were calculated (Equation 2.2.). Knowing that $\alpha > 1.2$ is considered sufficient for the chromatographic separation of two enantiomers, the same criteria were applied to α^* values.

Considering the different enantiomer retention behaviours previously observed on **CR-I** (+) and **(-)** CSPs, based on mobile phase composition (e.g., Fig. 2.6.), experiments were performed in both – low (15% ACN or 40% MeOH) and high (95% ACN or 90% MeOH) organic modifier content MPs. Data representing **CR-I** CSP enantioselectivity towards tetrapeptides **1** – **11** are summarized in Table 2.1.

Table 2.1.

Experimentally obtained retention factor *k* and calculated α^* values for tetrapeptides 1 - 11 (LLLL-enantiomers) on CR-I (+) and (-) CSPs

Compound (Fig. 2.4.)		ACN (%) in 50 mM $HClO_4$ in ACN/water MPs				MeOH (%) in 50 mM $HClO_4$ in MeOH/water MPs			
	CR-I	15		95		40		90	
		k	α^*	*k*	α^*	*k*	α^*	*k*	α^*
1	(+)	50.27	2.54	53.74	6.45	42.78	2.85	31.01	4.27
	(-)	19.82		8.33		15.00		7.27	
2	(+)	5.95	1.94	20.88	4.68	5.80	1.26	17.95	3.16
	(-)	3.07		4.46		4.61		5.69	
3	(+)	7.31	2.53	57.55	7.62	7.87	1.79	29.99	4.64
	(-)	2.89		7.55		4.41		6.47	
4	(+)	11.91	2.19	57.16	5.91	15.93	2.46	29.08	3.98
	(-)	5.45		9.67		6.48		7.31	
5	(+)	28.71	2.75	52.14	6.10	28.38	2.84	24.08	3.96
	(-)	10.46		8.54		10.00		6.08	
6	(+)	93.09	2.70	64.33	7.34	65.30	2.05	56.25	6.97
	(-)	34.43		8.77		31.90		9.42	
7	(+)	15.95	2.62	52.27	6.82	20.38	3.19	41.63	4.53
	(-)	6.08		7.67		6.39		9.21	
8	(+)	28.74	6.22	111.61	11.30	28.14	4.43	85.39	22.63
	(-)	4.62		9.88		6.35		7.35	
9	(+)	20.00	3.46	79.70	7.81	18.77	3.15	46.72	5.78
	(-)	5.78		10.21		5.96		8.08	
10	(+)	40.01	4.00	64.55	10.26	35.60	4.36	27.51	5.75
	(-)	10.01		6.29		8.17		4.78	
11	(+)	159.21	2.78	65.30	7.43	100.00	2.18	57.60	5.25
	(-)	57.28		8.79		45.88		10.97	

Based on Table 2.1. data, it can be seen that for all studied tetrapeptides, regardless of the mobile phase used, the calculated α^* values exceed 1.2. Furthermore, particularly high enantioselectivity was observed with high organic modifier content MPs.

In order to characterize the influence of different amino acid residue (as well as the position of the amino acid residue in tetrapeptide sequence) in modified tetrapeptide **1** structure, a parameter $\frac{\alpha*_{(1)}}{\alpha*_{(2-11)}}$, which compares the apparent enantioseparation that was obtained for tetrapeptide **1** (α*(1)) against α* (obtained for structural analogues **2** – **11**) was introduced (Table 2.2.). Calculated parameter values lower than 1 represents tendencies in increase, while values above 1 indicate a decrease in the enantioselectivity, caused by the amino acid residue in tetrapeptide **2** – **11** structures.

Table 2.2.

Calculated values of $\frac{\alpha*_{(1)}}{\alpha*_{(2-11)}}$ parameter

Residual amino acid (Fig. 2.4.)	Position (in tetrapeptide **1** sequence)	ACN (%) in 50mM $HClO_4$ in ACN/water MPs		MeOH (%) in 50mM $HClO_4$ in MeOH/water MPs	
		15%	95%	40%	90%
His (**2**)	Phe	1.31	1.38	2.26	1.35
Glu (**3**)		1.00	0.85	1.59	0.92
Cys (**4**)		1.16	1.09	1.16	1.07
Leu (**5**)		0.92	1.06	1.00	1.08
Trp (**6**)		0.94	0.88	1.39	0.72
His (**7**)	N-terminus	0.97	0.95	0.89	0.94
Glu (**8**)		0.41	0.57	0.64	0.37
Cys (**9**)		0.73	0.83	0.90	0.74
Leu (**10**)		0.64	0.63	0.65	0.74
Trp (**11**)		0.91	0.87	1.31	0.81

According to Table 2.2. data, the effects, caused by the introduction of a different substituent in tetrapeptide **1** Phe position seems to be less significant, than modifications at the N-terminus. According to assumption, that N-terminal amino group in tetrapeptide **1** structure might be responsible for the complexation with the chiral crown ether selector (Section 2.1.), steric effects caused by the size of substituents at the α-carbon sidechains at the chiral centre may be an important factor for analyte chiral recognition. The 5-membered imidazole ring in His (**7**) may provide similar steric environment as the 6-membered phenol ring in Tyr (**1**) residue, explaining the similar enantioselectivity of both tetrapeptides, whereas smaller, noncyclic, flexible substituents in tetrapeptides **8**, **9** and **10** seem to improve tetrapeptide enantioseparation.

Based on these observations it can be concluded, that tetrapeptide **1** – **11** enantioseparation on **CR-I** (+) and **(-)** CSPs strongly depends on steric effects, caused by the size of the α-carbon sidechains at the N-terminus, rather than the polarity or nature (charged/ noncharged) of the residual amino acid itself.

2.4. Chiral recognition mechanism studies of tetrapeptide 1 on CR-I CSPs[4,5]

The LLLL and DDDD-enantiomers of tetrapeptide **1** were selected as model compounds for further studies on the chiral recognition mechanism. To better understand the intermolecular binding underlying the chromatographic separation of the tetrapeptide **1** enantiomers on **CR-I** chiral stationary phases, the complexation between the crown ether selectors and LLLL-**1** and DDDD-**1** was investigated by HPLC, HRMS and NMR. To ensure similar conditions in both the NMR tube and the chromatographic system, all experiments were performed in 50 mM $HClO_4$ in methanol solutions.

2.4.1. Structure – chromatographic behaviour relationship studies for binding site identification

In theory, three functional groups may be involved in the binding between tetrapeptide **1** and the crown ether selector: the *α*-amino group in Tyr residue, the *ε*-amino group Lys, and the guanidine fragment in Arg (Fig. 2.7.).

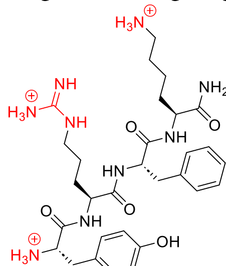


Fig. 2.7. **Tetrapeptide 1 functional groups capable of interacting.** Potential H-bond donors are highlighted in red.

To determine which of the three possible binding sites in Tyr-Arg-Phe-Lys-NH_2 are responsible for chiral resolution, seven compounds **1a** – **1g** (Fig. 2.8.) were synthesized with the aim to systematically exclude potentially interacting amino groups in tetrapeptide **1** sequence by replacing them with OH-groups or excluding them altogether, while maintaining stereochemistry of the molecule similar to that of tetrapeptide **1**. To exclude N-terminal amino group in Tyr, chemical structure of tetrapeptide **1** was altered by introducing 3-(4-hydroxyphenyl) propanoic acid in Tyr position (compounds **1a** - **1d**). To avoid the possible interaction sites in Lys (compounds **1a**; **1b**; **1e** and **1f**) and Arg (compounds **1a**; **1d**; **1f** and **1g**) moieties, these amino acids were replaced with 6-hydroxynorleucine. Considering, that only in case of enantiomeric resolution, retention times of single enantiomer observed on **CR-I** (+) and **CR-I (-)** columns, under the same chromatographic conditions, would differ from each other (thus, indicating a stereoselective binding), retention behaviour of seven tetrapeptide **1** structural analogues **1a** – **1g** was evaluated on **CR-I** (+) and **(-)** CSPs.

Based on Figure 2.8. data, no retention ($t_R \sim t_0$) was observed for tetrapeptide **1** structural analogue **1a** as the complexation between primary ammonium ion ($R\text{-}NH_3^+$) of the analyte and crown ether ring of the CSP in given case was eliminated by

[4] **Upmanis, T**.; Kažoka, H. Mechanistic insights in chiral recognition of μ-opioid receptor agonist tetrapeptide on crown ether chiral stationary phase. *J. Chromatogr. Open* **2021**, *1*, 100016 (Appendix IV)
[5] **Upmanis, T.**; Sevostjanovs, E.; Kažoka, H. Chiral recognition mechanism studies of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide on crown ether based chiral stationary phase. *Chirality* **2024**, *36(1)*, e23619 (Appendix V)

excluding all three NH_2 groups in the Tyr-Arg-Phe-Lys-NH_2 structure. The lack of chromatographic retention observed for compound **1b**, suggests that the guanidine moiety in Arg has no significant effect on retention or chiral recognition accordingly. In contrast to **1a** and **1b**, stronger retention was shown for compounds **1c** and **1d**. Both compounds share a common primary ε-amino group in Lys moiety, able to participate in hydrogen bonding with the crown ether selector, thus, explaining the observed retention. However, given the similar retention behaviour (e.g., **1c**: $k_{(-)} \sim k_{(+)} = 8.9$) obtained on both **CR-I** (+) and **CR-I** (**-**) columns, it appears that this binding is non-stereoselective. Likely, due to the ε-amino group being located four C atoms away from the chiral centre, where sterically bulky aromatic groups in crown ether selectors (Fig. 1.5.) are unable to provide a chiral environment.

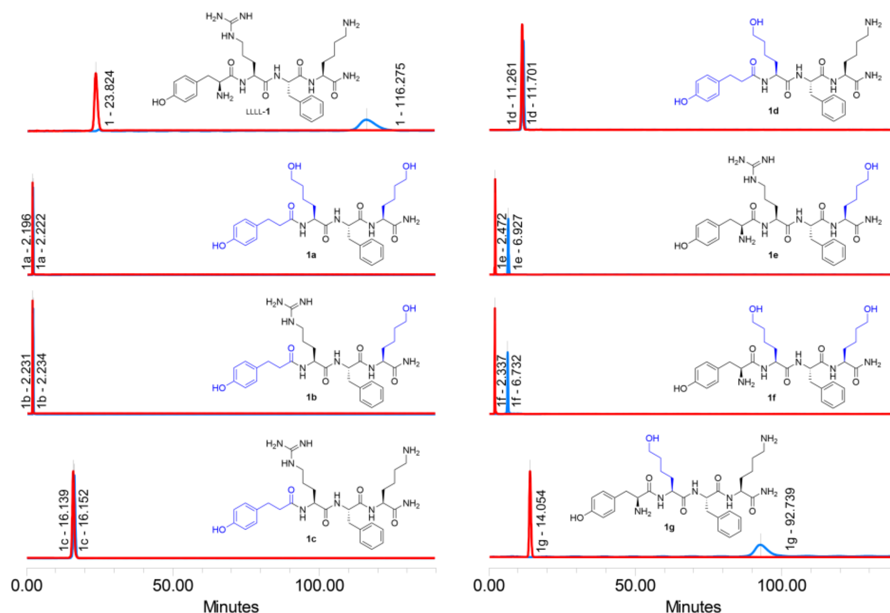


Fig. 2.8. **Overlay of chromatograms representing retention of LLLL-1 and structural analogues 1a – 1g on CR-I** (+) (***represented in blue***) **and CR-I (-)** (***in red***) **CSPs.** Mobile phase: 50mM $HClO_4$ in MeOH.

A possible stereoselective binding occurred for tetrapeptide **1** analogues **1e** – **1g**, where, similarly to LLLL-**1**, different retention behaviour on **CR-I** (+) and **(-) CSPs** was observed for the injected single enantiomers**.** By comparing chemical structures of **1e** – **1g** (Fig. 2.8.) the presence of N-terminal α-amino group in tyrosine was found to be the unique feature common in all three compounds, that way indicating the importance of this amino group in chiral recognition.

Interestingly, out of these three compounds, different retention behaviour can be seen between **1e** ($k_{(-)} = 0.53$; $k_{(+)} = 3.28$); **1f** ($k_{(-)} = 0.44$; $k_{(+)} = 3.15$) and **1g** ($k_{(-)} = 7.67$; $k_{(+)} = 56.2$), where, the apparent introduction of ε-amino group in Lys have led to significant increase in retention of **1g** and LLLL-**1**. This observation may indicate on a deviation from the generally acceptable 1:1 stoichiometry, meaning that both N-terminal α-amino group in Tyr and ε-amino group in Lys may participate in tetrapeptide **1** chiral recognition. To confirm this assumption further HRMS and NMR experiments were performed.

2.4.2. High resolution MS experiments for tetrapeptide 1 – crown selector complex determination

To demonstrate the tetrapeptide **1** enantiomer ability to form complexes with **CR** selectors outside the chromatographic column, high resolution mass spectrometry (HRMS) operated in positive electrospray ionization mode was used. Description of the experimental work can be found in Appendix V, Section 2.6.

Optically pure enantiomers of (*S*)- and (*R*)-(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 (**CR (+)** and **(-)** respectively; Fig. 1.2.) chiral selectors were synthesized and mixed in five-fold (5:1) excess with the appropriate LLLL or DDDD-enantiomers of tetrapeptide **1** in methanol containing 50 mM $HClO_4$. The prepared solutions were then injected directly into time-of-flight (TOF) mass spectrometer. The excess of the crown ether selector corresponds to intense signals at *m/z* 641 and 663 (for the Na^+ adduct; Fig. 2.9.). A closer inspection of the obtained spectra reveals several lower intensity signals, among which, the most important can be found at *m/z* 626 and 631, corresponding to 1:1 and 1:2 stoichiometry complexes between tetrapeptide **1** enantiomers and crown selectors (Fig. 2.9., **A**). In addition, 1:3 stoichiometry representing signal was observed at *m/z* 845 (Fig. 2.9., **B**), indicating the ability of the crown ether selector to bind to all three available amino moieties in tetrapeptide **1** structure in gas phase. Interestingly, the formation of all three above mentioned complexes with similar intensities also occurs for DDDD-**1** enantiomer in the presence of **CR** (+) selector.

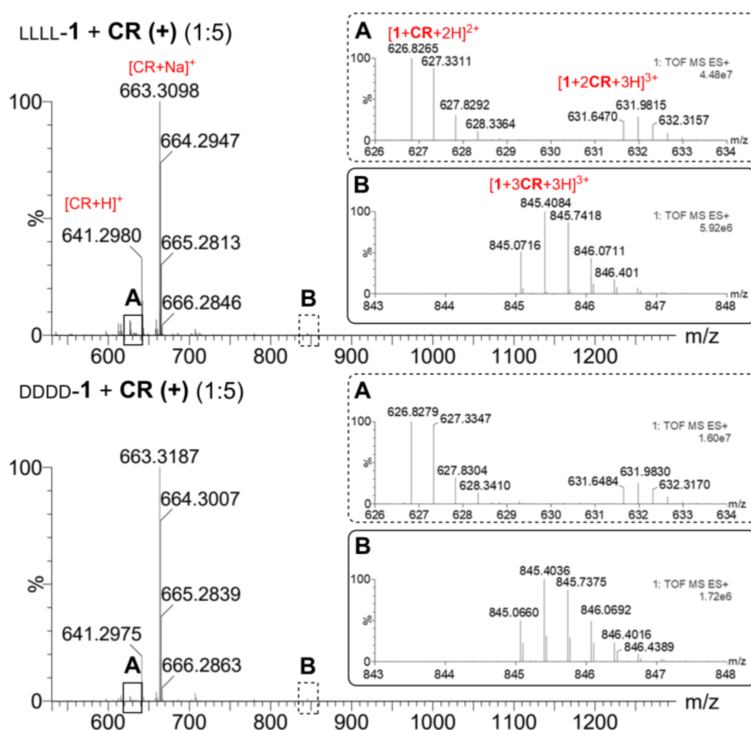


Fig. 2.9. **Partial ESI-TOF mass spectra of tetrapeptide 1 upon complexation:** LLLL-**1** with **CR** (+) chiral selector (in fivefold excess; *represented on top*); DDDD-**1** with **CR** (+) chiral selector (in fivefold excess; *bottom*). Mass signals corresponding to 1:1 and 1:2 complex adduct are represented in cut-out (**A**); Mass signals corresponding to 1:3 adduct are represented in cut-out (**B**).

This may point to a different (non-stereospecific) binding mechanism in gas phase, limiting the advantages of this technique in chiral recognition studies. Nevertheless, the use of ESI-HRMS confirmed the formation of non-covalent complexes between LLLL and DDDD-enantiomers of tetrapeptide **1** and crown selectors as well as provided us with valuable information on binding stoichiometry.

2.4.3. NMR study of complexation induced shifts upon enantioselective binding between Tyr-Arg-Phe-Lys-NH_2 enantiomers and crown ether selectors

In order to better understand the binding between synthesized **CR** (+) and **(-)** chiral selectors and the LLLL and DDDD-enantiomers of tetrapeptide **1** a series of studies were carried out by employing several 1D and 2D NMR techniques (instruments used in the experiments as well as sample preparation are described in Appendix V, Section 2.7.). Tetrapeptide **1** pseudoracemate (LLLL:DDDD = 2:1 for identification) in its free base form was mixed with **CR** (+) and **(-)** crown ether selectors in two-fold excess (Fig. 2.10) in methanol-d_4 containing 50 mM $HClO_4$ to generate conditions similar to those used in the chromatographic separations (Section 2.4.1.). It was experimentally determined that addition of the crown selector in higher excess did not reflect to any significant changes in the observed chemical shifts.

The ^{1}H-NMR chemical shifts of tetrapeptide **1** (labelled according to the numbering scheme shown in Figure 2.10.) were assigned by a combination of ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) methods. The formation of diastereomeric complexes were comfirmed by the migration of tetrapeptide **1** proton signals observed in presence of the crown ether selectors as shown in the ^{1}H-NMR spectra in Figure 2.10. The overlapped signals in the spectrum were assigned using total correlation spectroscopy (TOCSY).

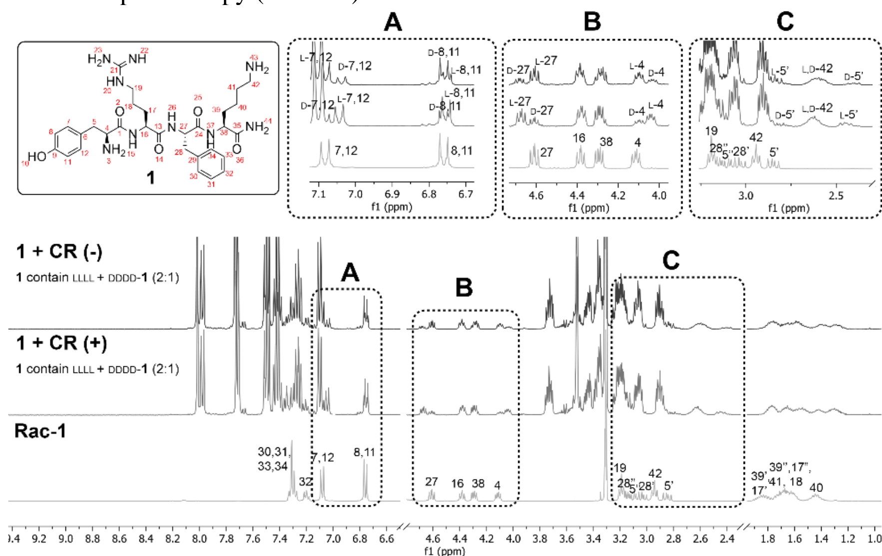


Fig. 2.10. **^{1}H-NMR (400 MHz) spectra:** of free 5 mM pseudoracemic tetrapeptide **1** (Rac-**1;** LLLL:DDDD = 2:1; *represented in bottom*); complexes with 10 mM **CR** (+) (*middle*) and 10mM **CR (-)** selectors (*top*). All spectra are obtained in methanol-d_4 containing 50 mM $HClO_4$. Signal splitting of the Tyr aromatic protons is highlighted in cut-out (**A**); Signal splitting for the α-protons in Phe in Tyr residues is highlighted in (**B**); Highlighted chemical shift changes in β-protons in Tyr residue and Lys ε-protons (**C**).

It was observed that upon complexation with **CR** (+) selector, protons of LLLL-**1** enantiomer exhibited greater chemical shift changes (relative to the unbound tetrapeptide **1**) than its DDDD-antipode. In contrast, the opposite behaviour was observed for pseudoracemic **1** upon complexation with **CR (-)** selector, where DDDD-**1** signals were correspondingly more strongly shifted. Additionally, it is evient from both obtained spectra (Fig. 2.10.) that they are the mirror images of each other, supporting our previous assumption that LLLL-**1** binding to the **CR** (+) and DDDD-**1** binding to the **CR (-)** selector (and vice versa) undergo identical complexation mechanisms. Therefore, the following discussion on the chiral recognition mechanism will focus only on complexation between LLLL and DDDD-**1** with **CR** (+).

For both enantiomers a pronounced upfield shifts were observed for H42 proton in tetrapeptide **1** Lys residue. This behaviour is indicative of intermolecular interactions, likely H-bond formation between tetrapeptide **1** and crown ether selector. This assumption is supported by previous studies [79], where similar shielding effect was observed for amino acid binding with **CR** (+) selector via H-bonds. However, given the almost identical values of the proton chemical shift changes between complexes formed by LLLL-**1** and DDDD-**1** with **CR** (+) selector, it can be assumed that both enantiomers undergo similar (non-enantioselective) complexation pattern between Lys ε-NH_3^+ group and the oxygens of the crown ether cycle.

Significant differences (Fig. 2.10., **C**) in proton chemical shifts were observed for Tyr residue β-protons H5'' and H5' of LLLL-**1**, which, similarly to protons in Lys residue shifted upfield, as well as for the Tyr α-proton H4 (Fig. 2.10., **B**) and the aromatic protons H7 and H12 (Fig. 2.10., **A**). At the same time, these Tyr residue proton shifts were practically unaffected in DDDD-enantiomer upon binding. Based on these observations, it can be concluded, that in addition to non-enantioselective hydrogen bonding between NH_3^+ group in tetrapeptide **1** Lys residue and **CR** (+), secondary binding also occurs between α-NH_3^+ group of the Tyr residue of LLLL-**1** and additional **CR** (+) selector molecule (DDDD-**1** with another **CR (-)** molecule), which could be responsible for chiral recognition of tetrapeptide **1**. Such an assumption would be consistent with both the results of the chromatographic separations of tetrapeptide **1** (Section 2.1.) and the analysis of chromatographic behaviour of structural analogues **1a** – **1g** (Section 2.4.1.).

Unlike previously discussed hydrogen bonding induced upfield shifts in tetrapeptide **1** Tyr and Lys residues, weak deshielding of Phe α-proton H27 (Fig. 2.10., **B**), β-protons H28'', H28' and aromatic H30, H31, H33 and H34 protons were observed for LLLL-**1** enantiomer in presence of **CR** (+). Based on the different character of chemical shift changes, it can be assumed that in this case other types of intermolecular interactions may take part, possibly involving π-systems of Phe residue in and the aromatics of **CR** (+) selector.

In summary, chromatographic retention analysis of tetrapeptide **1** and its structural analogues **1a** - **1g** on **CR-I** (+) and **(-)** CSPs indicates that enantioseparation of LLLL/DDDD-**1** might be possible due to significant differences in the enantiomeric binding mechanisms. From chromatographic point of view, for the stronger retained tetrapeptide **1** enantiomer [LLLL-**1** - **CR-I** (+)] and [DDDD-**1** - **CR-I (-)**], the NH_3^+ groups in the Tyr and Lys residues can bind simultaneously to two crown ether selector molecules on the CSP surface, while for the weaker retained tetrapeptide **1** enantiomer [DDDD-**1** - **CR-I** (+)] and [LLLL-**1** - **CR-I (-)**], only the NH_3^+ group on the Lys residue is responsible for binding to the crown ether selector (and hence the weaker retention).

CONCLUSIONS

1. The application of commercially available crown ether CSPs in short peptide enantioseparation was investigated by using Tyr-Arg-Phe-Lys-NH_2 tetrapeptide as a model compound (all 16 stereoisomers were synthesized). The best enantioselectivity was observed on CROWNPAK **CR-I** (+) CSP: all eight enantiomer pairs were resolved with ACN rich mobile phase.
2. The chromatographic behaviour of ten newly synthesized structural analogues of Tyr-Arg-Phe-Lys-NH_2 (N-terminal and Phe positions modified with different amino acids) was evaluated on **CR-I** phases. Modifications of the tetrapeptide structure at the Phe position were found to have less significant effect on enantioselectivity than modifications at the N-terminus. The effect of the amino acid residue on enantioselectivity depends largely on steric effects caused by the size of the α-carbon side chains at the N-terminus rather than the polarity or nature of the introduced amino acid.
3. Seven structural analogues were synthesized with the aim of systematically excluding the tetrapeptide amino groups that may interact with the chiral selector by replacing them with OH groups (or excluding them completely) and studying their chromatographic behaviour on **CR-I** (+) and **(-)** phases containing opposite chirality selectors. It was concluded that:
   a. The guanidine fragment in Arg residue does not bind to the crown ether selectors;
   b. The primary ε-amino group in the Lys residue is able to participate in non-enantioselective $^{+}N\text{-}H\cdots O$ hydrogen bond formation with the crown ether selector;
   c. It is likely that the α-amino group in the Tyr residue is responsible for chiral recognition.
4. HRMS experiments revealed that LLLL and DDDD-enantiomers of Tyr-Arg-Phe-Lys-NH_2 can form 1:1, 1:2, and 1:3 stoichiometry complexes upon binding with crown ether selectors.
5. NMR studies of the complexation between LLLL and DDDD-enantiomers and the crown ether selectors showed that for one tetrapeptide enantiomer, NH_3^+ groups in Tyr and Lys residues can bind simultaneously to two crown ether selector molecules on the sorbent surface, while for the other enantiomer, only the NH_3^+ group in Lys residue is responsible for binding to the crown ether selector, which is also supported by the HPLC data.

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PIELIKUMI / APPENDICES

I

Upmanis, T.; Kažoka, H.; Arsenyan, P.

A study of tetrapeptide enantiomeric separation on crown ether based chiral stationary phases

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A study of tetrapeptide enantiomeric separation on crown ether based chiral stationary phases

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ABSTRACT

The chiral separations of small peptides is an important challenge in the biological and medical sciences, because different stereoisomers of chiral drugs can often possess different pharmacological, pharmacokinetic, and/or toxicological activities. Commercially available crown ether chiral stationary phases based on *S*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 (CROWNPAK CR-I (+)) and (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (ChiroSil RCA (+)) have been successfully used for separating enantiomers of various racemic compounds containing primary amino groups. In this investigation, enantioresolution of more complex model analyte - tetrapeptide Tyr-Arg-Phe-Lys-NH_2, has been reported on crown ether chiral stationary phases. Organic and acidic modifier content in aqueous mobile phase was tested. All Tyr-Arg-Phe-Lys-NH_2 stereoisomers showed U-shaped retention plots, based on ACN content in mobile phase. Increased retention of tetrapeptide stereoisomers was observed at low (<35%) and at high (>70%) acetonitrile content in the mobile phase, indicating that different separation mechanisms are most likely involved. As a result, baseline separation of all eight tetrapeptide enantiomer pairs was achieved under isocratic elution mode on both chiral columns.

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1. Introduction

Over the past decade, peptide drug discovery has experienced a revival of interest as the pharmaceutical industry has come to appreciate how this class of compounds can be an excellent complement or even a preferable alternative to small molecule drugs [1]. The essential biological functions of peptides depend on peptide stereochemistry. Amino acids exist in D and L forms (with an exception of glycine) and as a result the peptide can exist as several stereoisomers with different biological properties [2,3]. During synthesis, storage or metabolic processes stereoisomers may experience racemization (or epimerization depending on the position of the stereogenic centers involved) [4,5], resulting in complex enantiomeric / epimeric compositions, therefore, enantiomeric purity control of peptide analytes is an important challenge in the biological and medical sciences and is necessary for the pharmaceutical industry.

Two most commonly used approaches for optical purity control of chiral amino acids using liquid chromatography (LC) are direct (without derivatization) and indirect methods (with chiral derivatization) [6,7]. Indirect methods are based on the formation of diastereomeric derivatives through reaction between the analyte enantiomers and homochiral derivatizing reagents. After the reaction diastereomeric derivatives can be separated under achiral conditions. However, following the introduction of new chromatographic and capillary electrophoresis techniques, the importance of chiral derivatization has decreased [6]. For direct separation methods, chiral stationary phases (CSPs) based on different types of chiral selectors have been developed to separate amino acid enantiomers using LC. However, there is a limited amount of existing publications related to LC chiral separations of short peptides. For example, enantioseparation of glycyl dipeptides has been achieved by ligand exchange chromatography [8]. Some unprotected nonproteinogenic amino acids and peptide like analyte enantioseparation has been performed on polysaccharide derivatives [9,10]. Unprotected dipeptide and tripeptide chiral resolution has been previously reported on CSPs based on cinchona alkaloids [11–13], macrocyclic glycopeptides [14–16], cyclodextrins [17,18].

Two types of chiral crown ethers (Fig. 1), incorporating an optically active 1,1′-binaphthyl unit first introduced by Cram and coworkers [19] and a tartaric acid unit developed by Behr and coworkers [20], have been successfully utilized for the separation of enantiomers of various racemic compounds containing a primary amino group, e.g. α- and β-amino acids [21–23], dipeptides [24–26] and tripeptides [27]. Resolution in more complex systems, e.g. tetrapeptides, to the best of our knowledge, has not yet been reported.

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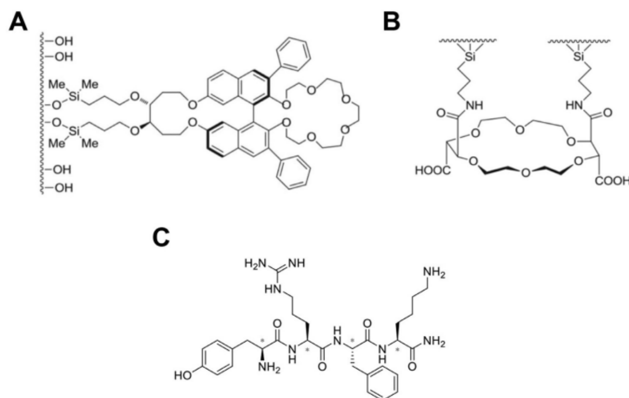

Fig. 1. Structures of chiral stationary phase in CROWNPAK CR-I (+) [*S*-(3,3′-diphenyl-1,1′-binaphthyl)–20-crown-6 immobilized on silica] - (A); ChiroSil RCA (+) column [(+)-(18-crown-6)–2,3,11,12-tetracarboxylic acid immobilized on silica] - (B) and Tyr-Arg-Phe-Lys-NH_2 tetrapeptide structure - (C).

To clarify the prospects of using crown ether-based CSPs for chiral resolution in tetrapeptides, Tyr-Arg-Phe-Lys-NH_2 (Fig. 1; LDLL isomer is also known as μ-opioid receptor agonist DALDA [28]), was chosen as a model structure and all 16 stereoisomers of Tyr-Arg-Phe-Lys-NH_2 were synthesized in order to study chromatographic behavior on two commercially available crown ether CSPs CROWNPAK CR-I (+) and ChiroSil RCA (+). Since chiral separation mechanisms involved may differ on both columns [29], different separation ability was expected for the chiral selectors in the series of experiments. Also adjusting chromatographic conditions, e.g. organic and acidic modifier content in aqueous mobile phase, were tested in order to improve Tyr-Arg-Phe-Lys-NH_2 enantiomeric separation. Efforts to extend the use of chiral crown ether-based CSPs in resolution of all sixteen tetrapeptide stereoisomers were made.

2. Material and methods

2.1. Chemicals and materials

All 16 stereoisomers of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide were synthesized at the Latvian Institute of Organic Synthesis (Riga, Latvia). Gradient grade acetonitrile (ACN) for LC was obtained from Merck (Darmstadt, Germany). Deionized water ($R \geq 18$ MΩ/cm, TOC ≤ 3 ppb) was produced by Milli-Q system (Millipore, Darmstadt, Germany). HPLC grade perchloric acid (60%) was purchased from Fisher Scientific (Loughborough, Leicestershire, United Kingdom), Sulfuric acid (96%) was purchased from Stanlab (Lublin, Poland).

2.2. Instrumentation

Chromatographic measurements were performed on Waters Alliance (Waters Corporation, Milford, MA, USA) LC systems equipped with 2695 separations module consisting of quaternary pump, degasser, autosampler and column heater, Waters 2489 dual λ absorbance detector was used for detection of analytes. The output signal was monitored and processed using Waters Empower 2 software. CROWNPAK CR-I (+) column (3.0 mm (i.d.) × 150 mm, 5 μm particle size) based on *S*-(3,3′-diphenyl-1,1′-binaphthyl)–20-crown-6 immobilized on silica was purchased from Chiral Technologies Europe (Illkirch, France). ChiroSil RCA (+) (4.6 mm (i.d.) × 250, 5 μm particle size) based on (+)-(18-crown-6)–2,3,11,12-tetracarboxylic acid immobilized on silica was purchased from Regis technologies inc. (Morton Grove, IL, USA). Mixtures of acetonitrile and water with perchloric or sulfuric acids as acidic modifier were used as a mobile phases (MP). For every experiment, columns were conditioned with MP for not less than 1 h. The proportion of each MP component was always measured by volume. Chromatographic runs were performed at flow rate either 1.0 mL/min (ChiroSil RCA (+)) or 0.4 mL/min (CROWNPAK CR-I (+)). Detection was accomplished via measurement of UV absorption at 220 nm and injection volume was set at 5 μl.

2.3. Preparation of tetrapeptide stereoisomer standard solution

The analytical samples were prepared by dissolving 5 mg of each Tyr-Arg-Phe-Lys-NH_2 stereoisomer in 50 mM $HClO_4$ resulting a stock solution with a concentration of 5 mg/mL. Single tetrapeptide stereoisomer standard solutions were prepared by diluting 100 μl of stock solution of each stereoisomer in 1 mL of mobile phase (0.5 mg/mL).

2.4. Preparation of mixed solution of tetrapeptide enantiomers

The analytical samples of tetrapeptide enantiomer pairs were prepared by mixing 100 μl of both corresponding enantiomer stock solutions in 1 mL of mobile phase (0.5 mg/mL). Elution order was determined by injecting configurationally known samples.

2.5. Preparation of mixed standard solution of all 16 tetrapeptide stereoisomers

The mix injection sample of all 16 stereoisomers was prepared by transferring 100 μl of each stock solution in an autosampler vial (0.31 mg/mL). For experiments involving high percentage of ACN in mobile phase, mix solution was evaporated under reduced pressure and dissolved in 1 mL of ACN/50 mM $HClO_4$ 95/5 (v/v).

Table 1
Influence of the mobile phase acidic modifier concentration on chromatographic behavior of tetrapeptide Tyr-Arg-Phe-Lys-NH_2 enantiomers on CROWNPAK CR-I (+).

Enantiomers	15%ACN + 16 mM $HClO_4$ (pH 2.0)				15%ACN + 50 mM $HClO_4$ (pH 1.5)				15%ACN + 160 mM $HClO_4$ (pH 1.0)			
	k_1	k_2	α	R_s	k_1	k_2	α	R_s	k_1	k_2	α	R_s
DLDL/LDLD	0.96	1.43	1.46	2.02	9.79	14.28	1.48	4.68	29.58	45.73	1.55	5.97
DDLL/LLDD	1.00	2.47	2.29	4.43	9.91	23.50	2.39	9.46	30.07	83.64	2.74	11.95
DLLL /LDDD	1.19	3.36	2.54	5.46	11.82	29.94	2.56	10.62	38.27	101.93	2.69	12.49
DLDD/LDLL	1.16	1.19	1.03	<0.5	11.89	12.41	1.04	<0.5	37.40	39.58	1.07	<0.5
DDLD/LLDL	1.40	2.57	1.58	2.59	12.51	20.79	1.65	5.75	36.81	69.55	1.88	8.19
DDDD /LLLL	1.53	4.45	2.58	5.50	13.97	37.08	2.65	11.00	43.33	129.86	2.98	13.05
DLLD/LDDL	1.62	3.74	2.13	4.41	15.39	32.87	2.10	8.59	50.32	112.67	2.23	10.18
DDDL/LLLD	2.19	5.43	2.47	5.42	17.12	44.53	2.63	11.47	51.65	156.50	3.03	13.31

Detection: UV 220 nm; column temperature: 25 °C; k_1: Retention factor of the first eluted enantiomer; α: Separation factor. R_s: Resolution; Elution time of the 1st unretained peak was set as a t_0 for each chromatogram; flow rate: 0.4 mL/min.

Table 2
Influence of the mobile phase acidic modifier concentration on chromatographic behavior of tetrapeptide Tyr-Arg-Phe-Lys-NH_2 enantiomers on ChiroSil RCA (+).

Mobile phase	20%ACN + 16 mM $HClO_4$ (pH 2.0)				20%ACN + 50 mM $HClO_4$ (pH 1.5)				20%ACN + 100 mM $HClO_4$ (pH 1.3)			
	k_1	k_2	α	R_s	k_1	k_2	α	R_s	k_1	k_2	α	R_s
LDLD/ DLDL	2.10	3.50	1.66	2.03	5.72	8.62	1.51	3.08	9.59	13.94	1.45	3.34
LLDD/DDLL	1.19	2.23	1.89	2.50	3.08	5.87	1.92	4.76	4.78	9.63	2.01	6.08
LDDD/DLLL	2.07	3.80	1.82	2.41	5.31	8.63	1.61	3.51	8.70	13.68	1.57	3.96
LDLL/DLDD	1.77	3.08	1.74	2.22	4.30	6.50	1.52	3.00	6.92	10.02	1.47	3.27
LLDL/DDLD	1.24	1.83	1.45	1.45	3.62	5.48	1.51	3.14	5.65	8.88	1.57	3.98
LLLL/DDDD	1.43	2.05	1.43	1.43	3.66	5.48	1.49	3.12	5.70	8.63	1.52	3.82
LDDL/DLLD	2.23	3.49	1.58	1.89	6.62	9.71	1.45	2.76	11.16	15.67	1.40	3.01
LLLD/DDDL	1.59	2.18	1.37	1.25	4.40	6.40	1.45	2.84	6.90	10.25	1.48	3.44

Detection: UV 220 nm; column temperature: 25 °C; k_1: Retention factor of the first eluted enantiomer; α: Separation factor. R_s: Resolution; Elution time of the 1st unretained peak was set as a t_0 for each chromatogram; flow rate: 1 mL/min.

3. Results and discussion

3.1. Optimization of the LC conditions

For separation of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide stereoisomers on two commercially available crown ether chiral columns CROWNPAK CR-I (+) and ChiroSil RCA (+) acetonitrile was chosen as the mobile phase organic modifier. Acetonitrile as aprotic solvent cannot interfere with hydrogen bonding type interactions between chiral selector and analytes. Added water acts as a highly competing species for the hydrogen bonding sites on the chiral selector surface and displaces the analyte from hydrogen bonding between chiral selector and selectand, reducing the hydrogen bond induced analyte retention with increasing the content of water in the mobile phase. Thus, hydrogen bonding type interactions are prevalent at low water content in acetonitrile (typically < 20%) while hydrophobic interactions take over commonly above 20% [30].

U-shape dependence of retention on the composition of binary acetonitrile-water mobile phases have been previously reported for various compounds on polysaccharide based CSPs [30,31], and only recently for enantioseparation of proteinogenic amino acids on crown ether CSP [21], In achiral reversed-phase chromatography, retention increases with increasing water content in the mobile phase. For polar analytes, opposite behavior, where retention increases with the decrease of water content in MP has been extensively researched in LC technique called hydrophilic interaction liquid chromatography (HILIC) and similar behavior, contrary to RP retention, has been reported for chiral separations on polysaccharide based CSPs [32,33]. Analyte retention can be considered as function of balance between hydrophilic (hydrogen bonding) and hydrophobic interactions taking place between the chiral selector and analyte [30], therefore, based on analyte characteristics and chromatographic conditions, CSPs may act either as RP-like or HILIC-like stationary phases. Similar principles can be applied to U-shaped retention dependency on crown ether based CSPs.

It is well known that separations on crown ether CSPs are carried out under acidic conditions, where the generated analyte ammonium ions can bind enantioselectively to the macrocyclic crown ether through inclusion complexation driven via the formation of three $^{+}N–H\cdots O$ hydrogen bonds between the ammonium ion in analyte structure and oxygens of the crown ether [34]. Strong acids such as CF_3COOH, HCl, H_2SO_4, HNO_3, H_3PO_4 and $HClO_4$ are required to ensure full protonation/ionization of the primary amino groups. Due to low ultraviolet (UV) absorption and great chaotropic character, perchloric acid is usually used as a mobile phase additive for CROWNPAK CR-I (+) column [35]. A chaotropic agent is an ion that can disrupt the hydrogen bonding network or solvation between the molecules of analyte and water. The chaotropic ion (ClO_4^-) can interact with the protonated amino enantiomers to form an ion pair. Since the ion pair is more lipophilic than the unpaired analyte, it is more strongly retained by lipophilic stationary phase. Consequently, the retention of the two enantiomers should increase [36].

In spite of sulfuric acid being the most frequently used acidic modifier in enantioseparations on crown ether-based CSPs with incorporated tartaric acid units [22,37], application of perchloric acid was found to afford, in general, better resolution of tetrapeptide Tyr-Arg-Phe-Lys-NH_2 enantiomers on ChiroSil RCA (+) column (See Tables S1 vs. S2 in electronic supplementary material).

Influence of perchloric acid concentrations in the aqueous mobile phase on chromatographic behavior of tetrapeptide Tyr-Arg-Phe-Lys-NH_2 enantiomers in order to find the optimal conditions was investigated on CROWNPAK CR-I (+) (Table 1) and ChiroSil RCA (+) columns (Table 2). Increasing the perchloric acid content in MP from 16 mM (pH 2.0) to 50 mM (pH 1.5) and then to 160 mM (pH 1.0), lead to notable increase in retention. As the concentration of ClO_4^- anion increases in the mobile phase, the

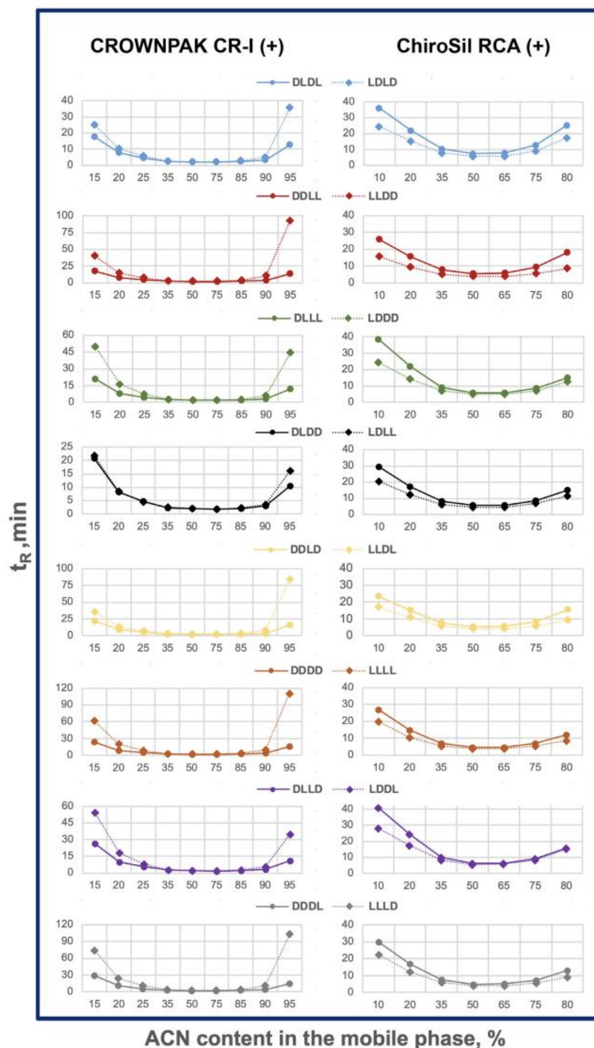

Fig. 2. Plots of the retention time (t_R) of eight pairs of Tyr-Arg-Phe-Lys-NH_2 enantiomers against the ACN content. Mobile phases: ACN/50 mM $HClO_4$ = 15/85 to 95/5 (v/v) on CROWNPAK CR-I (+); 10/90 to 80/20 (v/v) on ChiroSil RCA (+).

retention of the protonated analyte increases. Hence, the retention time of analytes can be manipulated by varying the concentration of chaotropic anion based on the requirement. For example, change in retention factor (k) from 2.19 to 17.12 to 51.65 for DDDL stereoisomer was observed on CROWNPAK CR-I (+) column in the studied acidic modifier concentration range, while generally no significant improvements in separation were achieved (α values ranged from 1.37 to 1.45 to 1.48; Table 1). Therefore, mixture

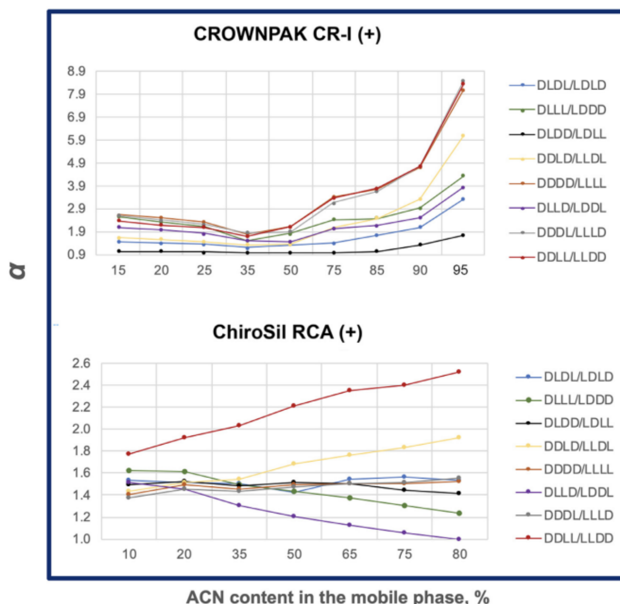

Fig. 3. Effect of ACN content in mobile phase on Tyr-Arg-Phe-Lys-NH_2 enantiomer separaition.

of acetonitrile and 50 mM $HClO_4$ was chosen as the optimal mobile phase for further chiral separation studies for Tyr-Arg-Phe-Lys-NH_2 tetrapeptide.

3.2. Effect of the ACN content in the MP on the retention

Retention profiles of sixteen Tyr-Arg-Phe-Lys-NH_2 stereoisomers were studied on both crown ether CSPs by injecting standard solutions of tetrapeptide enantiomer pairs into LC system and varying ACN/50 mM $HClO_4$ compositions in MP from 15/85 to 95/5 (v/v) for CHIRALPAK CR-I (+) and from 10/90 to 80/20 (v/v) for ChiroSil RCA (+) columns. Plots of the retention times (t_R) of eight pairs of tetrapeptide enantiomers against the ACN content are represented in Fig. 2. All Tyr-Arg-Phe-Lys-NH_2 stereoisomers showed U-shaped curves with a minimum within 50–75% ACN range for CROWNPAK CR-I (+) and 50% ACN for ChiroSil RCA (+) columns.

Increased retention of stereoisomers was observed at lower (<25% for CROWNPAK CR-I (+) and <35% for ChiroSil RCA (+)) and at higher (>90% for CROWNPAK CR-I (+) and >75% for ChiroSil RCA (+)) acetonitrile content in the MP, indicating that different mechanisms are most likely employed. Reversed-phase mode, based on inclusion complexation, followed by enantioselective hydrophobic interactions between the solute and the binaphthyl and two phenyl groups of the stationary phase plays the primary role in analyte retention at low ACN content (increased retention of stereoisomers below 25% ACN content; Fig. 2). Meanwhile, with ACN rich mobile phases, HILIC like partition mechanism (increased retention of stereoisomers starting at 90% ACN content; Fig. 2) contributing additional polar interactions, possibly with silanols or water absorbed to the stationary phase is believed to be responsible for the increase in analyte retention.

Two possible separation mechanisms have also previously been suggested for ChiroSil RCA (+) column [38,39]. In lower ACN content mobile phases (increased retention of stereoisomers starting at 35% ACN content; Fig. 2), in addition to the complexation of primary ammonium ion inside the cavity of the 18-crown-6 ring, additional hydrophobic interactions between the two carboxylic acid groups in the crown ether selector (Fig. 1B) and the sterically bulky side groups in α-amino compounds, are expected to be necessary for chiral resolution. In ACN rich mobile phases (increased retention of stereoisomers starting at 75% ACN content; Fig. 2), the side two carboxylic acid groups in chiral selector can act as hydrogen bonding donor or acceptor groups.

Tetrapeptide enantiomers possessing LXXX configuration (L-Tyr- at the N-terminus, Fig. 1C) retained stronger than their D-antipodes (DXXX) on CROWNPAK CR-I (+) column (Fig. 2). Moreover, different retention behavior between tetrapeptide enantiomers were observed for N-terminal L-Tyr-stereoisomers showing a sharp increase in retention at high ACN content in mobile phase, indicating more favourable association of the ammonium ion and the crown ether binding site. In contrast, retention of N-terminal D-Tyr-tetrapeptide enantiomers did not show a significant increase in retention with the same change in ACN composition of the mobile phase. According to Fig. 2, opposite elution order of DXXX/LXXX enantiomers was observed on ChiroSil RCA (+) and steady increase of retention was noticed for both corresponding tetrapeptide enantiomers on ChiroSil RCA (+) column in the high ACN range.

Fig. 4. Overlay of chromatograms of mixed standard solution of all 16 Tyr-Arg-Phe-Lys-NH_2 stereoisomers (in black) and enantiomeric pairs (in color) on CROWNPAK CR-I (+) with mobile phases ACN/50 mMH ClO_4.

Even though no research has been done in order to characterize elution order in compounds consisting of multiple binding sites, e.g. multiple amino groups in short peptides, this particular elution order might indicate that the α-amino acid at the N-terminus - Tyr-could be responsible for chiral complexation between the crown ether ring of the CSP and the primary ammonium ions (R-NH_3^+) of the analyte. This hypothesis is supported by previous studies, regarding enantioseparation of α-amino acids and esters, reviewed by Hyun [34]. For CSPs based on *S*-(3,3′-diphenyl-1,1′-binaphthyl)–20-crown-6, stronger retention has always been observed for L-amino acids. Such elution order has been rationalized by chiral recognition model suggested in [40,41], and simplified by Newman projection in Fig. S1. The analyte is held in the crown ether ring by the three NH⁺• • •O hydrogen bonds, where the 3-phenylnaphthyl rings provide chiral barriers. In this instance, the largest substituent (three residual amino acid chain) at the chiral center of analytes is located at the most remote position from the sterically most demanding 3-phenylnaphthyl group and the other two groups at the chiral center are positioned automatically. In the more stable complex (Fig. S1), the small substituent (hydrogen) in L-enantiomer is directing to the sterically less demanding 3-phenylnaphthyl group, forming more stable complexes and resulting in longer retaintion times for LXXX-enantiomers.

For CSPs based on (+)-(18-crown-6)–2,3,11,12-tetracarboxylic acid, stronger retention has always been observed for D-amino acids (with the exception of serine and threonine) [34]. A chiral recognition mechanism proposed for crown ether CSPs incorporating tartaric acid units [39] suggests that, in addition to the tripodal complexation of the primary ammonium ions with the crown ether ring of the CSP, hydrogen bonding between the carboxylic acid group of the chiral selector and the carbonyl oxygen of α-amino acid (the same principles would apply for the tetrapeptide) is involved in the chiral recognition (Fig S2). For D-enantiomer the 4-hydroxybenzyl group at the chiral center is directed away from the chiral barrier (-COOH) of the chiral selector, where the 4-hydroxybenzyl group for the L-enantiomer is facing the carboxyl group of the chiral selector, resulting in steric hindrance. According to this suggested model, tetrapeptide DXXX-enantiomers would form more stable complexes, resulting in longer retention times.

3.3. Effect of the ACN content of the MP on the enantioselectivity

According to data shown in Fig. 3 and retention profiles illustrated in Fig. 2, all Tyr-Arg-Phe-Lys-NH_2 tetrapeptide DXXX/LXXX enantiomers could be separated on CROWNPAK CR-I (+) column. Seven out of eight tetrapeptide enantiomeric pairs (poor resolution was observed for LDLL/DLDD enantiomers) could be separated under reversed-phase conditions (low ACN range; Fig. 3; Table S3). However, increase in separation factor values for all eight enantiomeric pairs was achieved with ACN rich mobile phases (starting at 85% ACN). Moreover, the use of mobile phase with ACN/50 mM $HClO_4$ = 90/10 (v/v) allowed the separation (α > 1.2) of all eight pairs of tetrapeptide enantiomers (Table S3).

Interestingly, all eight enantiomeric pairs were baseline separated on ChiroSil RCA (+) column with low ACN content in

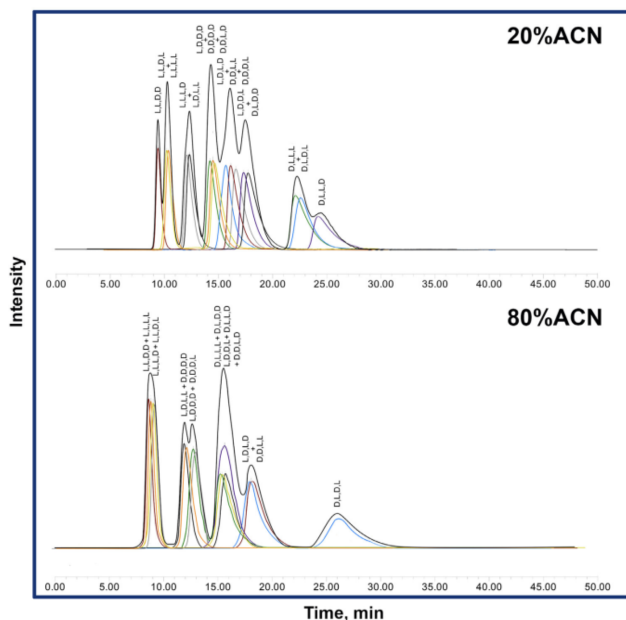

Fig. 5. Overlay ofchromatograms of mixed standard solution of all 16 Tyr-Arg-Phe-Lys-NH_2 stereoisomers (in black) and enantiomeric pairs (in color) on ChiroSil RCA (+) with mobile phases ACN/50 mM $HClO_4$.

mobile phase (Table S4). According to data shown in Fig. 3, separation factor for DDLL/LLDD enantiomers increased, when ACN content in mobile phase was increased. In contrast, α value for DLLL/LDDD decreased with the increase in ACN content. Almost no significant changes in selectivity were observed for the other 4 enantiomeric pairs in the studied ACN range. It can be well seen (Fig. 3) that enantiomers possessing DDLX/LLDX configuration are better separated in ACN rich mobile phase, while enantiomers possessing DLLX/LDDX configuration are better separated in low ACN content mobile phases. The results obtained indicate that multiple chiral recognition mechanisms are possible within a single molecule and steric arrangement of the substituents in tetrapeptide structure is crucial for enantiomer separation and both elution modes are worth investigating.

3.4. Application of crown ether CSPs for chiral resolution of tetrapeptide stereoisomers

A mixed standard solution of all sixteen Tyr-Arg-Phe-Lys-NH_2 tetrapeptide stereoisomers was injected into LC system for evaluation of stereoisomer separation ability on both crown ether CSPs. Since different selectivity was previously observed, chromatographic behavior of tetrapeptide stereoisomers was investigated in mobile phases consisting of high and low ACN content (Table 3). Changes in elution order were observed not only from using different CSPs, but also depended on ACN content in mobile phase. As a general trend, retention and separation of tetrapeptide isomers possessing DXXX configuration was weaker than isomers possessing LXXX configuration on CROWNPAK CR-I column. Opposite retention trends were observed on ChiroSil RCA (+) where stereoisomers with LXXX configuration eluted earlier.

Table 3
Elution order of tetrapeptide Tyr-Arg-phe-lys-NH_2 stereoisomers on crown ether CSPs with mobile phases ACN/50 mM $HClO_4$.

#	CROWNPAK CR-I (+)		ChiroSil RCA (+)	
	15%ACN	95%ACN	20%ACN	80%ACN
1	DLDL	DLDD	LLDD	LLDD
2	DDLL	DLLD	LLDL	LLLL
3	DLLL	DLLL	LLLL	LLLD
4	DLDD	DLDL	LDLL	LLDL
5	LDLL	DDLL	LLLD	LDLL
6	DDLD	DDDL	LDDD	DDDD
7	DDDD	DDDD	DDDD	LDDD
8	LDLD	DDLD	DDLD	DDDL
9	DLLD	LDLL	LDLD	LDDL
10	DDDL	LDDL	DDLL	DLLL
11	LLDL	LDLD	DDDL	DLDD
12	LLDD	LDDD	DLDD	DDLD
13	LDDD	LLDL	LDDL	DLLD
14	LDDL	LLDD	DLDL	LDLD
15	LLLL	LLLD	DLLL	DDLL
16	LLLD	LLLL	DLLD	DLDL

A noticeable relation between the stereochemistry of the tetrapeptide isomer and its retention was observed under high (95%) ACN mode only on CROWNPAK CR-I (+), where the retention

of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide stereoisomers can be sorted in order of DLXX > DDXX > LDXX > LLXX (Table 3), indicating, that configuration of the first two amino acids (Tyr-and Arg) in the tetrapeptide sequence could determine the binding affinity with the 1,1′-binaphthyl crown ether selector under high ACN content in mobile phase.

According to chromatogram shown in Fig. 4, LXXX stereoisomers (with exception of LDLL and LDLD) can be separated, if mobile phase with low (15%) ACN content was used on CROWNPAK CR-I (+) column. With high (95%) ACN content, weak stereoisomer separation was observed. According to data represented in Fig. 4, seven stereoisomers were baseline separated. Much weaker stereoselectivity for tetrapeptide isomers was observed on ChiroSil RCA (+) column (Fig. 5). It can be concluded, that for Tyr-Arg-Phe-Lys-NH_2 tetrapeptide stereoisomer separations, *S*-(3,3′-diphenyl-1,1′-binaphthyl)−20-crown-6 chiral selector is more preferable to (+)-(18-crown-6)−2,3,11,12-tetracarboxylic acid selector.

4. Conclusions

Separation of tetrapeptide Tyr-Arg-Phe-Lys-NH_2 on commercially available crown ether columns CROWNPAK CR-I (+) and ChiroSil RCA (+) was investigated. All eight pairs of tetrapeptide enantiomers could be baseline resolved on both columns. The best overall enantioseparation was achieved on CROWNPAK CR-I (+) column with mobile phases consisting of high ACN content. Efforts to extend the use of chiral crown ether-based CSPs in resolution of all sixteen tetrapeptide stereoisomers resulted in baseline separation of seven stereoisomers on CROWNPAK CR-I (+) column.

U-shaped plots, based on ACN content in mobile phase, with an increase in retention both in highly aqueous and ACN rich mobile phases were observed on both crown ether CSPs. The differences in tetrapeptide chromatographic behavior points out that stereochemistry of Tyr-Arg-Phe-Lys-NH_2 structure play an important role in chiral recognition mechanisms.

In general, *S*-(3,3′-diphenyl-1,1′-binaphthyl)−20-crown-6 chiral selector is more preferable to (+)-(18-crown-6)−2,3,11,12-tetracarboxylic acid selector for enantiomer stereoisomer separations of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

T. Upmanis: Conceptualization, Methodology, Investigation, Data curation, Funding acquisition, Writing - original draft. **H. Kažoka:** Conceptualization, Methodology, Supervision, Writing - review & editing, Project administration. **P. Arsenyan:** Conceptualization, Methodology, Supervision, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461152.

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II

Upmanis, T.; Kažoka, H.

Application of Commercially Available Crown Ether Chiral Stationary Phases for Separation of Tetrapeptide Stereoisomers

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P-80

Application of Commercially Available Crown Ether Chiral Stationary Phases for Separation of Tetrapeptide Stereoisomers

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Keywords: *Tetrapeptide, enantioseparation, crown ether chiral stationary phases, chiral chromatography*

1. Introduction

Over the past decade, peptide drug discovery has experienced a revival of interest as the pharmaceutical industry has come to appreciate how this class of compounds can be an excellent complement or even a preferable alternative to small molecule drugs [1]. The essential biological functions of small peptide drugs often depend on peptide stereochemistry. With the exception of glycine, all other amino acids exist as D and L enantiomers, therefore, peptides can exist as several stereoisomers, possessing different biological properties [2,3]. During synthesis, storage or metabolic processes stereoisomers may experience racemization, resulting in complex enantiomeric / epimeric mixtures, therefore, enantiomeric purity control of peptide analytes is an important challenge in the biological and medical sciences and is necessary for the pharmaceutical industry.

Among the numerous commercially available chiral stationary phases (CSPs), crown ether-based CSPs have been proven to be very effective for the resolution of chiral analytes containing primary amino groups [4].

This work is an extension to our previous research [5] regarding the chiral resolution of tetrapeptides, using Tyr-Arg-Phe-Lys-NH_2 (see ***Figure 1A***; LDLL isomer is also known as μ-opioid receptor agonist DALDA), as a model structure.

Efforts to extend the use of chiral crown ether-based CSPs in resolution of all sixteen tetrapeptide stereoisomers were made by employing the opposite characteristics of *S*- and *R*-3,3′-diphenyl-1,1′-binaphthyl crown ether selectors (see ***Figure 1B***, commercially sold as CROWNPAK CR-I columns).

2. Materials and methods

All 16 stereoisomers of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide were synthesized at the Latvian Institute of Organic Synthesis by the author. Chromatographic measurements were performed on Waters Alliance (Waters Corporation, Milford, MA, USA) LC systems equipped with 2695 separations module consisting of quaternary pump, degasser, autosampler and column heater, Waters 2489 dual λ absorbance detector was used for detection of analytes. The output signal was monitored and processed using Waters Empower 2 software. CROWNPAK CR-I (+) and CR-I (-) columns (3.0 mm (i.d.) × 150 mm, 5 μm particle size) based on *S- and R*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 immobilized on silica was purchased from Chiral Technologies Europe (Illkirch, France). Mixtures of acetonitrile and 50mM $HClO_4$/water solution were used as a mobile phase. Chromatographic runs were performed at flow rate of 0.4 mL/ min. Detection was accomplished via measurement of UV absorption at 220 nm and injection volume was set at 5 μl. The analytical samples consist of mixtures eight LXXX stereoisomers and a mixture of their corresponding DXXX enantiomers (0.5 mg/ mL of each stereoisomer in mobile phase). Elution order was determined by injecting configurationally known samples.

3. Results

It is well known, that the chiral selector in CROWNPAK CR-I columns - *S- and R*-(3,3′-diphe-

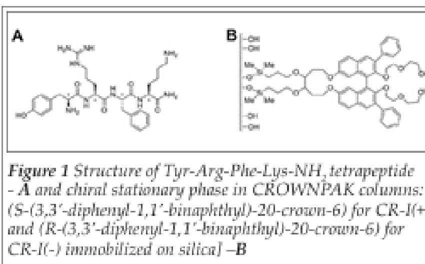

Figure 1 *Structure of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide - A and chiral stationary phase in CROWNPAK columns: (S-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6) for CR-I(+) and (R-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6) for CR-I(-) immobilized on silica]* –**B**

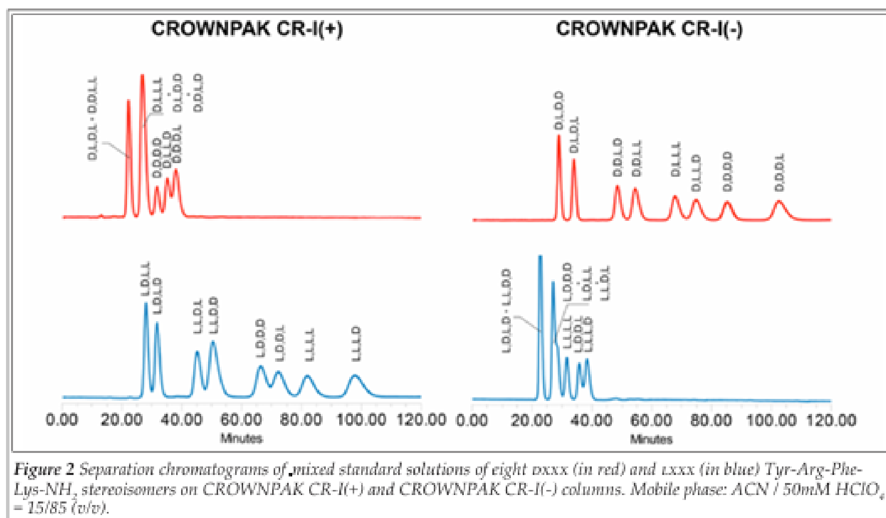

Figure 2 *Separation chromatograms of mixed standard solutions of eight* DXXX *(in red) and* LXXX *(in blue) Tyr-Arg-Phe-Lys-NH_2 stereoisomers on CROWNPAK CR-I(+) and CROWNPAK CR-I(-) columns. Mobile phase: ACN / 50mM $HClO_4$ = 15/85 (v/v).*

nyl-1,1′-binaphthyl)-20-crown-6 are both each other enantiomers, therefore, opposite elution order is expected for analyte enantiomers. A mixed standard solution of eight LXXX stereoisomers and a mixture of their corresponding DXXX enantiomers of Tyr-Arg-Phe-Lys-NH_2 was injected into LC system for evaluation of stereoisomer separation ability on both crown ether CSPs.

It was determined that the best stereoisomer separation can be achieved under reversed phase mode (15% ACN). Tetrapeptide enantiomers possessing LXXX configuration (L-Tyr at the N-terminus, see ***Figure 1A***) retained stronger than their D-antipodes (DXXX) on CROWNPAK CR-I (+) column and vice versa for CR-I (-) column.

As shown in ***Figure 2***, a mixture of eight LXXX tetrapeptide stereoisomers was separated on CROWNPAK CR-I (+) column. It appears, that dxxx tetrapeptide stereoisomers, that could not be resolved on the CR-I (+) column can be resolved on CR-I (-) column. This property could be used in the analysis of a real life samples in order to be able to identify the optical impurities.

4. Conclusions

Reversed phase LC conditions were found to achieve the best separation for a mixture of Tyr-Arg-Phe-Lys-NH2 stereoisomers. The combined analysis on both columns of the Tyr-Arg-Phe-Lys-NH2 sample is a convenient approach that would provides a broader insight within real life sample optical purity. These LC conditions allow the identification and quantification of sixteen Tyr-Arg-Phe-Lys-NH2 from the mixture.

5. Acknowledgements

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III

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Influence of amino acid residue on chromatographic behaviour of *μ*–opioid receptor agonist tetrapeptide analogue on crown ether based chiral stationary phase

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Research article

Influence of amino acid residue on chromatographic behaviour of μ-opioid receptor agonist tetrapeptide analogue on crown ether based chiral stationary phase

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The influence of amino acid residue on μ-opioid receptor agonist tetrapeptide Tyr-Arg-Phe-Lys-NH_2 analogue chromatographic behaviour on crown ether based chiral stationary phases has been investigated. *S*- and *R*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 chiral selectors in commercially available CROWNPAK CR-I (+) and (-) columns are both each other enantiomers, thus, under the same LC conditions, retention of Tyr-Arg-Phe-Lys-NH_2 DXXX enantiomers (fixed in D-tyrosine position) on *S*-chiral selector do not significantly differ from the retention times of their LXXX antipodes, obtained on *R*-chiral selector (and vice versa), allowing us to study the apparent separation of a specific tetrapeptide enantiomeric pair, without obtaining the actual racemate. Ten tetrapeptides (LLLL-isomers) have been synthesized with the aim to cover a wider range of different amino acid classes. Histidine, glutamic acid, cysteine, leucine and tryptophan were introduced at the N-terminus or Phe position of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide structure. The effects of the amino acid residue with emphasis on retention, enantioseparation, as well as the influence of position of the amino acid residue in tetrapeptide sequence, are discussed.

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1. Introduction

With the rapid development of biological and medical sciences over the past decades, the use and research of peptide therapeutics is continuously evolving [1]. The key contributor to this trend is the potent and specific, yet safe, mode of action [2], allowing this class of compounds to be an excellent complement or even a preferable alternative to small molecule drugs [3]. Currently there are several hundred peptide drugs undergoing clinical developments, with over 80 already approved for clinical use worldwide, covering a wide range of therapeutic areas, including variety of metabolic and autoimmune diseases, oncology and chronic pain [4].

Chirality plays one of major roles in the function of peptides, as the biological activity [5,6] and physical properties depend on conformation, which is inherently controlled by the configuration of the amino acid components [7]. Therefore, development of accurate and rapid analytical techniques to determine enantiomeric purity in peptides is of paramount importance for pharmaceutical industry as well as chemical and biological research.

Liquid chromatography (LC) using chiral stationary phases (CSPs) has demonstrated to be extremely useful, accurate, versatile, and widely used technique in diverse fields of applications [8–11]. Amongst the numerous commercially available CSPs used in LC, two types of chiral crown ethers, incorporating an optically active 1,1′-binaphthyl unit first introduced by Cram and co-workers [12] and a tartaric acid unit developed by Behr and co-workers [13], have been successfully utilized for the separation of enantiomers of various racemic compounds containing primary amino groups [14,15].

We have previously reported on the use of crown ether based CSPs for chiral resolution of tetrapeptides [16]. By choosing a known μ-opioid receptor agonist [17] tetrapeptide Tyr-Arg-Phe-Lys-NH_2 (1; Fig. 1A) as a model compound, enantiorecognition capability of two commercially available crown ether CSPs was investigated. Application of *S*-(3,3′-diphenyl-1,1′-binaphthyl)–20-crown-6 chiral selector (Fig. 1B) in CROWNPAK CR-I (+) column (CR-I (+) in further text), in general, was proven more suitable in tetrapeptide 1 enantiomer and stereoisomer separations. A broader insight of the chiral impurity (stereoisomer) composition in complex isomeric mixtures (e.g. peptides) can be achieved by employing the opposite chirality of chiral selectors (Fig. 1B). As a result, an inverse of elution order for the same chiral compound occurs. This way (Fig. 2), analysis of a mixture of tetrapeptide 1 stereoisomers

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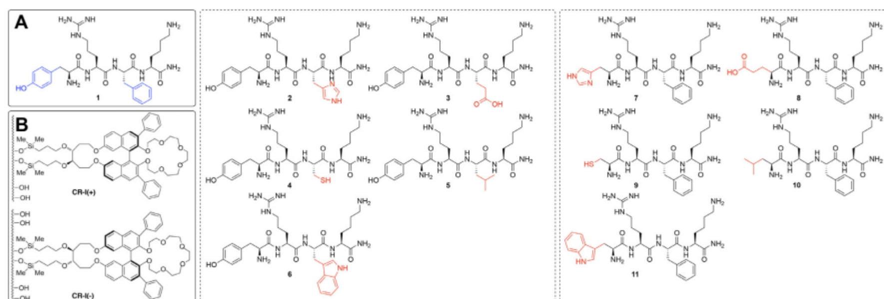

Fig. 1. Chemical structure Tyr-Arg-Phe-Lys-NH_2 tetrapeptide – A; Structures of chiral selectors in CROWNPAK CR-I (+) [*S*-(3,3′-diphenyl-1,1′-binaphthyl)–20-crown-6 immobilized on silica] and CROWNPAK CR-I (-) [*R*-(3,3′-diphenyl-1,1′-binaphthyl)–20-crown-6 immobilized on silica] – B; structures of studied tetrapeptides 2 - 11.

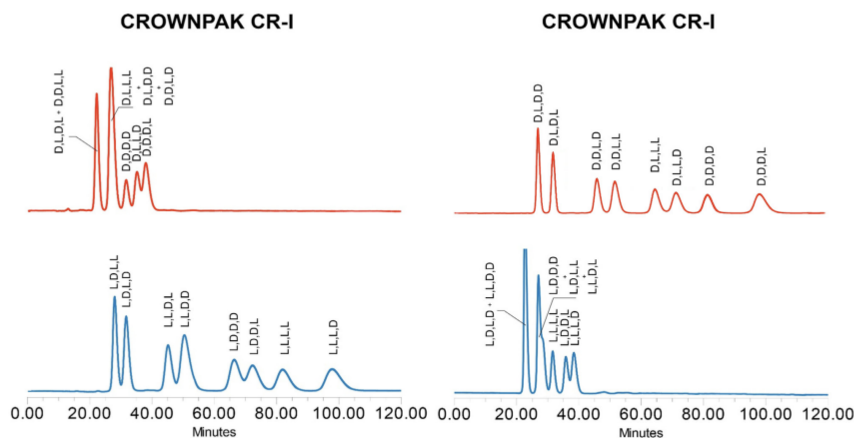

Fig. 2. Separation chromatograms of mixed standard solutions of eight DXXX (in red) and LXXX (in blue) Tyr-Arg-Phe-Lys-NH_2 stereoisomers on CROWNPAK CR-I(+) and CROWNPAK CR-I(-) columns. Mobile phase: ACN / 50 mM $HClO_4$ = 15/85 (v/v).

on both CR-I (+) and (-) column allowed the separation of all sixteen tetrapeptide stereoisomers [18].

It is well known, that even slight changes in chiral analyte structure can completely change its chromatographic behaviour. Thus, the aim of this work is to investigate the influence of amino acid residue on μ-opioid receptor agonist tetrapeptide 1 analogue (compounds 2 – 11; Fig. 1) chromatographic behaviour on crown ether based chiral stationary phases CR-I (+) and CR-I (-).

2. Material and methods

2.1. Chemicals and materials

Tyr-Arg-Phe-Lys-NH_2 (1), Tyr-Arg-His-Lys-NH_2 (2), Tyr-Arg-Glu-Lys-NH_2 (3), Tyr-Arg-Cys-Lys-NH_2 (4), Tyr-Arg-Leu-Lys-NH_2 (5), Tyr-Arg-Trp-Lys-NH_2 (6), His-Arg-Phe-Lys-NH_2 (7), Glu-Arg-Phe-Lys-NH_2 (8), Cys-Arg-Phe-Lys-NH_2 (9), Leu-Arg-Phe-Lys-NH_2 (10) and Trp-Arg-Phe-Lys-NH_2 (11; Fig. 1) were synthesized (as hydrochloride salts) by the authors at the Latvian Institute of Organic Synthesis (Riga, Latvia). Solution phase synthesis of tetrapeptides 1 - 11 consisted of multiple steps. First, amide coupling of H-Lys(Boc)-NH_2 and commercially available N-protected amino acids using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) / Hydroxybenzotriazole (HOBt) / N-methylmorpholine in ACN was performed to afford the C-terminal dipeptide building blocks (Fig S1A in electronic supplementary material). N-terminal tetrapeptide building blocks (Fig S1B) were synthesized by coupling N-protected amino acids and arginine using dicyclohexylcarbodiimide (DCC) / N-hydroxysuccinimide in THF / $NaHCO_3$ (aq). The resulting C-terminal dipeptide building blocks were selectively deprotected and used in coupling reaction with the N-terminal building blocks (EDC/HOBt, DMF). The obtained N-protected tetrapeptides (Fig S2) were then deprotected and purified via column chromatography (purity > 95%) giving tetrapeptdies 1 – 11.

Gradient grade acetonitrile (ACN) and methanol (MeOH) for LC were obtained from Merck (Darmstadt, Germany). Gradient grade deionized water ($R \geq 18$ MΩ•cm, total organic carbon (TOC) concentration ≤ 3 ppb) was produced by Milli-Q system (Millipore,

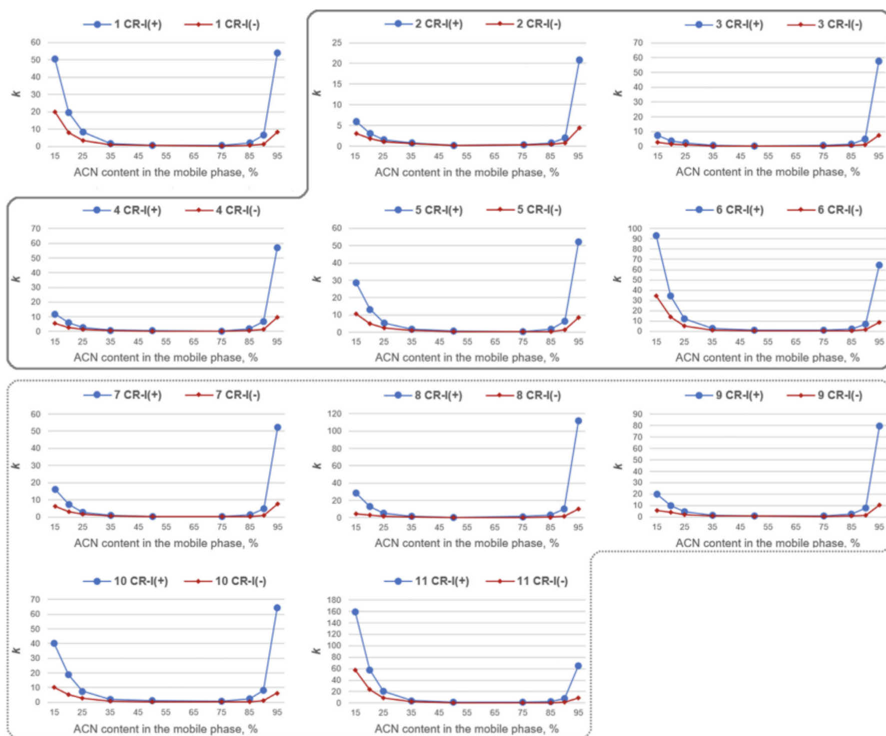

Fig. 3. Plots of tetrapeptide 1 - 11 (LLLL-isomer) retention factor (k) against the ACN content on CROWNPAK CR-I (+) – in blue; and CR-I (-) - in red. Mobile phases: 50 mM $HClO_4$ in ACN/water = 15/85 to 95/5 (v/v).

Darmstadt, Germany). HPLC grade perchloric acid (60%) was purchased from Fisher Scientific (Loughborough, Leicestershire, United Kingdom).

2.2. Instrumentation

Experiments were performed on Waters Alliance (Waters Corporation, Milford, MA, USA) instrument equipped with 2695 separations module, consisting of quaternary pump, degasser, autosampler and column heater, Waters 2489 dual wavelength absorbence detector was used for detection of analytes. The output signal was monitored and processed using Waters Empower 2 software.

2.3. Preparation of tetrapeptide standard solutions

The stock solutions were prepared by dissolving 5 mg of each compound in 1 mL 50 mM $HClO_4$ giving a solution with a concentration of 5 mg/mL. Tetrapeptide 1 - 11 standard solutions were prepared by diluting 100 µL of stock solution of each compound in 1 mL of mobile phase (0.5 mg/mL), to avoid peak distortion caused by mismatch between the MP and diluent.

2.4. Chromatographic conditions

CROWNPAK CR-I (+) and CR-I (-) columns (3.0 mm (I.D.) × 150 mm, 5 µm particle size) based on *S*- and *R*-(3,3′-diphenyl-1,1′-binaphthyl)–20-crown-6 immobilized on silica were purchased from Chiral Technologies Europe (Illkirch, France). Mixtures of 50 mM $HClO_4$ in acetonitrile (or methanol) / water solution was used as a mobile phase (MP). The proportion of each MP component was always measured by volume. Columns were conditioned with MP for not less than 1 h, prior to every experiment. Chromatographic runs were performed at flow rate of 0.4 mL/min and the injected volume of tetrapeptide sample solutions was set at 5 µL. The column oven was maintained at 25 °C and the UV absorption was measured at 220 nm.

3. Results and discussion

To extend our knowledge in peptide chromatographic behaviour on crown ether CSPs, ten tetrapeptides 2 – 11 (Fig. 1; LLLL-isomers) have been synthesized. To cover a wider range of different amino acid classes, histidine (His – polar basic); glutamic acid (Glu – polar acidic); cysteine (Cys – polar neutral; S-containing); leucine

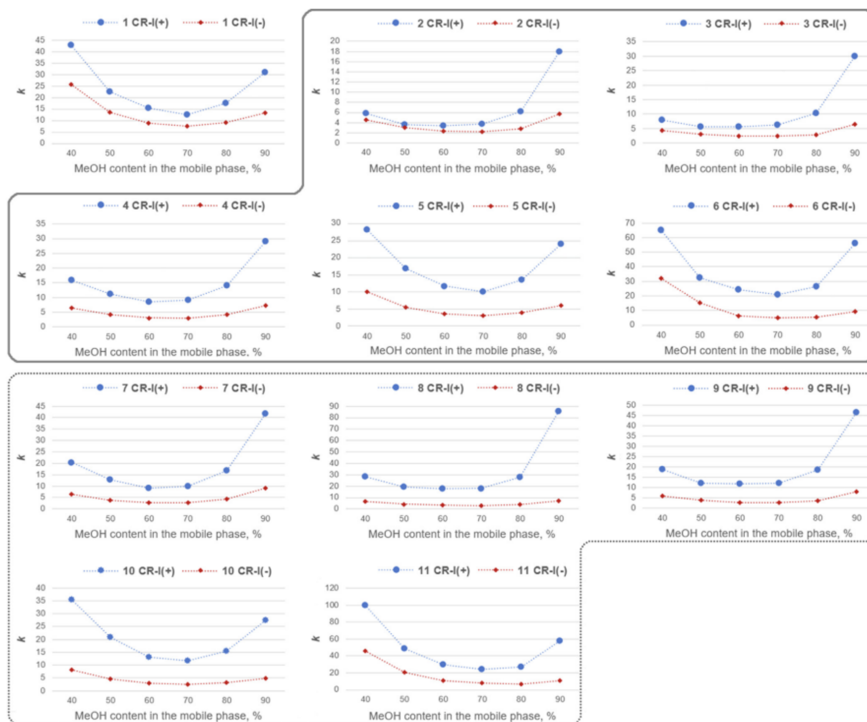

Fig. 4. Plots of the tetrapeptide 1 - 11 (LLLL-isomer) retention factor (k) against the MeOH content on CROWNPAK CR-I (+) – in blue; and CR-I (-) - in red. Mobile phases: 50 mM $HClO_4$ in MeOH/water = 40/60 to 90/10 (v/v).

(Leu - nonpolar; aliphatic) and tryptophan (Trp – nonpolar; aromatic) were introduced at the N-terminus or Phe position of tetrapeptide 1 structure. The effects of the amino acid residue, as well as the influence of position of the amino acid residue in tetrapeptide sequence, on chromatographic behaviour are further discussed.

3.1. *Retention of model compounds on crown ether stationary phases*

In general, retention of primary amino compounds is achieved through inclusion complexation, driven via triple $^{+}N–H\cdots O$ hydrogen bond formation between the ammonium ion in the protonated analyte and oxygens of the crown ether selector [19]. Perchloric acid was used as the acidic additive (with constant concentration of 50 mM in all mobile phases, studied; apparent pH* 1.5), to ensure full protonation of the amino functionalities in compounds 1 – 11. Retention profiles of eleven tetrapeptides 1 - 11 were studied on both CR-I (+) and (-) CSPs by injecting tetrapeptide standard solutions into LC system and varying ACN compositions in MP from 15/85 to 95/5 (v/v); MeOH from 40/60 to 90/10 (v/v). Information regarding chromatographic parameters (retention time – t_R; retention factor - k) are summarized in Tables S1 - S4 found in electronic supplementary material.

Plots of k of tetrapeptide 1 - 11 LLLL-enantiomers against the ACN content are represented in Fig. 3 (against MeOH content in Fig. 4). All studied tetrapeptides showed U-shaped curves with a minimum within the range of 50–75% (v/v) ACN (60–80% MeOH) on CR-I (+) and (-) columns. Retention for all eleven studied tetrapeptides is lost (an example of tetrapeptide 2 is shown in Fig. S3A), when 50–75% (v/v) ACN content is used (k < 1; Tables S1 - S2). In contrast to ACN containing mobile phases, weaker nature of MeOH as an eluent caused significantly stronger retention even at the U-shape curve minimum (k > 3; Tables S3 - S4), providing optimal conditions for tetrapeptide 1 – 11 analysis within the entire studied mobile phase range (e.g., Fig. S3B).

Similar U-shaped retention dependence on the eluent composition has been previously observed for methyl-substituted anilines [20] and different proteinogenic amino acids [21] and is explained by different retention mechanisms in aqueous and organic solvent rich mobile phases. Analyte retention can be considered as a function of balance between hydrophilic (polar e.g., hydrogen bonding) and hydrophobic interactions taking place between the chiral selector and the solute [22], therefore, based on analyte charac-

Table 1
Obtained retention factors k on CR-I (+) and (-) columns and calculated $\frac{k_{(+)}}{k_{(-)}}$ values ("apparent" separation factors) for tetrapeptides 1 - 11 (LLLL-enantiomers) with acetonitrile-containing mobile phases.

Compound	CR-I	ACN (%) in 50 mM $HClO_4$ in ACN/water (v/v): 15		25		50		85		95	
		k	$\frac{k_{(+)}}{k_{(-)}}$	k	$\frac{k_{(+)}}{k_{(-)}}$	k	$\frac{k_{(+)}}{k_{(-)}}$	K	$\frac{k_{(+)}}{k_{(-)}}$	k	$\frac{k_{(+)}}{k_{(-)}}$
Tyr-Arg-Phe-Lys-NH_2 (1)	(+)	50.27	2.54	8.20	2.46	0.72	1.48	2.03	3.74	53.74	6.45
	(-)	19.82		3.33		0.49		0.54		8.33	
Tyr-Arg-His-Lys-NH_2 (2)	(+)	5.95	1.94	1.54	1.46	0.22	1.15	0.71	1.83	20.88	4.68
	(-)	3.07		1.05		0.19		0.39		4.46	
Tyr-Arg-Glu-Lys-NH_2 (3)	(+)	7.31	2.53	2.27	2.18	0.42	1.75	1.44	2.54	57.55	7.62
	(-)	2.89		1.04		0.24		0.57		7.55	
Tyr-Arg-Cys-Lys-NH_2 (4)	(+)	11.91	2.19	2.59	1.70	0.41	1.57	1.73	3.15	57.16	5.91
	(-)	5.45		1.53		0.26		0.55		9.67	
Tyr-Arg-Leu-Lys-NH_2 (5)	(+)	28.71	2.75	5.24	2.09	0.72	1.94	1.82	3.37	52.14	6.10
	(-)	10.46		2.50		0.37		0.54		8.54	
Tyr-Arg-Trp-Lys-NH_2 (6)	(+)	93.09	2.70	12.37	2.31	0.92	1.53	2.35	3.10	64.33	7.34
	(-)	34.43		5.36		0.60		0.76		8.77	
His-Arg-Phe-Lys-NH_2 (7)	(+)	15.95	2.62	2.78	1.80	0.31	1.44	1.11	3.71	52.27	6.82
	(-)	6.08		1.54		0.21		0.30		7.67	
Glu-Arg-Phe-Lys-NH_2 (8)	(+)	28.74	6.22	5.13	3.33	0.40	1.49	3.04	5.14	111.61	11.30
	(-)	4.62		1.54		0.27		0.59		9.88	
Cys-Arg-Phe-Lys-NH_2 (9)	(+)	20.00	3.46	4.64	2.36	1.07	1.67	2.47	3.41	79.70	7.81
	(-)	5.78		1.96		0.64		0.73		10.21	
Leu-Arg-Phe-Lys-NH_2 (10)	(+)	40.01	4.00	7.51	2.84	1.03	2.01	2.26	5.32	64.55	10.26
	(-)	10.01		2.65		0.51		0.43		6.29	
Trp-Arg-Phe-Lys-NH_2 (11)	(+)	159.21	2.78	20.35	2.32	1.49	1.93	2.71	3.32	65.30	7.43
	(-)	57.28		8.77		0.77		*(0.82)*		*(8.79)*	

teristics and chromatographic conditions, CSPs may act either as reversed-phase (RP) like or HILIC-like stationary phases.

Reversed-phase mode, based on inclusion complexation, followed by enantioselective hydrophobic interactions between the solute and the binaphthyl and two phenyl groups of the stationary phase (Fig. 1B) plays the primary role in analyte retention at low organic modifier content and can be observed as an increase in analyte retention when aqueous mobile phases with less than 25% (v/v) ACN (Fig. 3) or 60% MeOH (Fig. 4) are used. With organic solvent rich mobile phases, in addition to inclusion complexation, secondary polar interactions, are believed to be responsible for the increase in analyte retention and can be seen as an increase in retention of compounds 1 - 11 in ACN (HILIC-like conditions; ACN > 90% (v/v); Fig. 3), whereas additional analyte solubility effects may be attributed to the increased tetrapeptide 1 – 11 retention in mobile phases with high MeOH (> 80%; Fig. 4) content.

Due to a variety of possible co-existing interactions between the analyte and crown ether CSP, the exact retention mechanism of tetrapeptides 1 – 11 is very complex. The studied analytes bind differently to the chiral selectors in CR-I (+) and (-) phases as their retention on the latter phase (obtained under the same LC conditions), in general, is weaker. Furthermore, based on U-shape plots, represented in Fig. 3, sharp increase in tetrapeptide 1 – 11 retention was observed on CR-I (+) column, when 95% (v/v) ACN mobile phase was used, while only moderate increase in retention was observed for the same compounds on CR-I (-). Similar phenomenon has been previously reported for L-amino acid retention on CR-I (+) column, while D-amino acids showed much smaller increases in retention within the same range, thus greatly improving the selectivity in organic solvent rich phases [23].

3.2. Approach of employing the opposite chiralities of crown ether CSPs for evaluation of enantioseparation

Usually, the racemic form of the analyte is easier available compared to its enantiomerically pure form, therefore, common way of studying, whether the CSP is enantioselective towards the chiral analyte, employs the analyte in a racemic form and a CSP in a "single enantiomeric form". However, especially for amino acids and their derivatives (amongst them peptides), enantiomerically pure forms are often easier available than the racemates. Therefore, opposite concept, specifically, using a single enantiomer of a chiral compound and a "racemic form" of the CSPs (with opposite stereochemical configuration as it is in chiral selector in CR-I (+) and (-) phases) may be used [24]. Based on our previous study, under the same LC conditions, retention times of tetrapeptide 1 DXXX enantiomers (fixed in D-tyrosine position) on CR-I (+) column can be roughly estimated by injecting their LXXX antipodes into the CR-I (-) column (and vice versa) [18]. Furthermore, as shown in example of tetrapeptide 1 LLLL/DDDD enantiomers represented in Fig. 5, selectivity α, obtained on CR-I (+) (e.g. 15% (v/v) ACN - $\alpha = 2.58$; 95% (v/v) ACN - $\alpha = 6.61$), is comparable with the ratio of retention factors $\frac{k_{(+)}}{k_{(-)}}$ (15% (v/v) ACN - $\frac{k_{(+)}}{k_{(-)}} = 2.54$; 95% (v/v) ACN - $\frac{k_{(+)}}{k_{(-)}} = 6.45$), obtained for single LLLL-1 enantiomer on CR-I (+) and CR-I (-) columns, and may be used in estimating the "apparent" enantioselectivity of a specific enantiomeric pair.

3.3. Evaluation of enatioselectivity towards model compounds

The approach, described above, was used to investigate, whether the chiral selector in CR-I CSPs is enantioselective towards modified tetrapeptide 1 analogues. The $\frac{k_{(+)}}{k_{(-)}}$ values or "apparent" separation factors (not directly measured by injection of tetrapeptide 1 – 11 LLLL/DDDD pairs) were used to evaluate the influence of mobile phase type and composition on enantioselectivity of LLLL/DDDD pairs. The significant difference in retention obtained on CR-I (+) and CR-I (-) CSPs (Tables 1 and 2) indicates that the LLLL/DDDD enantiomers of the studied tetrapeptides can be separated on CR-I CSPs.

With 50 mM $HClO_4$ in ACN/water containing mobile phases (overlaid chromatograms, representing model compound 1 – 11 "apparent" separation with ACN mobile phases are summarized in Fig. S4), increasing the content of organic modifier in mobile phase, calculated $\frac{k_{(+)}}{k_{(-)}}$ values (along with analyte retention,

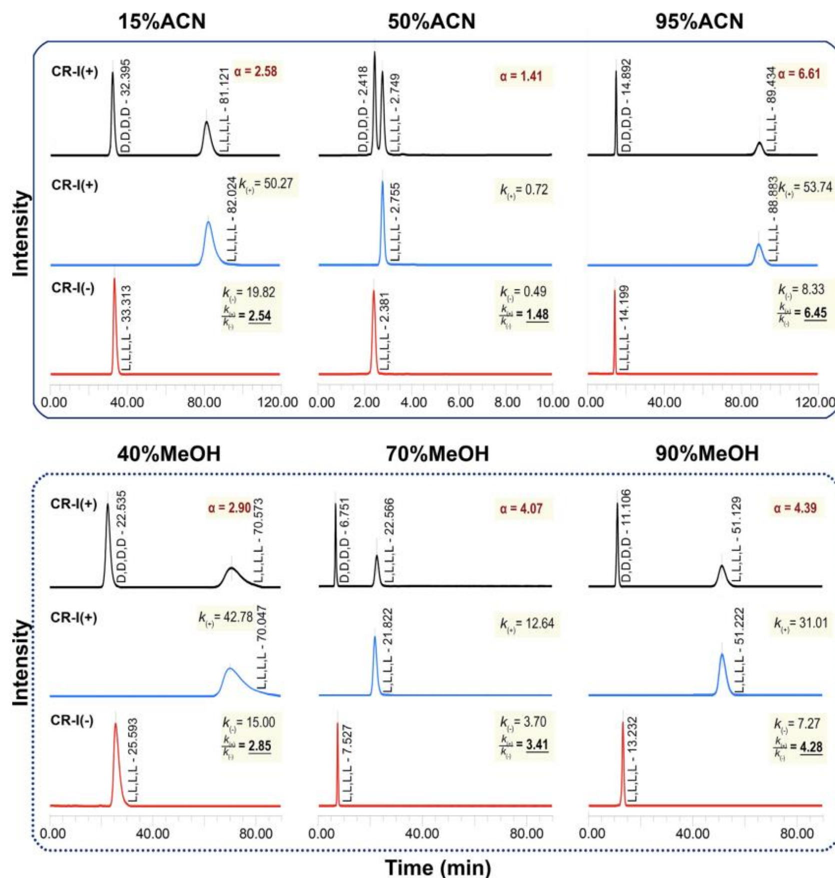

Fig. 5. Chromatograms of LLLL/DDDD-Tyr-Arg-Phe-Lys-NH_2 separation on CROWNPAK CR-I (+) – represented in black; Retention of LLLL enantiomer of Tyr-Arg-Phe-Lys-NH_2 on CR-I (+) – in blue ; Retention of LLLL enantiomer of Tyr-Arg-Phe-Lys-NH_2 on CR-I (-) - in red. Mobile phases: ACN (MeOH)/ 50 mM $HClO_4$.

Table 1) initially decrease (until 50–75% (v/v) ACN). Further increasing the amount of ACN improves the apparent enantioseparation of all eleven tetrapeptides (an example of tetrapeptide 2 is represented by overlaying chromatograms, obtained on CR-I (+) and (-) columns, under the same chromatographic conditions; Fig. S3A). From a mechanistic point of view, it can be assumed, that secondary polar interactions (which are eliminated by competing water molecules in RP mode) provide such stereospecific environment where increasingly favourable association can occur between LLLL-enantiomer of tetrapeptides 1 – 11 and the chiral selector in CR-I (+) column, resulting in the improved selectivity in HILIC-like (> 90% (v/v) ACN) conditions (indicated by increased $\frac{k_{(+)}}{k_{(-)}}$ values).

Even though a common belief is that MeOH, as a protic solvent and H-bond donor, may impair the chiral separation, a closer comparison of tetrapeptide 1 – 11 chromatographic behaviour shows (chromatograms, representing model compound 1 – 11 “apparent” separation with MeOH mobile phases are summarized in Fig. S5), that, similar to ACN containing mobile phases, the highest $\frac{k_{(+)}}{k_{(-)}}$ values for tetrapeptides 1 – 11 with 50 mM $HClO_4$ in MeOH/water mobile phases were obtained under organic solvent rich conditions (90% (v/v) MeOH; Table 2). In contrast to ACN (comparison of tetrapeptide 2 separation, based on organic modifier type shown in Fig. S3), the apparent enantioseparation of model compounds steadily improves, with the increase of MeOH content.

Table 2
Obtained retention factors k on CR-I (+) and (-) columns and calculated $\frac{k_{(+)}}{k_{(-)}}$ values ("apparent" separation factors) for tetrapeptides 1 - 11 (LLLL-enantiomers) with methanol-containing mobile phases.

Compound	CR-I	MeOH (%) in 50 mM $HClO_4$ in MeOH/water (v/v) 40		60		80		90	
		k	$\frac{k_{(+)}}{k_{(-)}}$	k	$\frac{k_{(+)}}{k_{(-)}}$	k	$\frac{k_{(+)}}{k_{(-)}}$	k	$\frac{k_{(+)}}{k_{(-)}}$
Tyr-Arg-Phe-Lys-NH_2 (1)	(+)	42.78	2.85	15.39	3.40	17.43	3.70	31.01	4.27
	(-)	15.00		4.53		4.71		7.27	
Tyr-Arg-His-Lys-NH_2 (2)	(+)	5.80	1.26	3.36	1.45	6.20	2.19	17.95	3.16
	(-)	4.61		2.32		2.83		5.69	
Tyr-Arg-Glu-Lys-NH_2 (3)	(+)	7.87	1.79	5.55	2.31	10.26	3.65	29.99	4.64
	(-)	4.41		2.40		2.81		6.47	
Tyr-Arg-Cys-Lys-NH_2 (4)	(+)	15.93	2.46	8.47	2.79	14.04	3.41	29.08	3.98
	(-)	6.48		3.03		4.12		7.31	
Tyr-Arg-Leu-Lys-NH_2 (5)	(+)	28.38	2.84	11.73	3.29	13.50	3.46	24.08	3.96
	(-)	10.00		3.56		3.90		6.08	
Tyr-Arg-Trp-Lys-NH_2 (6)	(+)	65.30	2.05	24.31	3.93	26.42	4.86	56.25	6.97
	(-)	31.90		6.18		5.43		9.42	
His-Arg-Phe-Lys-NH_2 (7)	(+)	20.38	3.19	9.22	3.32	16.78	3.90	41.63	4.53
	(-)	6.39		2.77		4.31		9.21	
Glu-Arg-Phe-Lys-NH_2 (8)	(+)	28.14	4.43	17.81	5.45	27.93	6.93	85.39	22.63
	(-)	6.35		3.27		4.03		7.35	
Cys-Arg-Phe-Lys-NH_2 (9)	(+)	18.77	3.15	11.69	4.22	18.67	5.13	46.72	5.78
	(-)	5.96		2.77		3.64		8.08	
Leu-Arg-Phe-Lys-NH_2 (10)	(+)	35.60	4.36	13.20	4.46	15.43	4.94	27.51	5.75
	(-)	8.17		2.96		3.12		4.78	
Trp-Arg-Phe-Lys-NH_2 (11)	(+)	100.00	2.18	29.91	2.80	27.09	4.01	57.60	5.25
	(-)	45.88		10.67		6.76		10.97	

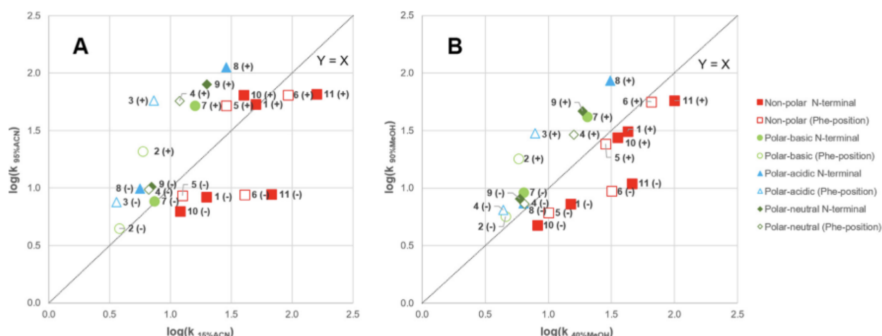

Fig. 6. Plots of log (k $_{15\%ACN}$) vs. log (k $_{95\%ACN}$) – for 50 mM $HClO_4$ in ACN/water containing mobile phases – A; log (k $_{40\%MeOH}$) vs. log (k $_{90\%MeOH}$) – for 50 mM $HClO_4$ in MeOH/water containing mobile phases – B; obtained on CR-I (+) and (-) columns, grouped by the nature of substituents (nonpolar/polar - acidic, polar neutral, basic) in model compounds 1 – 11.

3.4. Influence of the amino acid residue on chromatographic behaviour

To rationalize the relationship between analyte retention and the nature of amino acid residue on CR-I CSPs, log k values obtained at 95% (v/v) ACN (90% MeOH) content were plotted against the log k values at 15% (v/v) ACN (40% MeOH) content and represented in Fig. 6. In other words, area beneath $X = Y$ trendline describes hydrophobic interactions as the primary contributor to the analyte retention, whereas hydrophilic interactions are considered dominant in the area above the trend line. According to Fig. 6 data, as a general trend, tetrapeptides containing nonpolar residues (represented as data points in red) are retained stronger under RP mode, while, analytes containing polar residues (blue and green data points), are retained stronger in organic solvent rich mobile phases. This distribution is even more pronounced, when using MeOH containing mobile phases.

For characterization of influence of different amino acid residue (as well as the position of the amino acid residue in tetrapeptide sequence) in modified tetrapeptide 1 structure, the ratio of "apparent" separation factors $\frac{k_{(+)}}{k_{(-)}}$ (obtained for tetrapeptide 1) / $\frac{k_{(+)}}{k_{(-)}}$ (2 – 11) was introduced (Table 3). Calculated values below 1 represents tendencies in increase, while values above 1 indicate a decrease in the enantioselectivity, caused by the amino acid residue in tetrapeptide 2 – 11 structures.

In general, separation can be achieved for all studied compounds, regardless of amino acid (and its position) introduced into the tetrapeptide structure (Tables 1 – 2). According to Table 3 data, no clear relationship, between the introduced amino acid polarity and its nature on enantioselectivity can be observed. However,

Table 3
Influence of amino acid residue on enantioseparation* of model compounds 2 - 11 in comparison to tetrapeptide 1.

Residual amino acid	Position (in tetrapeptide 1 sequence)	ACN (%) in 50 mM $HClO_4$ in ACN/water (v/v) 15%	95%	MeOH (%) in 50 mM $HClO_4$ in MeOH/water (v/v) 40%	90%
His (2)	Phe	1.31	1.38	2.26	1.35
Glu (3)		1.00	0.85	1.59	0.92
Cys (4)		1.16	1.09	1.16	1.07
Leu (5)		0.92	1.06	1.00	1.08
Trp (6)		0.94	0.88	1.39	0.72
His (7)	N-terminus	0.97	0.95	0.89	0.94
Glu (8)		0.41	0.57	0.64	0.37
Cys (9)		0.73	0.83	0.90	0.74
Leu (10)		0.64	0.63	0.65	0.74
Trp (11)		0.91	0.87	1.31	0.81

*Effect of different amino acid residue on tetrapeptide enantioseparation is characterized by the ratio of $\frac{k_{(+)}}{k_{(-)}}(1)$ / $\frac{k_{(+)}}{k_{(-)}}$ (2 – 11).

the effects, caused by the introduction of a different substituent in tetrapeptide 1 Phe position seems to be less significant, than modifications at the N-terminus (Table 3). According to assumption, that N-terminal amino group in tetrapeptide 1 structure is responsible for the complexation with the chiral crown ether selector [18], steric effects, caused by the size of substituents at the α-carbon sidechains at the chiral centre may be an important factor for analyte chiral recognition. The 5-membered imidazole ring in His may provide similar steric environment as the 6-membered phenol ring in Tyr, explaining the similar enantioselectivity of both tetrapeptides. Larger indole cycle at the α-carbon in tetrapeptide 11 seems to follow this pattern with ACN as the mobile phase modifier, whereas smaller, noncyclic, flexible substituents in tetrapeptides 8, 9 and 10 seem to improve tetrapeptide synthetic analogue enantioseparation.

Based on these observations it can be concluded, that tetrapeptide 1 – 11 enantioseparation on CR-I (+) and (-) crown ether CSPs strongly depends on steric effects, caused by the size of the α-carbon sidechains at the N-terminus, rather than the polarity or nature (charged/ noncharged) of the residual amino acid itself.

4. Conclusions

The effects of the amino acid residue, as well as the influence of position of the amino acid residue in tetrapeptide sequence, on chromatographic behaviour has been investigated for μ-opioid receptor agonist tetrapeptide Tyr-Arg-Phe-Lys-NH_2 analogues, by modifying its structure at the N-terminus and Phe position. Retention dependence of the organic modifier content in mobile phase showed U-shaped curves for all eleven studied tetrapeptides on CR-I (+) and (-) columns indicating, that, based on analyte characteristics and chromatographic conditions, CSPs may act either as reversed-phase or HILIC-like. As a general trend, tetrapeptides containing nonpolar residues are retained stronger under RP mode, while analytes containing polar residues are retained stronger in organic solvent rich mobile phases.

The opposite chirality of selectors in CR-I (+) and (-) phases was employed to characterize selectivity of a specific tetrapeptide LLLL/DDDD enantiomeric pairs, by injecting a tetrapeptide 2 - 11 in single enantiomeric form (LLLL-enantiomer) into LC system and comparing their retention on both columns.

It was concluded, that modifying tetrapeptide structure at Phe position has far less significance in enantioselectivity than modification at the N-terminus. The effect of amino acid residue on tetrapeptide 1 enantioseparation strongly depends on steric effects, caused by the size of the α-carbon sidechains at the N-terminus, rather than the polarity or nature of the residual amino acid itself.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

T. Upmanis: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Funding acquisition, Writing – original draft. **H. Kažoka:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463059.

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IV

Upmanis, T.; Kažoka, H.

Mechanistic insights in chiral recognition of µ-opioid receptor agonist tetrapeptide on crown ether chiral stationary phase

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Short communication

Mechanistic insights in chiral recognition of μ-opioid receptor agonist tetrapeptide on crown ether chiral stationary phase

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Mobile phase
Chiral recognition

A B S T R A C T

Chiral separation of short peptides is of great interest, due to different pharmacological, pharmacokinetic, and/or toxicological activities often possessed by different stereoisomers of chiral drugs. Crown ether chiral stationary phases have been successfully used for separating enantiomers of various racemic compounds containing primary amino groups. Even though chiral recognition mechanism for crown-ethers CSPs is generally understood, on a molecular level, the exact chiral recognition mechanisms for the resolution are still unclear. Furthermore, short peptide analytes often contain multiple amino moieties capable of binding to the crown ether selector. A research of relationship between peptide chemical structure and chiral chromatographic interactions was performed, by comparing the retention profiles of μ-opioid receptor agonist tetrapeptide Tyr-Arg-Phe-Lys-NH_2 and its structural analogues on two commercially available *S*- and *R*-(3,3'-diphenyl-1,1′-binaphthyl)-20-crown-6) stationary phases [CROWNPAK CR-I (+) and (-)], in order to clarify, which of the potential interaction sites are responsible for retention and chiral recognition in Tyr-Arg-Phe-Lys-NH_2 tetrapeptide.

1. Introduction

The growing interest in peptide drug discovery field within the pharmaceutical industry has become evident over the past decades, reaching the market for a wide range of diseases, including diabetes, cancer, osteoporosis, multiple sclerosis, HIV and chronic pain [1]. As intrinsic signalling molecules for a multitude of physiological functions, peptide drugs (either in their native or modified form) present an opportunity for therapeutic intervention that closely mimics the natural pathways [2]. Among the factors that have contributed to this trend are the high specificity and low toxicity profile (deriving from their extremely tight binding to their targets), allowing this class of compounds to be an excellent complement or even a preferable alternative to small molecule drugs [3].

The essential biological functions of peptides depend on peptide stereochemistry. Amino acids exist in D and L forms (with an exception of glycine) and, as a result the peptide can exist as several stereoisomers with different biological properties [4,5]. However, peptide stereoisomers often experience racemization (or epimerization depending on the position of the stereogenic centers involved) during synthesis, storage or metabolic processes [6,7], resulting in complex enantiomeric / epimeric compositions, therefore, determining enantiomeric purity in peptide analytes is an important challenge in the biological and medical sciences and is necessary for the pharmaceutical industry.

In our previous work [8], we reported on a study on chiral resolution for model tetrapeptide Tyr-Arg-Phe-Lys-NH_2 (Fig. 1A; LDLL isomer is also known as μ-opioid receptor agonist DALDA [9]) on two commercially available crown ether chiral stationary phases [*S*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 (CROWNPAK CR-I (+)) and (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (ChiroSil RCA (+))]. It was concluded, that the chiral selector in CROWNPAK CR-I (+) column (in further text **CR-I (+)**) was the most suitable for both Tyr-Arg-Phe-Lys-NH_2 (**1**) enantiomer and stereoisomer separations.

Even though the concept of chiral recognition mechanism for crown-ethers CSPs is generally understood [10] on a molecular level, the exact mechanisms for the resolution of primary or non-primary amino compounds on crown ether-based CSPs are still not clear. Tyr-Arg-Phe-Lys-NH_2 sequence consists of multiple amino moieties (N-terminal α-amino group in Tyr; ε-amino group in Lys and guanidine fragment in Arg; Fig. 1A), any of which can theoretically bind to the crown ether selector, therefore, an in-depth investigation is needed in order to clarify, which of these three potential interaction sites are responsible for the retention and chiral recognition in Tyr-Arg-Phe-Lys-NH_2 tetrapeptide.

In this short communication we try to rationalize the chiral recognition mechanism on **CR-I (+)** and **CR-I (-)** for tetrapeptide **1**, by comparing the retention behaviour of the LLLL-isomer of Tyr-Arg-Phe-Lys-NH_2 and its structural analogues (or model compounds) **1a–1h** (Fig. 1), which were synthesized with the aim to selectively exclude the inter-

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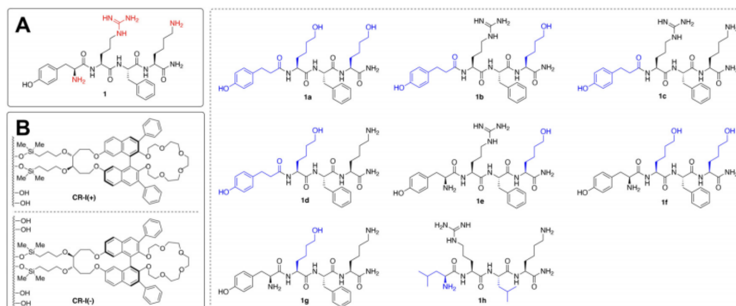

Fig. 1. Chemical structure Tyr-Arg-Phe-Lys-NH_2 tetrapeptide – A; Structures of chiral selectors in CROWNPAK CR-I (+) [*S*-(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 immobilized on silica] and CROWNPAK CR-I (-) [*R*-(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 immobilized on silica] - B and structures of studied Tyr-Arg-Phe-Lys-NH_2 tetrapeptide derivatives.

acting amino groups in tetrapeptide sequence (by replacing them with OH-groups or excluding them altogether), while maintaining the stereochemistry similar to the tetrapeptide **1** molecule.

2. Material and methods

2.1. Chemicals and materials

Both LLLL and DDDD enantiomers of tetrapeptide **1** (Fig. 1A) and eight model compounds **1a** - **1h** (Fig. 1) were synthesized (as hydrochloride salts) by the authors at the Latvian Institute of Organic Synthesis (Riga, Latvia). Gradient grade acetonitrile (ACN) and methanol (MeOH) for LC was obtained from Merck (Darmstadt, Germany). Gradient grade deionized water (R ≥ 18 MΩ/cm, TOC ≤ 3 ppb) was produced by Milli-Q system (Millipore, Darmstadt, Germany). HPLC grade perchloric acid (60%) was purchased from Fisher Scientific (Loughborough, Leicestershire, United Kingdom).

2.2. Instrumentation

Chromatographic measurements were performed on Waters Alliance (Waters Corporation, Milford, MA, USA) LC systems equipped with 2695 separations module consisting of quaternary pump, degasser, autosampler and column heater, Waters 2489 dual l absorbance detector was used for detection of analytes. The output signal was monitored and processed using Waters Empower 2 software. CROWNPAK CR-I (+) and CR-I (-) columns (3.0 mm (i.d.) × 150 mm, 5 µm particle size) based on *S*- and *R*-(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 immobilized on silica was purchased from Chiral Technologies Europe (Illkirch, France). Mixtures of acetonitrile (or methanol) and 50mM $HClO_4$/ water solution as acidic modifier were used as a mobile phases (MP). For every experiment, columns were conditioned with MP for not less than 1 h. The proportion of each MP component was always measured by volume. Chromatographic runs were performed at flow rate of 0.4 mL/ min. Detection was accomplished via measurement of UV absorption at 220 nm and injection volume was set at 5 µl.

2.3. Preparation of tetrapeptide derivative standard solutions

The stock solutions were prepared by dissolving 5 mg of each compound in 1mL 50 mM $HClO_4$ resulting solution with a concentration of 5 mg/mL. Single tetrapeptide **1** stereoisomer and derivative **1a** - **1h** standard solutions were prepared by diluting 100 µl of stock solution of each stereoisomer in 1 mL of mobile phase (0.5 mg/ mL).

3. Results and discussion

3.1. Retention of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide on crown ether stationary phases

It was considered [8] that the amino acid at the N-terminus - Tyr may be responsible for chiral complexation between the crown ether ring of the CSP and the primary ammonium ions (R-NH_3^+) of the analyte, which is also supported by chiral recognition model suggested in [11,12]. The use of 50 mM perchloric acid (pH 1.5) resulted in optimal retention for the tetrapeptide **1** stereoisomers on **CR-I (+)**. Therefore, the same mobile phases were chosen for separations on **CR-I (-)** in this investigation (Fig. S1).

S- and *R*-(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 chiral selectors (Fig. 1B) are both each other enantiomers, thus, analysis of the same chiral compound on both CSPs, should result in opposite elution order. It is well known, that on **CR-I (+)**, the D-form of amino acids always elutes in the first position and using **CR-I (-)** will result in an inversion of the elution order. The same chromatographic behavior was found to apply for tetrapeptide **1**, where under the same LC conditions, stereoisomers, possessing DXXX configuration (fixed in D-tyrosine position) eluted before LXXX stereoisomers on **CR-I (+)** and vice versa on **CR-I (-)**. This observation was used to address the weak separation of DXXX tetrapeptide **1** stereoisomers previously faced on **CR-I (+)** [8]. By switching to **CR-I (-)** column, all eight DXXX tetrapeptide **1** stereoisomers could be separated under the same conditions (Fig. 2).

The common way to examine, whether or not a chiral stationary phase is enantioselective towards a specific chiral analyte, the retention of a chiral analyte in a racemic form and a CSP in a "single enantiomeric form" is studied. However, in this work, opposite concept, specifically, using a single enantiomer of a chiral compound and virtually a "racemic form" of the CSPs (with opposite stereochemical configuration of a chiral selector) is introduced. Commonly the racemic form of the analyte is easier available compared to its enantiomerically pure form. However, sometimes (especially for amino acids and their derivatives, among them peptides) enantiomerically pure forms are easier available than the racemates.

According to Fig. 3, retention of DDDD-**1** enantiomer (RT = 31.9 min) on **CR-I (+)** column (Fig. 3A, 15%ACN) does not differ significantly from LLLL-**1** enantiomer retention time (RT = 32.2 min) observed on **CR-I (-)** (Fig. 3B, 15%ACN). It can be seen, that retention of LLLL/DDDD enantiomers of tetrapeptide **1** on **CR-I (+)** and **CR-I (-)** CSPs demonstrates the capability of roughly estimating the retention times of the corresponding enantiomers (Fig. 3C), observed on the opposite CSP.

Fig. 2. Separation of LLLL/DDDD enantiomers of Tyr-Arg-Phe-Lys-NH_2 on CROWNPAK CR-I (+) – A; Separation of LLLL/DDDD enantiomers of Tyr-Arg-Phe-Lys-NH_2 on CROWNPAK CR-I (-) – B; Overlay of chromatograms representing LLLL-Tyr-Arg-Phe-Lys-NH_2 retention on CR-I (+) (in blue) and CR-I (-) (in red) columns – C. Mobile phases: ACN / 50mM $HClO_4$.

Fig. 3. Separation chromatograms of mixed standard solutions of eight DXXX Tyr-Arg-Phe-Lys-NH_2 stereoisomers on CROWNPAK CR-I(+) and CROWNPAK CR-I(-) columns. Mobile phase: ACN / 50mM $HClO_4$ = 15/85 (v/v).

It can be assumed that only in case of enantiomeric resolution, retention times of single enantiomer observed on **CR-I (+)** and **CR-I (-)** columns, ran under the same conditions, would differ from each other, indicating a stereoselective binding. To confirm this hypothesis, model compounds **1a** - **1h** (Fig. 1) were synthesized within this study. To exclude potentially interacting N-terminal amino group in tyrosine, the chemical structure of tetrapeptide **1** was modified by introducing 3-(4-hydroxyphenyl) propanoic acid in tyrosine position (compounds **1a** - **1d**). To avoid the possible interaction sites in lysine (compounds **1a; 1b; 1e** and **1f**) and arginine (compounds **1a; 1d; 1f** and **1g**) moieties, these amino acids in tetrapeptide sequence were replaced with 6-hydroxynorleucine. Model compound **1h** was synthesized to evaluate the effect of the presence of aromatic moieties in tetrapeptide **1** (by replacing Tyr and Phe with Leu).

3.2. Retention of model compounds on crown ether stationary phases

Retention of model compounds **1a – 1h** was studied on **CR-I (+)** and **CR-I (-)** CSPs by injecting standard solutions of single compound into LC system. Mobile phases, ranging from 15/85 to 95/5 (acetonitrile/50 mM $HClO_4$; Tables S1 - S2 in electronic supplementary material) and 40/60 to 90/10 (v/v) (methanol/50 mM $HClO_4$; Tables S3 - S4) were tested. The key data obtained has been summarized as overlays in Fig. 4 (ACN/50 mM $HClO_4$; for broader variety of mobile phases see Fig. S2) and Fig. 5 (MeOH/50 mM $HClO_4$; see Fig. S3). It can be seen, that for the studied model compounds, in general terms, no significant changes in chiral recognition were observed by varying mobile phase organic modifier (ACN *vs.* MeOH).

Based on the retention data obtained on both columns, three groups of model compound **1a – 1h** retention behavior were characterized. The first group of compounds consists of tetrapeptide **1** structural analogues **1a** and **1b**. Compound **1a** was synthesized with the aim to exclude all three interacting NH_2 groups in the tetrapeptide 1 structure, by replacing Tyr with 3-(4-hydroxyphenyl) propanoic acid and replacing Arg and Lys with 6-hydroxynorleucine (Fig. 1). As the complexation between primary ammonium ion (R-NH_3^+) of the analyte and crown ether ring of the CSP in given case was eliminated, retention times around t_0 were expected. As shown in Figs. 4 - 5, retention ($k \sim 4$) was observed for both compounds **1a** and **1b** when lower content of organic modifier in mobile phases were used. However, retention for both compounds decreased, when organic modifier concentration was increased (95% ACN: $k < 1$). It can be assumed, that the hydrophobic moieties of crown ether selectors could perform the same way as an achiral RP stationary phase would, considering the relatively non-polar nature of analytes **1a** and **1b**. Chromatographic behavior observed for compound **1b** (Arg position in tetrapeptide sequence has been replaced with 6-hydroxynorleucine; Fig. 1), suggests that the guanidine moiety in Arg had no significant effect on tetrapeptide **1** retention and chiral recognition.

The second group includes compounds **1c** and **1d**, for which, contrary to structural analogues **1a** and **1b**, stronger retention (15% ACN: $k \sim 18$ for **1c**; $k \sim 14$ for **1d**; 95% ACN: $k \sim 3.5$ for **1c**; $k \sim 6.5$ for **1d**) was shown (Figs. 4 - 5). Structurally, both compounds share a common

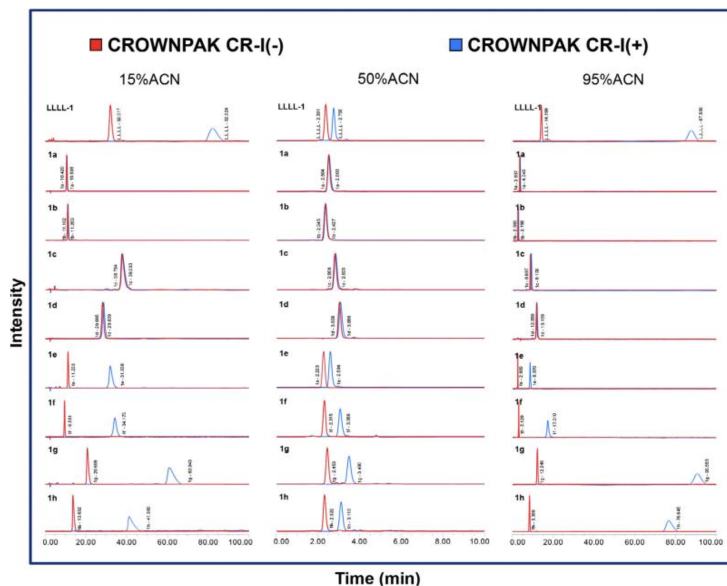

Fig. 4. Overlay of chromatograms representing retention profiles of LLLL-1 and structural analogues 1a - 1h on CR-I (+) (in blue) and CR-I (-) (in red) columns. Mobile phases: ACN / 50mM $HClO_4$.

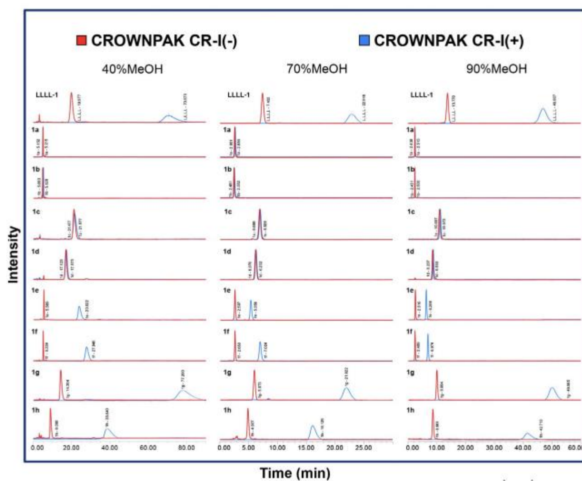

Fig. 5. Overlay of chromatograms representing retention profiles of LLLL-1 and structural analogues 1a - 1h on CR-I (+) (in blue) and CR-I (-) (in red) columns. Mobile phases: MeOH / 50mM $HClO_4$.

primary ε-amino group in Lys moiety (Fig. 1), able to form $^{+}$N–H···O hydrogen bonds with the crown ether selector and, thus, explaining the observed retention. However, given the similar retention times within the studied mobile phase range on both **CR-I (+)** and **CR-I (-)** columns, it can be assumed that this complexation is not stereoselective. Likely, due to the ε-amino group being located four C atoms away from the chiral center, where sterically bulky biphenyl and phenyl groups in crown ether selectors are unable to provide a chiral environment.

The third group consists of structural analogues **1e** - **1h**. For these model compounds, similarly to tetrapeptide **1** enantiomers, different retention times were observed, depending on the CSP used, indicating different chiral interactions between the analyte and the crown selectors in **CR-I (+)** and **CR-I (-)**. By comparing chemical structures of model compounds **1e** - **1h** (Fig. 1), the presence of α-amino group at N-terminus (Tyr) was found to be the unique feature common in all four compounds, indicating, that the N-terminal amino group could be the driving force in stereoselective $^{+}$N–H···O hydrogen bonding between the crown ether ring of the CSP and the chiral peptide. Because the interactive site in Tyr is located next to the chiral center, bulky hydrophobic groups in crown ether selectors could provide steric hindrance, resulting in chiral environment, where stereoselective binding could occur, explaining the chiral separation of tetrapeptide **1** previously observed. From the obtained retention plots of **1h**, it can be seen that eliminating the π-systems in tetrapeptide structure has very irrelevant effect on retention.

4. Conclusions

Enantioseparation of tetrapeptide Tyr-Arg-Phe-Lys-NH_2 on CROWNPAK CR-I (+) and CR-I (-) chiral stationary phases is reported. It was established, that on **CR-I (+)**, D-form of Tyr-Arg-Phe-Lys-NH_2 enantiomers always elutes in first position, where an inversion of elution order was observed on **CR-I (-)**. Under the same LC conditions, retention of tetrapeptide isomers, possessing DXXX configuration (fixed in D-tyrosine position) on **CR-I (+)** does not differ significantly from their corresponding LXXX enantiomer retention on **CR-I (-)** column and vice versa, demonstrating the capability of roughly estimating the retention times of the corresponding enantiomer, observed on the opposite CSP. It was assumed that only in case of enantiomeric resolution, retention times of single enantiomer observed on **CR-I (+)** and **CR-I (-)** columns, ran under the same conditions, would differ from each other, indicating stereoselective binding. Retention behavior of eight model compounds were studied on **CR-I (+)** and **CR-I (-)** and it was concluded, that N-terminal α-amino group in Tyr is responsible for chiral recognition of Tyr-Arg-Phe-Lys-NH_2.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

T. Upmanis: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Funding acquisition, Writing – original draft. **H. Kažoka:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2021.100016.

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Chiral recognition mechnism studies of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide on crown ether-based chiral stationary phase

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RESEARCH ARTICLE

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Chiral recognition mechanism studies of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide on crown ether-based chiral stationary phase

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Abstract

Even though chiral recognition for crown-ether CSPs is generally understood, on a molecular level, exact mechanisms for the resolution are still unclear. Furthermore, short peptide analytes often contain multiple amino moieties capable of binding to the crown ether selector. In order to extend the understanding in chiral recognition mechanisms, polar organic mode separation of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide LLLL/DDDD enantiomers on *S*- and *R*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 stationary phases was studied with 50-mM perchloric acid in methanol as mobile phase. Deviation from the generally acceptable 1:1 stoichiometry was supported by mass spectroscopy analysis of the formed complexes between tetrapeptide enantiomer and crown ether selectors, which revealed adducts possessing 1:1, 1:2, and 1:3 stoichiometry. Further investigation of complexation induced shifts by NMR indicated on different binding mechanisms between LLLL/DDDD enantiomers of Tyr-Arg-Phe-Lys-NH_2 and crown ether selectors. Enantioselective proton shifts were observed in studied tetrapeptide tyrosine and phenylalanine residues exclusively for LLLL enantiomer upon binding with *S*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 selector (and DDDD enanti omer with *R*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 selector), indicating that these two amino acid residues contribute to chiral recognition. The obtained results were in agreement with the LC data.

KEYWORDS
amino acids, chiral recognition, crown ether CSPs, enantioselectivity, tetrapeptide

1 | INTRODUCTION

Liquid chromatography (LC) using chiral stationary phases (CSPs) is known to be extremely convenient, accurate, versatile, and widely used technique in diverse fields of applications.[1–4] By choosing a known μ-opioid receptor agonist[5] tetrapeptide Tyr-Arg-Phe-Lys-NH_2 (1; Figure 1), we have previously observed that CSPs, based on *R* and *S*-(3, 3′-diphenyl-1, 1′-binaphthyl)-20-crown-6 chiral selectors (commercially available as CROWNPAK **CR-I (+)** or **(−)**; Figure 1), are optimal for tetrapeptide **1** chiral separations.[6] It was later found out that these stationary phases also work surprisingly well in other structurally similar tetrapeptide enantioseparations.[7,8] Despite being successfully used for the separation of enantiomers of various racemic compounds containing primary amino groups,[9,10] exact mechanisms governing the binding and chiral recognition of crown ethers, incorporating an optically active 1,1′-binaphthyl unit

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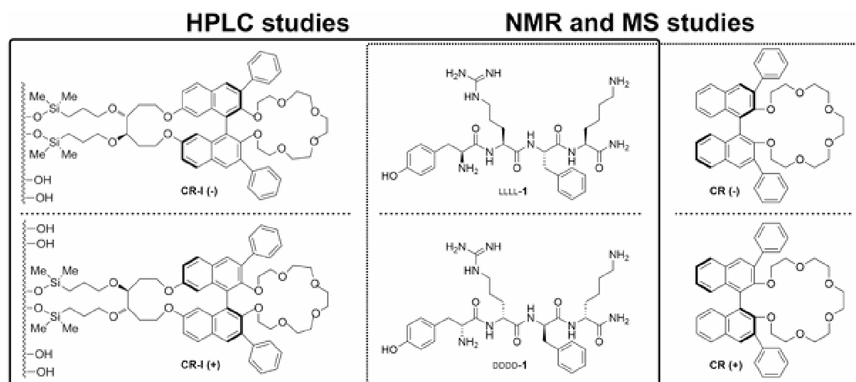

FIGURE 1 Chiral stationary phase in CROWNPAK columns: (*R*-[3,3′-diphenyl-1,1′-binaphthyl]-20-crown-6) for **CR-I (−)** and (*S*-[3,3′-diphenyl-1,1′- binaphthyl]-20-crown-6) for **CR-I (+)** immobilized on silica. Structures of LLLL/DDDD-Tyr-Arg-Phe-Lys-NH_2 tetrapeptide (**1**) enantiomers and crown ether selectors **CR (+)** and **CR (−)** used in chiral recognition studies.

first introduced by Cram and coworkers,[11] remain unclear. Therefore, creating the basis of an in-depth understanding of chiral recognition mechanisms is equally important as finding the right mobile phase and column to perform a specific chiral separation.

Many characterization methods have been employed to rationalize chiral recognition. Among numerous spectroscopic techniques,[12–14] nuclear magnetic resonance (NMR) has been extensively used to study chiral recognition of various classes of chiral selectors applied in LC[15–17] and capillary electrophoresis.[18,19] Mass spectrometry (MS) can also be useful for the formed host–guest complex detection, as well as proving stoichiometry of the complexation between molecules.[20,21] Another useful tool for gaining insight into different host–guest binding modes is X-ray diffraction by providing comprehensive information on the structural features of the formed complex structure in the solid state.[22,23] However, obtaining good-quality crystals, especially co-crystals of specific host–guest complexes, can often be problematic. Lately, computational methods have started gaining more acceptance in the community and various molecular modeling methods, together with other experimental techniques have been employed to characterize different selector-selectand complexes.[24,25]

In the present work, application of high-resolution MS and NMR is employed to study the complexes formed between crown ethers **CR (+)** and **(−)** (Figure 1) with the enantiomers of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide, with the aim to better understand the intermolecular processes behind our previously observed enantiomeric separation of tetrapeptide **1** on CROWNPAK **CR-I** CPSs.[6]

To simplify our proposed chiral recognition model, only two tetrapeptide **1** enantiomers (LLLL/DDDD) were chosen as model compounds for the study. To replicate similar conditions in NMR tube and compare the obtained chromatographic separations with the NMR chiral recognition data, experiments were performed in MeOH containing 50-mM $HClO_4$. Chiral separations under such conditions, also known as polar organic elution mode, are not typical for crown ether CSPs, and to our knowledge, no studies have been published on such applications. However, since the immobilization of chiral selector in CROWNPAK CR CSPs, no limits regarding mobile phase solvent choice exist, making the **CR-I** phases more versatile. To clarify which of the potential interaction sites are responsible for the retention and chiral recognition in tetrapeptide 1, structure–enantioselectivity relationship experiments were performed by comparing the retention behavior of the LLLL-isomer of Tyr-Arg-Phe-Lys-NH_2 and its structural analogues, synthesized with the aim to selectively exclude the interacting amino groups in tetrapeptide sequence (by replacing them with OH-groups or excluding them altogether), while maintaining the stereochemistry similar to the tetrapeptide 1 molecule.

2 | MATERIALS AND METHODS

2.1 | General procedures

Chemicals, reagents, and solvents were obtained from commercial suppliers (Acros, Sigma-Aldrich, Fluorochem, Alfa

Aesar, TCI with purity >95%) and were used without further purification. Both tetrapeptide **1** LLLL/DDDD enantiomers and all tetrapeptide **1** structural analogues **1a–1g** were synthesized (as hydrochloride salts) by solution phase peptide synthesis in place according to synthesis Schemes S1–S10. Chiral selectors **CR (+)** and **CR (−)** were synthesized following the procedure previously developed by Cram's group[26] (Scheme S11). Reactions were monitored by LCMS on a Waters Acquity H-Class UPLC system connected to a Waters Acquity QDa detector quadrupole mass spectrometer operating in the electrospray ionization (ESI) positive and negative ion mode and Waters Acquity PDA detector using reverse-phase Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm). ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a 400-MHz Bruker Avance Neo spectrometer (Bruker Biospin Gmbh) equipped with a 5-mm Double-Resonance Broadband CryoProbe with Z-gradients. Chemical shifts are referenced to the residual solvent signal. HRMS (ESI) was performed on a Waters Synapt G2-Si Mass Spectrometer in positive ionization mode. Reversed-phase chromatographic purifications of the synthesized compounds were performed using Biotage SNAP Ultra C18 cartridges on a Biotage Isolera One purification system.

2.1.1 | General procedure **P1** for *C*-terminal dipeptide building block synthesis

To a suspension of appropriate configuration N^{ε}-Boc-lysinamide (1 eq) and the appropriate N^{α}-protected amino acid (1.1 eq) in acetonitrile (MeCN, 10 mL per 1-mmol amino acid), hydroxybenzotriazole hydrate (HOBt·H_2O, 1.1 eq) is added. Reaction mixture is cooled to 0°C and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 2 eq) and *N*-methylmorpholine (NMM, 3 eq) are added. Reaction mixture is stirred at 0°C for 30 min and then at room temperature for additional 18 h. Distilled water is then added to the reaction mixture to precipitate the product, which is then filtered, washed with distilled water, and dried.

2.1.2 | General procedure **P2** for *N*-terminal dipeptide building block synthesis

To a solution of appropriate N^{α}-protected amino acid (1 eq) in dry tetrahydrofuran (THF, 10 mL per 1-mmol amino acid), *N*-hydroxysuccinimide (HOSu, 1.2 eq) is added. Reaction mixture is cooled to 0°C and *N,N′*-dicyclohexyl carbodiimide (DCC, 1.2 eq) is added. Reaction mixture is stirred at room temperature for 4 h. The appropriate configuration arginine (1 eq) is dissolved in saturated $NaHCO_3$ water solution (2 mL/1-mmol arginine) and added to the reaction mixture at 0°C. Reaction mixture is then stirred for additional 12 h, acidified with glacial acetic acid and concentrated in vacuo. Product was purified by reverse-phase flash chromatography.

2.1.3 | General procedure of desalting tetrapeptide **1** HCl salts

To a solution containing tetrapeptide **1**, equal amount by mass of Amberlite IRN-78 (OH form) ion-exchange resin was added. The solution was stirred for 30 min, the resin separated by filtering, and the solvent was evaporated under reduced pressure, resulting in tetrapeptide **1** in its free base form.

2.2 | Synthesis of Tyr-Arg-Phe-Lys-NH_2 (tetrapeptide 1) enantiomers

2.2.1 | *Tert*-butyl ((*S*)-6-amino-5-((*S*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-6-oxohexyl) carbamate (LL-**1.1**)

Synthesized following procedure **P1** for *C*-terminal dipeptide building block synthesis from 1000 mg N^{ε}-Boc-L-lysinamide hydrochloride L-**1ab** (3.55 mmol), 1160 mg N^{α}-Cbz-L-phenylalanine L-**1aa** (3.90 mmol), 543 mg HOBt·H_2O (3.55 mmol), 1360 mg EDC·HCl (7.10 mmol) and NMM (1.0 mL, 10.01 mmol) yielded 1200 mg (60%) product LL-**1.1** as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.36–7.16 (m, 10H), 5.03 (s, 2H), 4.40 (dd, *J* = 8.9, 5.9 Hz, 1H), 4.30 (dd, *J* = 9.1, 4.9 Hz, 1H), 3.13 (dd, *J* = 13.6, 6.1 Hz, 1H), 3.00 (t, *J* = 6.8, 2H), 2.89 (dd, *J* = 13.8, 8.9 Hz, 1H), 1.86–1.75 (m, 1H), 1.69–1.56 (m, 1H), 1.42 (s, 11H), 1.39–1.27 (m, 2H) ppm. HRMS (ESI/Q-TOF) m/z: [M + Na^+] calculated for $C_{28}H_{38}N_4O_6Na^+$: 549.2689, found: 549.2708.

2.2.2 | *Tert*-butyl ((*R*)-6-amino-5-((*R*)-2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-6-oxohexyl)carbamate (DD-**1.1**)

Synthesized following procedure **P1** for *C*-terminal dipeptide building block synthesis from 400 mg N^{ε}-Boc-D-lysinamide D-**1ab** (1.31 mmol), 430 mg N^{α}-Cbz-D-phenylalanine D-**1aa** (1.44 mmol), 200 mg HOBt·H_2O (1.31 mmol), 503 mg EDC·HCl (2.62 mmol) and NMM (0.4 mL, 3.93 mmol) yielded 850 mg (81%) product DD-**1.1** as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.37–7.17 (m, 10H), 5.04 (s, 2H), 4.39 (dd, *J* = 8.9, 5.9 Hz, 1H), 4.30 (dd, *J* = 9.1, 4.9 Hz, 1H), 3.13

(dd, J = 13.6, 6.1 Hz, 1H), 3.01 (t, J = 6.8, 2H), 2.89 (dd, J = 13.8, 8.9 Hz, 1H), 1.87–1.74 (m, 1H), 1.68–1.56 (m, 1H), 1.42 (s, 11H), 1.39–1.27 (m, 2H) ppm. HRMS (ESI/Q-TOF) m/z: $[M + Na^+]$ calculated for $C_{28}H_{38}N_4O_6Na^+$: 549.2689, found: 549.2703.

2.2.3 | (*tert*-butoxycarbonyl)-L-tyrosyl-L-arginine acetate (LL-**1.2**)

Synthesized following procedure **P2** for *N*-terminal dipeptide building block synthesis from 1000 mg N^α-Boc-L-tyrosine L-**1ac** (3.50 mmol), 480 mg HOSu (4.20 mmol), 860 mg DCC (4.20 mmol) and 674 mg L-arginine L-**1ad** (3.01 mmol). Product was purified by reverse phase flash chromatography (C18, MeCN/0.1% AcOH in water, 10%–90%) to give 780 mg (44%) *N*-terminal building block LL-**1.2** acetate as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.06 (d, J = 8.5 Hz, 2H), 6.70 (d, J = 8.4 Hz, 2H), 4.28–4.17 (dd, 2H), 3.29–3.13 (m, 2H), 3.06 (dd, J = 14.0, 4.7, 1H), 2.74 (dd, J = 14.0, 9.6, 1H), 1.95–1.82 (m, 1H), 1.80–1.67 (m, 1H), 1.66–1.54 (m, 2H), 1.37 (s, 9H) ppm. HRMS (ESI/Q-TOF) m/z: $[M + H^+]$ calculated for $C_{20}H_{32}N_5O_6^+$: 438.2353, found: 438.2361.

2.2.4 | (*tert*-butoxycarbonyl)-D-tyrosyl-D-arginine acetate (DD-**1.2**)

Synthesized following procedure **P2** for *N*-terminal dipeptide building block synthesis from 1000 mg N^α-Boc-D-tyrosine D-**1ac** (3.50 mmol), 480 mg HOSu (4.20 mmol), 860 mg DCC (4.20 mmol) and 670 mg D-arginine L-**1ad** (3.01 mmol). Product was purified by reverse phase flash chromatography (C18, MeCN/0.1% AcOH in water, 10%–90%) to give 1130 mg (65%) *N*-terminal building block DD-**1.2** acetate as white solid.^{1}H-NMR (400 MHz, CD_3OD) δ: 7.06 (d, J = 8.4 Hz 2H), 6.70 (d, J = 8.5 Hz, 2H), 4.29–4.17 (dd, 2H), 3.29–3.13 (m, 2H), 3.06 (dd, J = 14.0, 4.8, 1H), 2.74 (dd, J = 14.0, 9.5, 1H), 1.95–1.82 (m, 1H), 1.80–1.67 (m, 1H), 1.66–1.57 (m, 2H), 1.37 (s, 9H) ppm. HRMS (ESI/Q-TOF) m/z: $[M + H^+]$ calculated for $C_{20}H_{32}N_5O_6^+$: 438.2353, found: 438.2365.

2.2.5 | (*S*)-6-amino-2-((*S*)-2-((*S*)-2-((*S*)-2-amino-3-(4-hydroxyphenyl)propanamido)-5-guanidino-pentanamido)-3-phenylpropanamido) hexanamide hydrochloride (LLLL-**1**)

A suspension of 200-mg (0.38 mmol) *C*-terminal building block LL-**1.1** and 100-mg 10% Pd/C in 20-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 138 mg (92%) product, which, without any further purification, was used in the next step. To a solution of 138 mg (0.35 mmol) previously afforded dipeptide in 5-mL dry DMF, 153-mg *N*-terminal dipeptide LL-**1.2** (0.35 mmol) and 60-mg HOBt·H_2O (0.38 mmol) were added. Reaction mixture was cooled to 0°C, and 134-mg EDC·HCl (0.70 mmol) was added. Reaction mixture was stirred at room temperature for 2 h, diluted with 10 mL distilled water, and extracted with 3 × 35 mL EtOAc. The combined organics were washed 1 × 50 mL with brine and concentrated in vacuo. After evaporation, the residue was purified by reverse phase flash chromatography (C18, MeCN/0.1% AcOH, 10%–80%) to give 187-mg Boc-protected tetrapeptide acetate (66%), which was then suspended in 5-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 5%–55%) to give 138 mg (98%) LLLL-**1** hydrochloride as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.34–7.22 (m, 4H), 7.20 (m, 1H), 7.05 (d, J = 8.0 Hz, 2H), 6.76 (d, J = 8.5 Hz, 2H), 4.61 (dd, J = 8.5, 6.2 Hz, 1H), 4.37 (dd, J = 8.1, 5.6 Hz 1H), 4.28 (dd, J = 8.9, 5.1 Hz, 1H), 4.08 (dd, J = 8.6, 5.5 Hz, 1H), 3.23–3.12 (m, 3H), 3.09 (dd, J = 14.4, 5.5 Hz, 1H), 3.02 (dd, J = 13.8, 8.7 Hz, 1H), 2.97–2.89 (m, 2H), 2.86 (dd, J = 14.3, 8.7 Hz, 1H), 1.92–1.73 (m, 2H), 1.73–1.59 (m, 4H), 1.59–1.56 (m, 2H), 1.51–1.33 (m, 2H) ppm. ^{13}C-NMR (101 MHz, CD_3OD) δ: 176.29, 173.63, 170.01, 158.71, 138.23, 131.81, 130.56, 129.76, 128.10, 126.10, 116.98, 56.59, 55.87, 54.46, 54.24, 42.08, 40.59, 38.61, 37.94, 32.45, 30.35, 28.01, 26.17, 23.68 ppm. HRMS (ESI/Q-TOF) m/z: $[M + H^+]$ calculated for $C_{30}H_{46}N_9O_5^+$: 612.3616, found: 612.3623.

2.2.6 | (*R*)-6-amino-2-((*R*)-2-((*R*)-2-((*R*)-2-amino-3-(4-hydroxyphenyl)propanamido)-5-guanidino-pentanamido)-3-phenylpropanamido)hexanamide hydrochloride (DDDD-**1**)

A suspension of 200-mg (0.38 mmol) *C*-terminal building block DD-**1.1** and 100 mg 10% Pd/C in 20-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 145-mg (97%) product, which, without any further purification, was used in the next step. To a solution of 145-mg (0.37 mmol) previously afforded dipeptide in 5-mL dry DMF, 184-mg *N*-terminal dipeptide DD-**1.2** (0.37 mmol) and 61-mg HOBt·H_2O (0.40 mmol) were added. Reaction mixture was cooled to 0°C, and 142 mg EDC·HCl (0.74 mmol) was added. Reaction mixture was stirred at room temperature for 2 h, diluted with 10-mL

distilled water, and extracted with 3 × 35 mL EtOAc. The combined organics were washed 1 × 50 mL with brine and concentrated in vacuo. After evaporation, the residue was purified by reverse phase flash chromatography (C18, MeCN/0.1% AcOH, 10%–80%) to give 153-mg Boc-protected tetrapeptide acetate (48%), which was then suspended in 5-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 5%–55%) to give 126 mg (99%) DDDD-**1** hydrochloride as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.34–7.22 (m, 4H), 7.25–7.15 (m, 1H), 7.05 (d, J = 8.5 Hz, 2H), 6.75 (d, J = 8.5 Hz, 2H), 4.61 (dd, J = 8.5, 6.2 Hz, 1H), 4.37 (dd, J = 8.1, 5.5 Hz, 1H), 4.28 (dd, J = 8.9, 5.1 Hz, 1H), 4.08 (dd, J = 8.6, 5.5 Hz, 1H), 3.26–3.13 (m, 3H), 3.09 (dd, J = 14.4, 5.5 Hz, 1H), 3.02 (dd, J = 13.8, 8.7 Hz, 1H), 2.99–2.89 (m, 2H), 2.86 (dd, J = 14.3, 8.7 Hz, 1H), 1.92–1.73 (m, 2H), 1.73–1.61 (m, 4H), 1.60–1.55 (m, 2H), 1.50–1.36 (m, 2H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{30}H_{46}N_9O_5^+$: 612.3616, found: 612.362.

2.3 | Synthesis of tetrapeptide 1 structural analogues 1a–1g

2.3.1 | (*S*)-2-(((benzyloxy)carbonyl)amino)-6-((tert-butyldimethylsilyl)oxy)hexanoic acid (L-1**af**)

N^α-Cbz-L-lysine (2.5 g, 8.92 mmol) was dissolved in 100 mL of MeCN/H_2O (40:60) followed by 1 mL of glacial acetic acid. The solution was cooled to 0°C with ice-water bath. Sodium nitrite (2.5 g, 89.2 mmol) in 10-mL water was added dropwise to stirring N^α-Cbz-L-lysine solution over 30 min. The reaction was heated to 70°C over 20 min. The cooled reaction was concentrated in vacuo to a yellow oil, dissolved in H_2O, and acidified with glacial acetic acid. The aqueous solution was extracted 3 × 75 mL with EtOAc. The combined organics were washed 1 × 75 mL with brine. The organic phase was dried over Na_2SO_4, filtered, and concentrated in vacuo yielding 1.1 g (44%) technical N^α-Cbz-L-6-hidroxynorleucine, which was further used without purification. 1.1 g (3.91 mmol) of previously afforded N^α-Cbz-L-6-hidroxynorleucine was suspended in 50-mL dichloromethane followed by addition of 800 mg (11.73 mmol) of imidazole and 650 mg (4.30 mmol) of *tert*-butyldimethylsilyl chloride. After stirring for 16 h, reaction mixture was concentrated in vacuo to a pale yellow solid, dissolved in H_2O, and acidified with glacial acetic acid. The aqueous solution was extracted 3 × 25 mL with ethyl acetate. The combined organics were washed 1 × 75 mL with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. After evaporation, the residue was purified by reverse phase flash chromatography (C18, MeCN/H_2O, 35%–95%) to give the product L-**1af** as white solid (942 mg, 61%). ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.40–7.31 (m, 4H), 7.31–7.25 (m, 1H), 5.09 (d, J = 1.6 Hz, 2H), 4.15 (dd, J = 9.1, 4.8 Hz, 1H), 3.64 (t, J = 6.0 Hz, 2H), 1.93–1.80 (m, 1H), 1.75–1.62 (m, 1H), 1.60–1.51 (m, 2H), 1.51–1.39 (m, 2H), 0.90 (s, 9H), 0.05 (s, 6H) ppm.

2.3.2 | Benzyl (*S*)-(1-amino-6-((tert-butyldimethylsilyl)oxy)-1-oxohexan-2-yl) carbamate (L-**1ag**)

To a stirring solution of (*S*)-2-(([benzyloxy]carbonyl)amino)-6-([tert-butyldimethylsilyl]oxy)hexanoic acid L-**1af** (1 g, 2.53 mmol) in 5-mL dry dimethylformamide was added 451 mg 1,1′-carbonyldiimidazole (2.79 mmol) in one portion. The mixture was stirred at room temperature for 3 h, and then aqueous ammonia (25% solution, 0.1 mL, 3.80 mmol) was added dropwise. After stirring for an additional 1 h, reaction mixture was extracted 3 × 25 mL with ethyl acetate. The combined organics were washed 1 × 35 mL with brine, dried over Na_2SO_4, and concentrated in vacuo. After evaporation, the residue was purified by reverse phase flash chromatography (C18, MeCN/H_2O, 15–75%) to give 482-mg product L-**1ag** as white solid (48%). ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.40–7.31 (m, 4H), 7.31–7.24 (m, 1H), 5.09 (d, J = 6.4 Hz, 2H), 4.09 (dd, J = 9.1, 5.0 Hz, 1H), 3.63 (t, J = 6.1 Hz, 2H), 1.87–1.74 (m, 1H), 1.70–1.58 (m, 1H), 1.57–1.49 (m, 2H), 1.50–1.35 (m, 2H), 0.89 (s, 9H), 0.05 (s, 6H) ppm.

2.3.3 | Benzyl ((*S*)-1-(((*S*)-1-amino-6-([tert-butyldimethylsilyl]oxy)-1-oxohexan-2-yl) amino)-1-oxo-3-phenylpropan-2-yl)carbamate (LL-**1.1a**)

A suspension of 370-mg (1.1 mmol) benzyl (*S*)-(1-amino-6-([tert-butyldimethylsilyl]oxy)-1-oxohexan-2-yl)carbamate L-**1ag** and 100-mg 10% Pd/C in 15 mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 284-mg (99%) product, which, without any further purification, was used in the next step. To a suspension of previously afforded 284-mg product (1.09 mmol) in 25-mL MeCN, 326-mg N^α-Cbz-L-phenylalanine **1aa** and 167-mg HOBt·H_2O (1.09 mmol) were added. Reaction mixture was cooled to 0°C, and 419-mg EDC·HCl (2.18 mmol) and 0.3-mL NMM (3.27 mmol) were added. Reaction mixture was stirred at 0°C for 30 min and

then at room temperature for additional 18 h. Distilled water was then added to the reaction mixture to precipitate the product, which was then filtered, washed with distilled water, and dried, yielding 471-mg (80%) *C*-terminal dipeptide building block **1.1a** as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.37–7.16 (m, 10H), 5.03 (d, *J* = 3.0 Hz, 2H), 4.40 (dd, *J* = 8.9, 5.8 Hz, 1H), 4.30 (dd, *J* = 8.9, 5.2 Hz, 1H), 3.62 (t, *J* = 6.3 Hz, 2H), 3.12 (dd, *J* = 13.8, 8.8 Hz, 1H), 2.89 (dd, *J* = 13.8, 8.9 Hz, 1H), 1.91–1.76 (m, 1H), 1.70–1.57 (m, 1H), 1.57–1.45 (m, 2H), 1.45–1.31 (m, 2H), 0.89 (s, 9H), 0.06 (s, 6H) ppm.

2.3.4 | (3-(4-hydroxyphenyl)propanoyl) arginine (L-**1.2a**)

Synthesized following procedure **P2** for *N*-terminal dipeptide building block synthesis from 500-mg 3-(4-hydroxyphenyl) propanoic acid **1ah** (3.01 mmol), 415-mg HOSu (3.61 mmol), 744-mg DCC (3.61 mmol), and 524-mg L-arginine L-**1ad** (3.01 mmol). Product was purified by reverse phase flash chromatography (C18, MeCN/0.1% AcOH in water, 10%–90%) to give 527-mg (48%) *N*-terminal building block **1.2a** acetate as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.03 (d, *J* = 8.5 Hz, 2H), 6.69 (d, *J* = 8.5 Hz, 2H), 4.25 (dd, *J* = 7.7, 5.1), 3.22–3.04 (m, 2H), 2.82 (t, *J* = 7.6 Hz, 2H), 2.50 (t, *J* = 7.6 Hz, 2H), 1.85–1.72 (m, 1H), 1.68–1.55 (m, 1H), 1.53–1.35 (m, 2H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{15}H_{23}N_4O_4^+$: 323.1719, found: 323.1725.

2.3.5 | (*S*)-2-((*S*)-2-((*S*)-5-guanidino-2-(3-(4-hydroxyphenyl)propanamido) pentanamido)-3-phenylpropanamido)-6-hydroxyhexanamide hydrochloride (**1b**)

A suspension of 225-mg (0.47 mmol) *C*-terminal building block LL-**1.1a** and 100-mg 10% Pd/C in 20-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 152-mg (83%) product, which, without any further purification, was used in the next step. To a solution of 152-mg (0.39 mmol) previously afforded dipeptide in 3-mL dry DMF, 147-mg *N*-terminal dipeptide LL-**1.2a** (0.39 mmol) and 60-mg HOBt·H_2O (0.39 mmol) were added. Reaction mixture was cooled to 0°C, and 150-mg EDC·HCl (0.78 mmol) was added. Reaction mixture was stirred at room temperature for 2 h, diluted with 10 mL distilled water, and extracted with 3 × 35 mL EtOAc. The combined organics were washed 1 × 50 mL with brine and concentrated in vacuo. After evaporation, the residue was purified by reverse phase flash chromatography (C18, MeCN/0.1% AcOH, 10%–80%) to give 112-mg protected tetrapeptide acetate (61%), which was then suspended in 5-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 5%–55%) to give 90-mg (91%) tetrapeptide analogue LLL-**1b** hydrochloride as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.32–7.18 (m, 5H), 7.02 (d, *J* = 8.5 Hz, 2H), 6.70 (d, *J* = 8.5 Hz, 2H), 4.62 (dd, *J* = 8.6, 6.0 Hz, 1H), 4.28 (dd, *J* = 11.1, 5.2 Hz, 1H), 4.24 (dd, *J* = 7.9, 6.2 Hz, 1H), 3.55 (t, *J* = 6.5 Hz, 2H), 3.16 (dd, *J* = 13.9, 6.0 Hz, 1H), 3.10 (t, *J* = 7.1 Hz, 2H), 2.97 (dd, *J* = 14.0, 8.6 Hz, 1H), 2.78 (t, *J* = 7.6 Hz, 2H), 2.47 (td, *J* = 7.4, 1.7 Hz, 2H), 1.90–1.76 (m, 1H), 1.74–1.62 (m, 2H), 1.61–1.47 (m, 3H), 1.45–1.32 (m, 4H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{30}H_{44}N_7O_6^+$: 598.3353, found: 598.3367.

2.3.6 | (*S*)-2-((*S*)-2-((*S*)-2-((*S*)-2-amino-3-(4-hydroxyphenyl)propanamido)-5-guanidinopentanamido)-3-phenylpropanamido)-6-hydroxyhexanamide (**1e**)

A suspension of 280-mg (0.51 mmol) *C*-terminal building block LL-**1.1a** and 100-mg 10% Pd/C in 20-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 200-mg (95%) product, which, without any further purification, was used in the next step. To a solution of 200-mg (0.49 mmol) previously afforded dipeptide in 5-mL dry DMF, 214-mg *N*-terminal dipeptide LL-**1.2** (0.49 mmol) and 75-mg HOBt·H_2O (0.49 mmol) were added. Reaction mixture was cooled to 0°C, and 188-mg EDC·HCl (0.98 mmol) was added. Reaction mixture was stirred at room temperature for 2 h, diluted with 10-mL distilled water, and extracted with 3 × 35 mL EtOAc. The combined organics were washed 1 × 50 mL with brine and concentrated in vacuo. After evaporation, the residue was purified by reverse phase flash chromatography (C18, MeCN/0.1% AcOH, 15%–65%) to give 197-mg protected tetrapeptide acetate (49%), which was then suspended in 5-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 5%–55%) to give 50-mg (40%) tetrapeptide analogue LLL-**1e** hydrochloride as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.35–7.24 (m, 4H), 7.24–7.15 (m, 1H), 7.07 (d, *J* = 8.5 Hz, 2H), 6.76 (d, *J* = 8.5 Hz, 2H), 4.66 (dd, *J* = 8.4, 6.4 Hz, 1H), 4.37

(t, $J = 6.8$ Hz, 1H), 4.28 (dd, $J = 9.0$, 5.0 Hz, 1H), 4.07 (dd, $J = 8.6$, 5.4 Hz, 1H), 3.55 (t, $J = 6.4$ Hz, 2H), 3.22–3.15 (m, 3H), 3.09 (dd, $J = 14.5$, 5.4 Hz, 1H), 3.00 (dd, $J = 13.9$, 8.4 Hz, 1H), 2.84 (dd, $J = 14.4$, 8.8 Hz, 1H), 1.89–1.76 (m, 2H), 1.76–1.63 (m, 2H), 1.62–1.56 (m, 2H), 1.58–1.51 (m, 2H), 1.49–1.34 (m, 2H) ppm. m/z: $[M + H^+]$ calculated for $C_{30}H_{45}N_8O_6^+$: 613.3494, found: 613.3488.

2.3.7 | Benzyl ((9*S*,12*S*,15*S*)-12-benzyl-9-carbamoyl-2,2,3,3,21,21,22,22-octamethyl-11,14-dioxo-4,20-dioxa-10,13-diaza-3,21-disilatricosan-15-yl)carbamate (**1.1b**)

A suspension of 315-mg (0.58 mmol) *C*-terminal dipeptide building block LL-**1.1a** and 100-mg 10% Pd/C in 20-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 180-mg (79%) product, which, without any further purification, was used in the next step. To a suspension of 180-mg (0.46 mmol) previously afforded dipeptide in 25-mL MeCN, 180-mg (0.46 mmol) (*S*)-2-(([benzyloxy]carbonyl)amino)-6-([tert-butyldimethylsilyl]oxy)-hexanoic acid L-**1ab** and 70-mg HOBt·H_2O (0.46 mmol) were added. Reaction mixture was cooled to 0°C, and 177-mg EDC·HCl (0.92 mmol) was added. Reaction mixture was stirred at 0°C for 30 min and then at room temperature for additional 18 h. Distilled water was then added to the reaction mixture to precipitate the product, which was then filtered, washed with distilled water, and dried, yielding 250-mg (70%) *C*-terminal tripeptide building block **1.1b** as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.40–7.29 (m, 5H), 7.29–7.14 (m, 5H), 5.08 (d,$J = 16.4$ Hz, 2H), 4.58 (dd, $J = 8.3$, 6.2 Hz, 1H), 4.13–4.01 (dd, 2H), 3.63–3.50 (t, 4H), 3.17 (dd, $J = 14.0$, 6.1 Hz, 1H), 2.98 (dd, $J = 14.0$, 6.1 Hz, 1H), 1.88–1.75 (m, 2H), 1.72–1.59 (m, 2H), 1.58–1.43 (m, 6H), 1.42–1.28 (m, 2H), 0.89 (s, 18H), 0.05 (s, 12H) ppm.

2.3.8 | (*S*)-*N*-((*S*)-1-(((*S*)-1-amino-6-hydroxy-1-oxohexan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-6-hydroxy-2-(3-(4-hydroxyphenyl)propanamido)hexanamide (**1a**)

A suspension of 125-mg (0.16 mmol) *C*-terminal tripeptide building block LLL-**1.ba** and 100-mg 10% Pd/C in 15 mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 90-mg (87%) product, which, without any further purification, was used in the next step. To a suspension of 90-mg (0.14 mmol) previously afforded tripeptide in 20-mL MeCN, 23-mg (0.14 mmol) 3-(4-hydroxyphenyl) propanoic acid **1ah** and 21-mg HOBt·H_2O (0.14 mmol) were added. Reaction mixture was cooled to 0°C, and 54-mg EDC·HCl (0.28 mmol) was added. Reaction mixture was stirred at 0°C for 30 min and then at room temperature for additional 18 h. Distilled water was then added to the reaction mixture to precipitate the product, which was then filtered, washed with distilled water, and dried, yielding 40 mg (36%) protected tetrapeptide analogue, which was then suspended in 3-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 5%–55%) to give 21-mg (74%) tetrapeptide analogue LLL-**1a** as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.32–7.15 (m, 5H), 7.01 (d, $J = 8.5$ Hz, 2H), 6.69 (d, $J = 8.5$ Hz, 2H), 4.59 (dd, $J = 8.3$, 6.1 Hz, 1H), 4.27 (dd, $J = 9.2$, 4.9 Hz, 1H), 4.16 (dd, $J = 8.7$, 5.5 Hz, 1H), 3.54 (t, $J = 6.5$ Hz, 2H), 3.49 (t, $J = 6.6$ Hz, 2H), 3.15 (dd, $J = 13.9$, 6.1 Hz, 1H), 2.98 (dd, $J = 13.9$, 8.3 Hz, 1H), 2.78 (t, $J = 8.1$ Hz, 2H), 2.45 (td, $J = 7.7$, 1.9 Hz, 2H), 1.90–1.77 (m, 1H), 1.74–1.56 (m, 2H), 1.57–1.31 (m, 7H), 1.30–1.12 (m, 2H) ppm. HRMS (ESI/Q-TOF) m/z: $[M + H^+]$ calculated for $C_{30}H_{43}N_4O_6^+$: 571.3126, found: 571.3122.

2.3.9 | (*S*)-2-((*S*)-2-amino-3-(4-hydroxyphenyl)propanamido)-N-((*S*)-1-(((*S*)-1-amino-6-hydroxy-1-oxohexan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-6-hydroxyhexanamide (**1f**)

A suspension of 125-mg (0.16 mmol) *C*-terminal tripeptide building block LLL-**1.ba** and 100-mg 10% Pd/C in 15-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 90-mg (87%) product, which, without any further purification, was used in the next step. To a suspension of 90-mg (0.14 mmol) previously afforded tripeptide in 20 mL MeCN, 33-mg (0.14 mmol) N^{α}-Boc-L-tyrosine L-**1 ac** and 21-mg HOBt·H_2O (0.14 mmol) were added. Reaction mixture was cooled to 0°C, and 54-mg EDC·HCl (0.28 mmol) was added. Reaction mixture was stirred at 0°C for 30 min and then at room temperature for additional 18 h. Distilled water was then added to the reaction mixture to precipitate the product, which was then filtered, washed with distilled water, and dried, yielding 70-mg (50%) protected tetrapeptide analogue, which was

then suspended in 3-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 5%–80%) to give 30 mg (70%) tetrapeptide analogue LLLL-**1f** hydrochloride as white solid. ^{1}N-NMR (400 MHz, CD_3OD) δ: 7.33–7.24 (m, 4H), 7.24–7.17 (m, 1H), 7.08 (d, J = 8.5 Hz, 2H), 6.77 (d, J = 8.6 Hz, 2H), 4.65 (dd, J = 8.1, 6.5 Hz, 1H), 4.35 (dd, J = 8.6, 5.7 Hz, 1H), 4.29 (dd, J = 8.9, 5.1 Hz, 1H), 4.04 (dd, J = 8.9, 5.2 Hz, 1H), 3.54 (t, J = 6.4 Hz, 4H), 3.15 (dd, J = 13.8, 6.5 Hz, 1H), 3.11 (dd, J = 14.4, 5.1 Hz, 1H), 3.00 (dd, J = 13.9, 8.2 Hz, 1H), 2.84 (dd, J = 14.5, 8.8 Hz, 1H), 1.89–1.70 (m, 2H), 1.70–1.64 (m, 1H), 1.64–1.46 (m, 5H), 1.46–1.31 (m, 4H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{30}H_{44}N_5O_7^+$: 586.3241, found: 586.3235.

2.3.10 | (*S*)-6-amino-2-((*S*)-2-((*S*)-5-guanidino-2-(3-(4-hydroxyphenyl)propanamido)pentanamido)-3-phenylpropanamido)hexanamide (**1c**)

A suspension of 200-mg (0.38 mmol) *C*-terminal building block LL-**1.1** and 100-mg 10% Pd/C in 20-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 136 mg (92%) product, which, without any further purification, was used in the next step. To a solution of 136-mg (0.34 mmol) previously afforded dipeptide in 3-mL dry DMF, 136-mg *N*-terminal dipeptide LL-**1.2a** (0.34 mmol) and 52-mg HOBt·H_2O (0.34 mmol) were added. Reaction mixture was cooled to 0°C, and 131-mg EDC·HCl (0.68 mmol) was added. Reaction mixture was stirred at room temperature for 2 h, diluted with 10-mL distilled water, and extracted with 3 × 35 mL EtOAc. The combined organics were washed 1 × 50 mL with brine and concentrated in vacuo. After evaporation, the residue was purified by reverse phase flash chromatography (C18, MeCN/0.1% AcOH, 10%–80%) to give 130-mg protected tetrapeptide acetate (51%), which was then suspended in 5-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 5%–55%) to give 51-mg (40%) tetrapeptide analogue LLL-**1c** hydrochloride as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.32–7.16 (m, 5H), 7.01 (d, J = 8.5 Hz, 2H), 6.70 (d, J = 8.5 Hz, 2H), 4.59 (dd, J = 8.7, 5.9 Hz, 1H), 4.29 (dd, J = 9.4, 4.8 Hz, 1H), 4.18 (dd, J = 8.5, 5.7 Hz, 1H), 3.15 (dd, J = 13.9, 6.0 Hz, 1H), 3.08 (t, J = 7.0 Hz, 2H), 2.99 (dd, J = 13.9, 8.7 Hz, 1H), 2.91 (t, J = 7.6 Hz, 2H), 2.78 (t, J = 7.6 Hz, 2H), 2.49 (td, J = 7.4, 2.9 Hz, 2H), 1.95–1.80 (m, 1H), 1.76–1.60 (m, 4H), 1.59–1.49 (m, 1H), 1.48–1.32 (m, 4H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{30}H_{45}N_8O_5^+$: 597.3313, found: 597.3520.

2.3.11 | Benzyl *tert*-butyl ((9*S*,12*S*,15*S*)-12-benzyl-15-carbamoyl-2,2,3,3-tetramethyl-10,13-dioxo-4-oxa-11,14-diaza-3-silanonadecane-9,19-diyl)dicarbamate (**1.1c**)

A suspension of 225-mg (0.43 mmol) *C*-terminal dipeptide building block LL-**1.1** and 100-mg 10% Pd/C in 20-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 161-mg (95%) product, which, without any further purification, was used in the next step. To a suspension of 161-mg (0.41 mmol) previously afforded dipeptide in 25-mL MeCN, 160-mg (0.41 mmol) (*S*)-2-(([benzyloxy]carbonyl)amino)-6-([tert-butyldimethylsilyl]oxy)-hexanoic acid L-**1ab** and 63-mg HOBt·H_2O (0.41 mmol) were added. Reaction mixture was cooled to 0°C, and 157-mg EDC·HCl (0.82 mmol) and 0.15-mL NMM (1.23 mmol) were added. Reaction mixture was stirred at 0°C for 30 min and then at room temperature for additional 18 h. Distilled water was then added to the reaction mixture to precipitate the product, which was then filtered, washed with distilled water, and dried, yielding 240-mg (76%) *C*-terminal tripeptide building block **1.1c** as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.39–7.28 (m, 5H), 7.29–7.14 (m, 5H), 5.07 (d, J = 16.8 Hz, 2H), 4.61 (dd, J = 8.3, 6.1 Hz, 1H), 4.27 (dd, J = 9.2, 4.8 Hz, 1H), 4.02 (dd, J = 8.5, 5.2 Hz, 1H), 3.58 (t, J = 6.4 Hz, 2H), 3.16 (dd, J = 14.0, 6.0 Hz, 1H), 3.05–2.95 (m, 3H), 1.83–1.78 (m, 1H), 1.70–1.60 (m, 2H), 1.59–1.52 (m, 1H), 1.52–1.44 (m, 4H), 1.41 (s, 9H), 1.36–1.26 (m, 4H), 0.89 (s, 9H), 0.05 (s, 6H) ppm.

2.3.12 | (*S*)-*N*-((*S*)-1-(((*S*)-1,6-diamino-1-oxohexan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-6-hydroxy-2-(3-(4-hydroxyphenyl)propanamido)hexanamide (**1d**)

A suspension of 140-mg (0.18 mmol) *C*-terminal tripeptide building block LLL-**1.1c** and 100-mg 10% Pd/C in 15-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 102-mg (88%) product, which, without any further purification, was used in the next step. To a suspension of 102-mg (0.16 mmol) previously afforded tripeptide in

20-mL MeCN, 27-mg (0.16 mmol) 3-(4-hydroxyphenyl) propanoic acid L-**1ah** and 25-mg HOBt·H_2O (0.16 mmol) were added. Reaction mixture was cooled to 0°C, and 63-mg EDC·HCl (0.32 mmol) was added. Reaction mixture was stirred at 0°C for 30 min and then at room temperature for additional 18 h. Distilled water was then added to the reaction mixture to precipitate the product, which was then filtered, washed with distilled water, and dried, yielding 65-mg (51%) protected tetrapeptide analogue, which was then suspended in 3-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 5%–55%) to give 36-mg (72%) tetrapeptide analogue LLL-**1d** hydrochloride as white solid. ^{1}H-NMR (CD_3OD) δ: 7.32–7.17 (m, 5H), 7.02 (d, J = 8.5 Hz, 2H), 6.70 (d, J = 8.5 Hz, 2H), 4.57 (dd, J = 8.5, 6.3 Hz, 1H), 4.30 (dd, J = 9.6, 4.6 Hz, 1H), 4.12 (dd, J = 8.7, 5.6 Hz, 1H), 3.50 (t, J = 6.6 Hz, 2H), 3.15 (dd, J = 13.8, 6.2 Hz, 1H), 3.01 (dd, J = 13.8, 8.6 Hz, 1H), 2.92 (t, J = 7.5 Hz, 2H), 2.79 (t, J = 7.6 Hz, 2H), 2.50 (td, J = 7.4, 2.1 Hz, 2H), 1.94–1.81 (m, 1H), 1.76–1.55 (m, 4H), 1.55–1.49 (m, 1H), 1.49–1.36 (m, 4H), 1.28–1.12 (m, 2H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{30}H_{44}N_5O_6^+$: 570.3292, found: 570.3302.

2.3.13 | (*S*)-2-((*S*)-2-amino-3-(4-hydroxyphenyl)propanamido)-*N*-((*S*)-1-(((*S*)-1,6-diamino-1-oxohexan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-6-hydroxyhexanamide (**1g**)

A suspension of 300-mg (0.39 mmol) *C*-terminal tripeptide building block LLL-**1.1c** and 100-mg 10% Pd/C in 15-mL isopropanol was stirred under H_2 atmosphere for 90 min, and then the mixture was filtered through a PTFE filter. The filtrate was concentrated in vacuo to yield 216-mg (87%) product, which, without any further purification, was used in the next step. To a suspension of 216-mg (0.34 mmol) previously afforded tripeptide in 30-mL MeCN, 96-mg (0.34 mmol) N^α-Boc-L-tyrosine L-**1 ac** and 52-mg HOBt·H_2O (0.34 mmol) were added. Reaction mixture was cooled to 0°C, and 130-mg EDC·HCl (0.68 mmol) was added. Reaction mixture was stirred at 0°C for 30 min and then at room temperature for additional 18 h. Distilled water was then added to the reaction mixture to precipitate the product, which was then filtered, washed with distilled water, and dried, yielding 150-mg (44%) protected tetrapeptide analogue, which was then suspended in 4-mL dry DCM. 0.1-mL TFA (1.3 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 90 min. Product was concentrated in vacuo and purified by reverse phase flash chromatography (C18, MeCN/0.1% HCl, 2%–55%) to give 60-mg (68%) tetrapeptide analogue LLLL-**1g** hydrochloride as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.34–7.25 (m, 4H), 7.25–7.15 (m, 1H), 7.10 (d, J = 8.1 Hz, 2H), 6.77 (d, J = 8.5 Hz, 2H), 4.60 (dd, J = 8.2, 6.5 Hz, 1H), 4.35 (dd, J = 8.4, 5.7 Hz, 1H), 4.30 (dd, J = 9.3, 4.9 Hz, 1H), 4.11 (dd, J = 8.9, 5.2 Hz, 1H), 3.55 (t, J = 6.3 Hz, 2H), 3.20–3.11 (dd, 2H), 3.03 (dd, J = 13.8, 8.2 Hz, 1H), 2.92 (t, J = 7.5 Hz, 2H), 2.86 (dd, J = 14.4, 8.6 Hz, 1H), 1.93–1.82 (m, 1H), 1.82–1.71 (m, 1H), 1.70–1.59 (m, 4H), 1.59–1.49 (m, 2H), 1.48–1.32 (m, 4H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{30}H_{45}N_6O_6^+$: 585.3401, found: 585.3422.

2.4 | Synthesis of crown ether selectors CR (+) and CR (−)

2.4.1 | (*R*)-2,2′-dimethoxy-1,1′-binaphthalene (*R*-**3**)

To a solution of 5 g (*R*)-[1,1′-binaphthalene]-2,2′-diol *R*-**2** (17.0 mmol) in 100-mL dry acetone, 18.8-g K_2CO_3 (136 mmol) was added. Reaction mixture was brought to 70°C, and 4.20-mL methyl iodide (68 mmol) was added dropwise and then refluxed for 16 h. After full conversion, the reaction mixture was cooled to ambient temperature and volatile compounds were removed in vacuo. To the resulting white solid, 50-mL distilled water was added and extracted with 3 × 45 mL DCM. The combined organics were washed 1 × 35 mL with brine, dried over Na_2SO_4, and concentrated in vacuo to yield 4.0 g (75%) product *R*-**3**, which, without any further purification, was used in the next step. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.98 (d, J = 9.3 Hz, 2H), 7.87 (d, J = 7.7 Hz, 2H), 7.46 (d, J = 9.0 Hz, 2H), 7.32 (ddd, J = 8.1, 6.7, 1.3 Hz, 2H), 7.21 (ddd, J = 8.2, 6.7, 1.3 Hz, 2H), 7.11 (d, J = 3.9 Hz, 2H), 3.77 (s, 6H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{22}H_{19}O_2^+$: 315.1385, found: 315.1390.

2.4.2 | (*R*)-3,3′-dibromo-2,2′-dimethoxy-1,1′-binaphthalene (*R*-**4**)

In a *Schlenk*-flask, 2.2-mL TMEDA (14.6 mmol) was dissolved in 100-mL diethyl ether and 6.4-mL *n*-BuLi (c = 2.5 M in hexane, 14 mmol) was added at room temperature. The solution was stirred for 20 min, and 2 g (*R*)-2,2′-dimethoxy-1,1′-binaphthalene *R*-**3** (6.36 mmol) was then added in one portion and stirred for 16 h. The

resulting brownish suspension was cooled to −78°C, and 0.81-mL bromine (15.9 mmol) was slowly added. The reaction mixture was warmed to room temperature and stirred for additional 5 h. A saturated $Na_2S_2O_3$ solution was added cautiously, the reaction was acidified with HCl and extracted with with 3 × 45 mL DCM. The combined organics were washed 1 × 35 mL with brine, dried over Na_2SO_4, concentrated in vacuo, and purified by column chromatography (PE: Et_2O = 90:10) to afford 1.7-g (57%) product *R*-**4** as white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.28 (s, 2H), 7.83 (ddd, *J* = 8.7, 1.2, 0.5 Hz, 2H), 7.43 (ddd, *J* = 8.1, 6.8, 1.2 Hz, 2H), 7.27 (ddd, *J* = 8.2, 6.8, 1.3 Hz, 2H), 7.09 (d, *J* = 8.5 Hz, 2H), 3.52 (s, 6H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{22}H_{16}Br_2O_2^+$: 469.9517, found: 469.9514.

2.4.3 | (*R*)-2,2′-dimethoxy-3,3′-diphenyl-1,1′-binaphthalene (*R*-5)

In a *Schlenk*-flask, 1.7-g (*R*)-3,3′-Dibromo-2,2′-dimethoxy-1,1′-binaphthalene *R*-**4** (3.6 mmol) was dissolved in 40-mL 1,2-dimethoxyethane and 0.62 g Pd $(PPh_3)_4$ (0.54 mmol) was added. After the reaction mixture was stirred for 30 min, 1.3-g phenylboronic acid (10.8 mmol) and aqueous $NaHCO_3$ (4 g in 40 mL water) were added. The resulting suspension was then refluxed at 90°C for 16 h. Once cooled to room temperature, the mixture was extracted with 3 × 45 mL ethyl acetate. The combined organics were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by column chromatography (PE: Et_2O = 97:3), and 0.90-g (53%) product (*R*)-**5** was isolated as white solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.98 (s, 2H), 7.92 (d, *J* = 8.3 Hz, 2H), 7.81–7.74 (m, 4H), 7.51–7.44 (m, 4H), 7.44–7.36 (m, 4H), 7.32–7.20 (m, 4H), 3.19 (s, 6H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{34}H_{27}O_2^+$: 467.2011, found: 467.2010.

2.4.4 | (*R*)-3,3′-diphenyl-[1,1′-binaphthalene]-2,2′-diol (*R*-6)

Solution of 0.87-g (*R*)-2,2′-dimethoxy-3,3′-diphenyl-1,1′-bi naphthalene *R*-**5** (1.80 mmol) in 30-mL DCM was cooled to −78°C, and 6.7-mL BBr_3 (c = 1 M in hexane, 6.7 mmol) was slowly added. The reaction mixture was warmed to room temperature and stirred for 16 h and quenched with distilled water. The organic phase was separated, and the aqueous layer was extracted with 3 × 25 mL DCM. The combined organics were washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by column chromatography (PE: Et_2O = 97:3) and 0.85-g (99%) product (*R*)-**6** was isolated as pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.03 (s, 2H), 7.93 (d, *J* = 7.9 Hz, 2H), 7.78–7.70 (m, 4H), 7.55–7.45 (m, 4H), 7.45–7.36 (m, 4H), 7.33 (ddd, *J* = 8.2, 6.8, 1.4 Hz, 2H), 7.24 (d, *J* = 8.1 Hz, 2H), 5.35 (s, 2H) ppm. HRMS (ESI/Q-TOF) m/z: [M + H$^+$] calculated for $C_{32}H_{23}O_2^+$: 439.1698, found: 439.1691.

2.4.5 | (*R*)-(3,3′- diphenyl-1,1′-binaphthyl)-20-crown-16 (**CR (−)**)

Under Ar atmosphere, to a solution of 0.40-g (*R*)-3,3′-dip henyl-[1,1′-binaphthalene]-2,2′-diol *R*-**6** (0.90 mmol) in 14-mL dry THF, 0.50-g pentaethylene glycol di(p-tolue nesulfonate) (0.90 mmol) and 0.22-g potassium *tert*-buto xide (1.80 mmol) were added. The resulting bright yellow suspension was refluxed at 70°C for 72 h and then cooled to room temperature and extracted with 35-mL DCM. The extract was washed 1 × 35 mL with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by volume chromatography (DCM/EtOAc 10:90), and 0.167-g (29%) product **CR (−)** was isolated as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 8.01 (s, 2H), 7.97 (d, *J* = 8.0 Hz, 2H), 7.83–7.72 (m, 4H), 7.53–7.44 (m, 4H), 7.44–7.35 (m, 4H), 7.25 (ddd, *J* = 8.2, 6.8, 1.3 Hz, 2H), 7.11 (d, *J* = 9.0 Hz, 2H), 3.73 (ddd, *J* = 10.1, 6.3, 4.8 Hz, 2H), 3.61–3.50 (m, 2H), 3.47–3.37 (m, 4H), 3.36–3.30 (m, 4H), 3.25 (ddd, *J* = 1.11, 6.6, 2.9 Hz, 2H), 3.16 (ddd, *J* = 11.1, 5.6, 2.9 Hz, 2H), 3.06 (ddd, *J* = 10.6, 5.6, 4.8 Hz, 2H), 2.93 (ddd, *J* = 11.1, 6.4, 4.8 Hz, 2H) ppm. HRMS (ESI/Q-TOF) m/z: [M + Na$^+$] calculated for $C_{42}H_{40}NaO_6^+$: 663.2717, found: 663.2739.

2.4.6 | (*S*)-(3,3′- diphenyl-1,1′-binaphthyl)-20-crown-16 (**CR (+)**)

Under Ar atmosphere, to a solution of 0.50-g commercially obtained (*S*)-3,3′-diphenyl-[1,1′-binaphthalene]-2,2′-diol *S*-**6** (1.14 mmol) in 25-mL dry THF, 0.62-g pentaethylene glycol di(p-toluenesulfonate) (1.25 mmol) and 0.14-g potassium hydroxide (2.50 mmol) were added. The resulting bright yellow suspension was refluxed at 70°C for 72 h and then cooled to room temperature and extracted with 35-mL DCM. The extract was washed 1 × 35 mL with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by volume chromatography (DCM/EtOAc 10:90), and 0.205-g (26%) product **CR (+)** was isolated as white solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 8.01 (s, 2H), 7.96 (d, *J* = 8.1 Hz, 2H), 7.83–7.72 (m, 4H), 7.53–7.44 (m, 4H), 7.44–7.36 (m, 4H), 7.25 (ddd, *J* = 8.2, 6.8, 1.3 Hz, 2H), 7.11 (d, *J* = 8.2 Hz, 2H), 3.72 (ddd, *J* = 10.1, 6.4, 4.8 Hz,

2H), 3.66–3.50 (m, 2H), 3.47–3.37 (m, 4H), 3.36–3.30 (m,, 4H), 3.25 (ddd, $J = 1.11$, 6.6, 2.9 Hz, 2H), 3.16 (ddd, $J = 11.1$, 5.6, 2.9 Hz, 2H), 3.06 (ddd, $J = 10.6$, 5.6, 4.8 Hz, 2H), 2.92 (ddd, $J = 11.1$, 6.4, 4.8 Hz, 2H) ppm. HRMS (ESI/Q-TOF) m/z: $[M + Na^{+}]$ calculated for $C_{42}H_{40}NaO_6^{+}$: 663.2717, found: 663.2736.

2.5 | Chromatographic conditions

Experiments were performed on Waters Alliance (Waters Corporation, Milford, MA, USA) instrument equipped with 2695 separations module, consisting of quaternary pump, degasser, autosampler, and column heater. Waters 2489 dual wavelength absorbance detector was used for detection of analytes. The output signal was monitored and processed using Waters Empower 2 software.

CROWNPAK **CR-I** (+) and **CR-I** (−) columns (3.0 mm [I.D.] × 150 mm, 5 μm particle size) based on *S*- and *R*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 immobilized on silica were purchased from Chiral Technologies Europe (Illkirch, France). Gradient grade methanol (MeOH) for LC was obtained from Merck (Darmstadt, Germany). HPLC grade perchloric acid (60%) purchased from Fisher Scientific (Loughborough, Leicestershire, United Kingdom) was used as the acidic additive with a constant concentration of 50 mM in mobile phase (corresponding to 0.5% of 60% perchloric acid to the total volume) to ensure full protonation of the amino functionalities in studied compounds. Columns were conditioned with MP for no less than 3 h, prior to every experiment. Chromatographic runs were performed at a flow rate of 0.4 mL/min, and the injected volume of tetrapeptide sample solutions was set at 5 μL. The column oven was maintained at 25°C, and the UV absorption was measured at 220 nm.

2.5.1 | Preparation of tetrapeptide standard solutions

The stock solutions were prepared by dissolving 5 mg of each compound in 1 mL in methanol, giving a solution with a concentration of 5 mg/mL. Tetrapeptide 1 and its structural analogue 1a–1g standard solutions were prepared by diluting 100 μL of stock solution of each compound in 1 mL of 50-mM $HClO_4$ in MeOH (0.5 mg/mL).

2.6 | Mass spectroscopy

All data were obtained using Waters Synapt G2-Si (Waters Corporation, Milford, MA, USA) high-resolution electrospray mass spectrometer operated in positive ESI mode. The critical instrument settings that yield adduct formation include capillary voltage of 3.0 V and source temperature of 120°C. Desolvation temperature was kept at 500°C with the desolvation gas flow set to 800 L/h and cone voltage of −40 V with cone flow set to 100 L/h. Full scan MS was acquired in the m/z range of 50–1300 Da. MassLynx 4.1. software with the QuanLynx 4.1. module (Waters, Milford, MA, USA) was used for data acquisition and processing.

2.6.1 | Preparation of tetrapeptide—crown selector MS standard solutions

Four stock solutions for tetrapeptide **1** LLLL/DDDD enantiomers and **CR** (+) and (−) selectors were prepared by dissolving approximately 6.1 mg of the appropriate tetrapeptide **1** enantiomer and 6.4 mg of **CR** (+) or (−) crown selector in 1-mL methanol containing 50-mM $HClO_4$ with the resulting concentration of 10 mM. 100 μL of the appropriate tetrapeptide **1** enantiomer stock solution was mixed with 200 μL (to achieve 1:2 stoichiometry) or 500 μL (for 1:5 stoichiometry) of each crown selector **CR** (+) or (−) stock, and the mixture was diluted to the total volume of 1 mL of 50-mM $HClO_4$/MeOH. Prior injection, 5 μL of the formed complex solution was transferred to 1-mL MeOH/0.1% formic acid (1:1) to create eight MS standard solutions of tetrapeptide **1**–crown selector complexes containing 5 μM of each tetrapeptide **1** enantiomer, together with the appropriate crown ether selector **CR** (+) or (−) in 10- or 25-μM range.

2.7 | NMR spectroscopy

NMR spectra were recorded at 25°C on a 400-MHz Bruker Avance Neo spectrometer (Bruker Biospin Gmbh, Rheinstetten, Germany) equipped with a 5-mm Double-Resonance Broadband CryoProbe Prodigy probe using TMS or the residual solvent peaks as internal reference (methanol-d_4: 3.31 ppm for ^{1}H nuclei and 49.0 ppm for ^{13}C nuclei). All spectra were processed in MestReNova v. 12.0.2 (Mestrelab Research).

2.7.1 | Preparation of tetrapeptide NMR standard solutions

The stock solutions were prepared by dissolving 12.8 mg of **CR** (+) or (−) crown selector and 12.2 mg of tetrapeptide **1** enantiomer in 1-mL methanol-d_4 containing

50-mM $HClO_4$ resulting in 20-mM solution. Necessary concentrations and ratio for NMR analytical samples were prepared by pipetting the appropriate amounts of the studied compounds: 200 μL (for 10-mM sample concentration) and 100 μL (for 5-mM sample concentration) directly to 5-mm NMR tubes and diluting the mixture with methanol-d_4 containing 50-mM $HClO_4$ to a total volume of 400 μL.

3 | RESULTS AND DISCUSSION

3.1 | Polar organic mode separation of Tyr-Arg-Phe-Lys-NH_2 LLLL/DDDD enantiomers

In general, retention of primary amino compounds is achieved through inclusion complexation, driven via triple $^{+}$N–H··· O hydrogen bond formation between the ammonium ion in the protonated analyte and oxygens of the crown ether selector.[27] From a mechanistical perspective, retention of a certain chiral analyte depends on its ability to bind to the chiral selector in the CSP creating transient diastereomeric complexes. Usually, CSPs are designed to create a steric environment, where one of the isomers bind more favourably than the other. The weaker bound isomer eludes first, thus allowing us to separate the mixture. An important variable in chiral recognition is the interaction medium (mobile phase in LC), where, based on the solvent nature, mobile-phase modifiers not only compete for chiral bonding sites with the chiral solutes but also may alter the steric environment of the chiral selector.

We have previously reported[7] on Tyr-Arg-Phe-Lys-NH_2 tetrapeptide U-shape retention dependency against organic modifier content in binary MeOH containing mobile phases with constant $HClO_4$ concentration of 50 mM, ranging from 40/60 to 90/10 (v/v) methanol/water on **CR-I** phases. Thus, as a logical follow-up, we extended our investigation into how complete removal of water from the mobile phase would affect the chromatographic behavior of tetrapeptide **1** LLLL/DDDD enantiomers on both **CR-I** CSPs by assessing the application of polar organic conditions (mobile phase: 50 mM HClO4 in MeOH; Figure 2).

Decrease in retention of tetrapeptide **1** enantiomers occurred when MeOH content was first increased from 40 to 70% (Figure S1A for **CR-I (+)** and Figure S1B for **CR-I (−)**), reaching a minimum in the 70% to 80% range. However, increasing the mobile phase organic modifier content further (80%–90% MeOH range) leads to a steady increase in analyte retention pointing toward different retention mechanisms in aqueous and organic-rich mobile phases. Analyte retention can be considered as a function of balance between hydrophilic (dominant in high organic modifier content) and hydrophobic (at lower organic modifier concentration) interactions taking place between the chiral selector and the solute, therefore, based on analyte characteristics and chromatographic conditions, CSPs may act either as reversed-phase (RP) like or HILIC-like stationary phases.

By switching to polar organic conditions, it was established that tetrapeptide **1** LLLL/DDDD enantiomer chromatographic behavior follows the previously observed trend and the complete exclusion of water from mobile

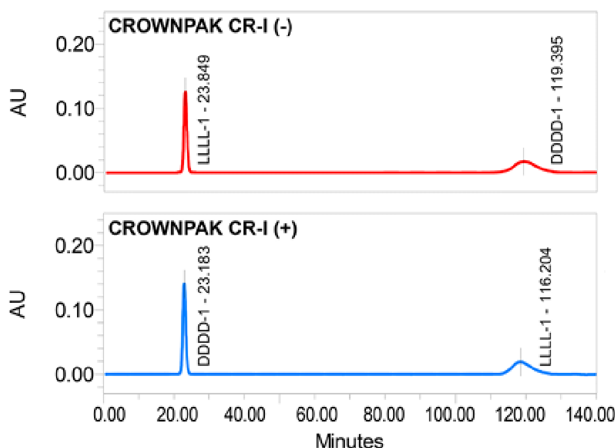

FIGURE 2 Chromatograms of LLLL/DDDD-**1** separation on CROWNPAK **CR-I** (+) (in blue) and (−) CSPs (in red); Mobile phase: 50-mM $HClO_4$ in MeOH.

phase has led to even greater analyte retention as well as the highest enantioselectivity (α; Table 1) with MeOH containing mobile phases. Moreover, α values steadily increase with the increase of MeOH concentration in mobile phase, and it is not affected by the decrease in retention in the 50% to 80% MeOH range (Figure S1). This observation might indicate on the complex competing interaction profiles between the chiral selector and tetrapeptide **1** enantiomers, based on the mobile phase composition within the whole studied range.

As a result of the reversed chirality of the selectors in **CR-I** CSPs, tetrapeptide **1** LLLL/DDDD enantiomers eluted in the opposite order: DDDD-**1** was eluted first on **CR-I (+)** CSP, whereas DDDD-**1** was retained stronger than its antipode on **CR-I (−)** CSP (Figure 2). The near identical k values obtained for LLLL-**1** and DDDD-**1** ($k = 70.7$ on **CR-I (+)** vs. $k = 72.7$ on **CR-I (−)**; Table 2), as well as DDDD-**1** ($k = 13.3$) on **CR-I (+)** and LLLL-**1** ($k = 13.7$) on **CR-I (−)** CSP, indicate on similar binding patterns between the of the stronger formed complexes (larger k values) of [LLLL-**1**–**CR-I (+)**] and [DDDD-**1**–**CR-I (−)**] and the weaker complexes of [DDDD-**1**–**CR-I (+)**] and [LLLL-**1**–**CR-I (−)**].

This observation can be particularly useful in cases where the analyte in its enantiomerically pure form is much easier accessible than racemate (e.g., amino acids, peptides, and their derivatives). Considering that other related compounds may follow similar binding patterns, retention times obtained from injecting a single enantiomer of a chiral compound separately on both **CR-I** CSPs with opposite stereochemical configuration can provide us with useful insight of the chromatographic behavior for a specific enantiomeric pair (LLLL/DDDD in this study). By assuming that only in the case of enantiomeric resolution, retention times of single enantiomer observed on **CR-I (+)** and **CR-I (−)** columns, under the same chromatographic conditions, would differ from each other (thus, indicating a stereoselective binding), the described approach also allows us to determine whether a CSP is enantioselective toward a specific chiral analyte.[7,8]

3.2 | Employing the opposite chiralities of crown ether CSPs for evaluation of chiral recognition

To determine which of the three possible binding sites in Tyr-Arg-Phe-Lys-NH_2 (N-terminal α-amino group in Tyr; ε-amino group in Lys and guanidine fragment in Arg, any of which can theoretically bind to the crown ether selector) are responsible for tetrapeptide **1** LLLL/DDDD chiral resolution, retention behavior of seven tetrapeptide **1** structural analogues **1a**–**1g** (as single enantiomers) was evaluated, by directly injecting standard solutions on opposite stereochemical configuration CSPs **CR-I (+)** and **(−)** under polar organic conditions (Figure 3) and comparing it with the known results obtained for tetrapeptide **1** LLLL enantiomer. Similar set of experiments was performed by our group previously,[7] by studying tetrapeptide **1** structural analogues **1a**–**1g** retention behavior in binary MeOH containing mobile phases, ranging from 40/60 to 90/10 (v/v) methanol/50 mM $HClO_4$ in water. However, we believe that the newly obtained results under polar organic conditions contain some important unpublished information that adds to the current understanding on tetrapeptide **1** chiral recognition.

Compounds **1a**–**1g** were synthesized with the aim to systematically exclude potentially interacting amino groups, while maintaining stereochemistry of the molecule similar to that of tetrapeptide **1**. To exclude N-terminal amino group in Tyr, chemical structure of tetrapeptide **1** was altered by introducing 3-(4-hydroxyphenyl) propanoic acid in Tyr position (compounds **1a**–**1d**). To avoid the possible interaction sites in Lys (compounds **1a**,**1b**, **1e**, and **1f**) and

TABLE 1 Obtained retention factors (k) and selectivity (α) of tetrapeptide **1** LLLL/DDDD enantiomers in mobile phases of different MeOH contents on CROWNPAK **CR-I (+)** and (−) CSPs.

		MeOH (%) in mobile phase[a]													
		40		50		60		70		80		90		100	
CSP	Enantiomer	k	α	k	α	k	α	k	α	k	α	k	α	k	α
CR-I (+)	LLLL-**1**	42.6	**3.30**	22.4	**3.50**	14.7	**3.69**	12.9	**4.08**	14.5	**4.37**	30.6	**4.91**	70.7	**5.31**
	DDDD-**1**	12.9		6.4		4.0		3.2		3.3		6.2		13.3	
CR-I (−)	LLLL-**1**	13.1	**3.16**	6.4	**3.47**	4.1	**3.75**	3.2	**3.93**	3.2	**4.46**	6.5	**4.85**	13.7	**5.30**
	DDDD-**1**	41.6		22.1		15.4		12.7		14.5		31.4		72.7	

Note: Column dimensions: 3.0 × 150 mm; the elution time of the 1st unretained peak was set as a t_0 for each chromatogram; temperature: 25°C; flow rate: 0.4 mL/min.

[a]Mobile phases consist of constant 50-mM $HClO_4$ in MeOH/water mixtures.

TABLE 2 ^{1}H chemical shifts (ppm) of pseudoracemic Tyr-Arg-Phe-Lys-NH_2 (**1**) LLLL/DDDD enantiomers in the absence and presence of **CR (+)** chiral selector at 25°C in methanol-d_4 with 50-mM $HClO_4$.

Residue	Atom*	Free[a]	[LLLL-1-CR (+)][b]	$\Delta\delta_{(LLLL)}$	[DDDD-1-CR (+)]	$\Delta\delta_{(DDDD)}$	$\Delta\Delta\delta_{(LLLL-DDDD)}$[c]
Tyr	H4	4.11	4.06	−0.05	4.10	−0.01	0.04
	H5′	3.10	2.92	−0.18	3.09	−0.01	0.17
	H5″	2.85	2.51	−0.34	2.82	−0.03	0.31
	H7	7.08	7.04	−0.04	7.07	−0.01	0.03
	H8	6.76	6.75	−0.01	6.74	−0.02	0.01
	H11	6.76	6.75	−0.01	6.74	−0.02	0.01
	H12	7.08	7.04	−0.04	7.07	−0.01	0.03
Arg	H16	4.38	4.38	0.00	4.38	0.00	0.00
	H17′	1.81	1.79	−0.02	1.81	0.00	0.02
	H17″	1.74	1.70	−0.04	1.71	−0.03	0.01
	H18	1.62	1.57	−0.05	1.61	−0.01	0.04
	H19	3.19	3.15	−0.04	3.18	−0.01	0.03
Phe	H27	4.61	4.68	0.07	4.63	0.02	0.05
	H28′	3.16	3.21	0.05	3.19	0.03	0.02
	H28″	3.03	3.05	0.02	3.04	0.01	0.01
	H30	7.30	7.35	0.05	7.31	0.01	0.04
	H31	7.30	7.35	0.05	7.31	0.01	0.04
	H32	7.20	7.21	0.01	7.20	0.01	0.01
	H33	7.30	7.35	0.05	7.31	0.01	0.04
	H34	7.30	7.35	0.05	7.31	0.01	0.04
Lys	H38	4.29	4.30	0.01	4.28	−0.01	0.02
	H39′	1.88	1.78	−0.10	1.81	−0.07	0.03
	H39″	1.70	1.65	−0.05	1.64	−0.06	0.01
	H40	1.44	1.31	−0.13	1.29	−0.15	0.02
	H41	1.67	1.44	−0.23	1.38	−0.29	0.06
	H42	2.94	2.65	−0.29	2.61	−0.33	0.04

*See Figure 5.
[a][rac-**1**] = 5 mM.
[b]The chemical shifts were based on the spectrum of pseudoracemate (LLLL:DDDD = 2:1) of **1** (5 mM) in the presence of **CR (+)** (10 mM).
[c]Obtained by subtracting [DDDD-**1**-(**CR+**)] value from the [LLLL-**1**-(**CR+**)] ones.

Arg (compounds **1a**, **1d**, **1f**, and **1g**) moieties, these amino acids were replaced with 6-hydroxynorleucine.

No retention ($t_R \sim t_0$) was observed for tetrapeptide **1** structural analogue **1a** as the complexation between primary ammonium ion (R-NH_3^+) of the analyte and crown ether ring of the CSP in the given case was eliminated by excluding all three NH_2 groups in the tetrapeptide **1** structure. The lack of chromatographic retention observed for compound **1b** suggests that the guanidine moiety in Arg has no significant effect on tetrapeptide **1** retention or chiral recognition accordingly.

In contrast to **1a** and **1b**, stronger retention was shown for compounds **1c** ($k \sim 8.9$; retention factors k of tetrapeptide **1** structural analogues **1a**–**1h** are summarized in Table S1) and **1d** ($k \sim 6.0$). Both compounds share a common primary ε-amino group in Lys moiety (Figure 3), able to participate in hydrogen bonding with the crown ether selector, thus explaining the observed retention. However, given the similar retention behavior obtained on both **CR-I (+)** and **CR-I (−)** columns, it appears that this binding is non-stereoselective. Likely, because of the ε-amino group being located four C atoms away from the chiral center, where sterically bulky aromatic groups in crown ether selectors (Figure 1) are unable to provide a chiral environment.

A possible stereoselective binding occurred for tetrapeptide **1** analogues **1e**–**1g**, where, similarly to tetrapeptide **1** enantiomers, different retention behavior on **CR-I (+)** and **(−) CSPs** was observed for the injected single

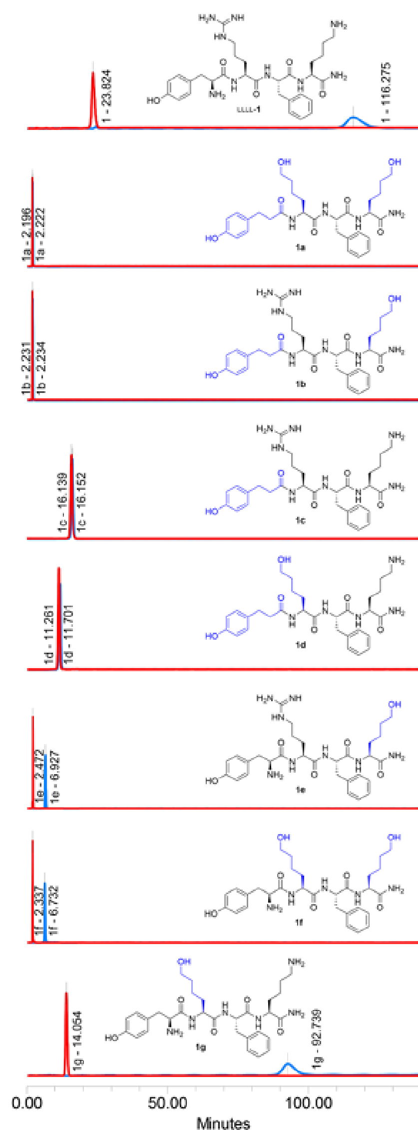

FIGURE 3 Overlay of chromatograms representing retention profiles of LLLL-**1** and tetrapeptide structural analogues **1a**–**1g** on **CR-I (+)** (in blue) and **CR-I (−)** (in red) columns. Mobile phase: 50 mM $HClO_4$ in MeOH.

enantiomers. By comparing chemical structures of **1e**–**1g** (Figure 3), the presence of N-terminal α-amino group in tyrosine was found to be the unique feature common in all three compounds, that way indicating the importance of this amino group in tetrapeptide **1** LLLL/DDDD enantiomer chiral recognition.

Interestingly, out of these three compounds, different retention behavior can be seen between **1e** (k = 0.53 on **CR-I (−)** and k = 3.28 on **CR-I (+)**); **1f** (k = 0.44 on **CR-I (−)** and k = 3.15 on **CR-I (+)**) and **1g** (k = 7.67 on **CR-I (−)** and k = 56.2 on **CR-I (+)**), where the apparent introduction of ε-amino group in Lys have led to significant increase in retention, even comparable with that of tetrapeptide **1** (k = 13.71 on **CR-I (−)** and k = 70.8 on **CR-I (+)**). This observation may indicate a deviation from the generally acceptable 1:1 stoichiometry, meaning that both N-terminal α-amino group in Tyr and ε-amino group in Lys may participate in tetrapeptide **1** chiral recognition.

This hypothesis is further supported by closer inspection of retention behavior of all Lys residue containing tetrapeptides LLLL-**1**, **1c**, **1d**, and **1g**. Based on analyte retention ($6 < k < 14$), we can assume that similar hydrogen bonding pattern through NH_3^+ in Lys moiety occurs for all four compounds on **CR-I (−)** CSP. In contrast, on **CR-I (+)** CSP, increased retention ($56 < k < 70$) was observed for compounds containing additional active site – α- NH_3^+ in Tyr moiety (in LLLL-**1** and **1g**), whereas only Lys containing tetrapeptide analogues **1c** and **1d** elu ted within the same range (as on **CR-I (−)**; $6 < k < 9$).

Analysis of tetrapeptide **1** analogue **1a**–**1g** retention behavior on **CR-I (+)** and **(−)** CSPs indicates that enantioseparation of LLLL/DDDD-**1** may be achieved because of the significant differences in binding patterns of each enantiomer. For the stronger formed [LLLL-**1**–**CR-I (+)**] and [DDDD-**1**–**CR-I (−)**], complexes, NH_3^+ in Tyr and Lys moieties may be involved in simultaneous binding with two crown ether selector molecules, whereas only NH_3^+ in Lys moiety is responsible for binding (and therefore retention) of the weaker [DDDD-**1**–**CR-I (+)**] and [LLLL-**1** and **CR-I (−)**] complexes. To support this theory, further experiments were performed.

3.3 | High-resolution MS experiments for tetrapeptide—crown selector complex determination

In order to confirm whether complexes responsible for chiral recognition of tetrapeptide **1** can be formed outside chromatographic column, high-resolution mass spectrometry (HRMS) operated in positive ESI mode was used. Optically pure *S*- and *R*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6

(**CR** (+) and (−) respectively) chiral selectors were mixed in two-fold (2:1) and five-fold (5:1) excess with the appropriate tetrapeptide **1** enantiomers and injected directly into mass spectrometer. As shown in Figure 4, the excess of the crown ether selector corresponds to intense signals at m/z 641 and 663 (for the sodium adduct).

A closer look to the obtained spectra reveals a number of low abundance signals, from which the most important can be found at m/z 626 and 631, corresponding to 1:1 and 1:2 complexes between tetrapeptide **1** enantiomers and crown selectors (Figure 4A). Furthermore, signal corresponding to 1:3 stoichiometry was observed at m/z 845 (Figure 4B), indicating that in gas phase, crown ether selector can simultaneously bind to all three amino moieties in tetrapeptide **1** structure. Interestingly, the formation of all three above-mentioned complexes also occurs for DDDD-**1** enantiomer upon complexation with **CR** (+) selector and further comparison of the intensity ratios for the formed complexes (Table S2) reveals that ratios between 1:1, 1:2, and 1:3 complexes remain similar irrespective of the quantity of the crown selector added. This may point to a different (non-stereospecific) binding pattern in gas phase than the one observed on LC columns, therefore limiting the use of this technique to study chiral recognition. Nonetheless, the use of ESI-HRMS proved the formation of non-covalent complexes between tetrapeptide

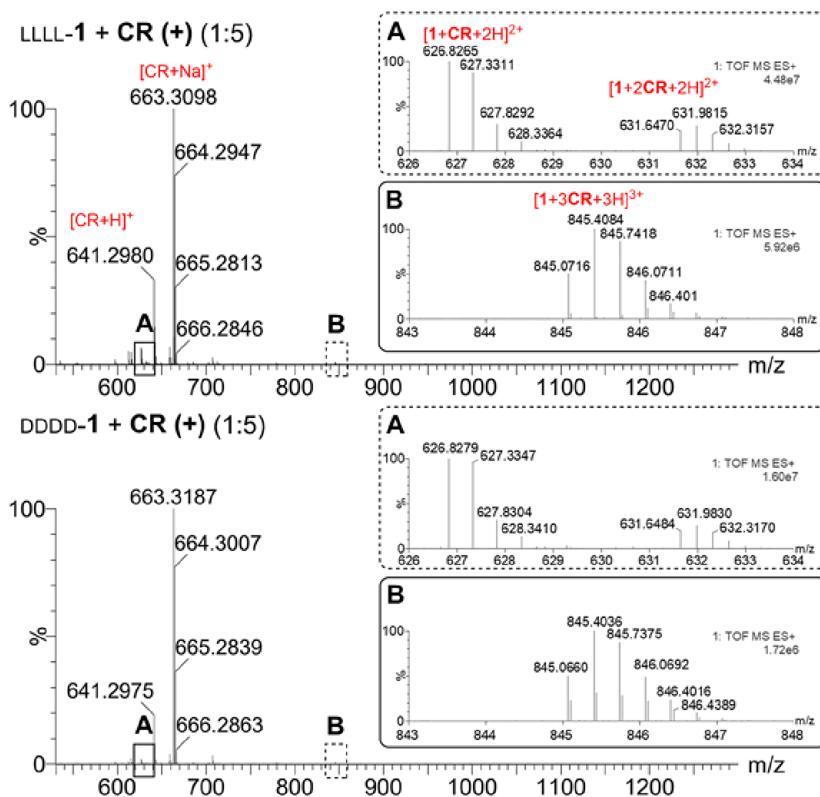

FIGURE 4 Partial ESI-HRMS spectra of tetrapeptide **1** LLLL enantiomer upon complexation with **CR** (+) chiral selector (in five-fold excess) (top); DDDD enantiomer with **CR** (+) chiral selector (in five-fold excess) (bottom). Mass signals corresponding to 1:1 and 1:2 complex adducts are represented in cut-out A; mass signals corresponding to 1:3 adduct are represented in cut-out B.

1 and crown selectors as well as provided us with valuable information on the different possible stoichiometry of complexes that was used in further research.

3.4 | NMR study of complexation induced shifts upon enantioselective binding between Tyr-Arg-Phe-Lys-NH_2 enantiomers and crown ether selectors

To study the binding properties of the formed diastereomeric complexes between synthesized **CR (+)** and **(−)** (chiral selectors in **CR-I** CSPs; Figure 1) and the LLLL/DDDD enantiomers of tetrapeptide **1**, several 1D and 2D NMR experiments were carried out. To generate conditions similar to those used in the chromatographic separations, all complexation experiments were carried out in 50 mM $HClO_4$ in methanol-d_4, where tetrapeptide **1** pseudoracemate (LLLL:DDDD = 2:1 for identification) in its free base form (preparation procedure in Section 2.1.3) was mixed with crown ether selectors **CR (+)** and **(−)** in two-fold excess (Figure 5). Increasing the amount of crown selector, past the 2:1 stoichiometry did not lead to any significant changes in the observed chemical shifts (Figures S2 and S3).

Tetrapeptide **1** NMR signals (referred to according to the numbering scheme shown in Figure 5) were assigned by combination of ^1H-^1H correlation spectroscopy (COSY), ^1H-^{13}C heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments (Figures S4–S6). Further, complexation-induced shifts of the Tyr-Arg-Phe-Lys-NH_2 signals upon enantioselective binding were assessed to discuss the possible intermolecular interaction profile, leading to the chiral recognition of tetrapeptide **1** LLLL/DDDD enantiomers.

Migration of tetrapeptide **1** proton signals, shown in Figure 5, indicates a successful formation of diastereomeric complexes between tetrapeptide **1** enantiomers and crown ether selectors. Upon complexation with **CR (+)** selector, tetrapeptide **1** LLLL-isomer exhibited greater chemical shift changes ($\Delta\delta$, relative to the free form of tetrapeptide **1**) than its DDDD antipode. The opposite behavior was observed for **CR (−)** selector, where, upon complexation, greater chemical shift changes were shown by DDDD-**1**. Moreover, both obtained spectra (Figure 5) are each other mirror images, supporting our previous assumption of identical binding patterns between LLLL-**1** and **CR (+)**, and DDDD-**1** and **CR (−)** selectors and vice versa, observed in LC. Therefore, only the case of complexation between LLLL-**1** and **CR (+)** is addressed in the following discussion.

Overlapped signals were assigned with the help of total correlation spectroscopy (TOCSY; Figure S7) and the ^1H shifts are summarized in Table 2. Pronounced upfield shifts (negative $\Delta\delta$ values; ~−0.3 ppm) were observed for H42 proton in tetrapeptide **1** Lys residue for both enantiomers.

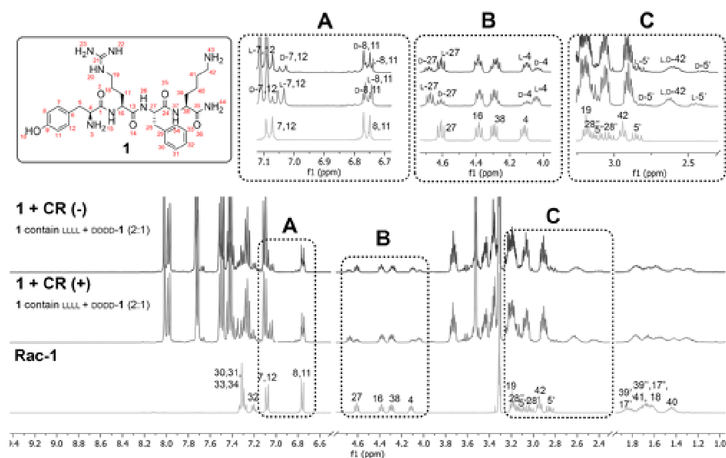

FIGURE 5 ^1H-NMR (400 MHz) spectra of free pseudoracemic tetrapeptide **1** (5 mM; LLLL:DDDD = 2:1) (bottom); complex with 10 mM **CR (+)** (middle), and 10 mM **CR (−)** (top). All spectra are obtained in methanol-d_4 containing 50-mM $HClO_4$. Signal splitting of the Tyr aromatic protons is highlighted in cut-out A; signal splitting for the α-protons in Phe in Tyr residues are highlighted in B; chemical shift changes in β-protons in Tyr residue and Lys ε-protons are highlighted in C.

The shielding effect was the strongest in close proximity to the ε-NH_2 group and weakened (Table 2; Δδ weakens in order H42 > H41 > H40 > H39″ > H39′ > H38) as the chiral center (H38, Δδ ~ 0, Table 2) was approached. This behavior indicates on some sort of intermolecular binding, possibly a hydrogen bonding between tetrapeptide **1** and crown ether selector. This assumption is supported by older studies,[28] where shielding effect has been observed for amino acid complexes upon hydrogen bonding with **CR (+)** selector. However, according to the near identical proton chemical shift differences ($\Delta\Delta\delta_{(LLLL\text{-}DDDD)}$ ~ 0) of formed complexes between LLLL and DDDD enantiomers of tetrapeptide **1**, represented in Table 2, it could be speculated that both enantiomers undergo similar (non-enantioselective) hydrogen bonding pattern between protonated ε-NH_2 group in Lys moiety and the oxygens of crown ether selectors.

A significant difference (Figure 5C; Table 2) in proton chemical shifts ($\Delta\Delta\delta_{(LLLL\text{-}DDDD)}$) upon complexation between LLLL and DDDD enantiomers of tetrapeptide **1** and the crown ether selector was observed for β-protons H5″ ($\Delta\Delta\delta_{(LLLL\text{-}DDDD)}$ ~ 0.3 ppm) and H5′ ($\Delta\Delta\delta_{(LLLL\text{-}DDDD)}$ ~ 0.2 ppm) in LLLL-**1** Tyr residue, which, similarly to protons in Lys moiety, shifted upfield. Weaker upfield shifts for LLLL enantiomer in presence of **CR (+)** were also observed in Tyr α-proton H4 (Figure 5B; $\Delta\delta_{(LLLL)} = -0.05$ ppm in Table 1) and aromatic protons H7 and H12 (Figure 5A; $\Delta\delta_{(LLLL)} = -0.04$ ppm in Table 2), whereas the same proton shifts in DDDD enantiomer Tyr moiety were practically unaffected ($\Delta\delta_{(DDDD)}$ ~ 0 ppm in Table 2). These observations strongly suggest, that in addition to non-enantioselective hydrogen bonding between tetrapeptide **1** Lys moiety and **CR (+)** selector, a secondary hydrogen bonding interaction occurs between protonated α-NH_2 in Tyr residue exclusively between LLLL-**1** and second **CR (+)** selector molecule (DDDD-**1** and second **CR (−)** selector molecule) that might be responsible for chiral recognition. This is also in agreement with the data obtained from chiral separations of tetrapeptide **1** and chromatographic behavior analysis of structural analogues **1a–1g** (see Section 3.2).

Minor differences in proton chemical shifts ($\Delta\Delta\delta_{(LLLL\text{-}DDDD)}$, Table 2) upon complexation between LLLL and DDDD enantiomers of tetrapeptide **1** and crown ether selectors were also observed for Phe residue of LLLL-**1**. However, unlike previously discussed upfield shifts, induced by hydrogen bonding, weak deshielding (positive $\Delta\delta_{(LLLL)}$ values in Table 2) of Phe α-proton H27 (Figure 5B; $\Delta\delta_{(LLLL)} = 0.05$ ppm in Table 2), β-protons H28″ ($\Delta\delta_{(LLLL)}$ ~ 0.02 ppm), H28′ ($\Delta\delta_{(LLLL)}$ ~ 0.05 ppm), and aromatic H30, H31, H33, and H34 protons ($\Delta\delta_{(LLLL)}$ ~ 0.05 ppm) occur exclusively for tetrapeptide **1** LLLL enantiomer in presence of **CR (+)** and likely also contribute to chiral recognition. Based on the different character of chemical shift change, we can speculate that a different type of intermolecular interaction, possibly involving π-systems of Phe moiety in LLLL enantiomer and the aromatics of **CR (+)** selector, occurs.

4 | CONCLUSIONS

Analysis of tetrapeptide **1** analogue **1a–1g** retention behavior on **CR-I (+)** and **(−)** CSPs with 50 mM perchloric acid in methanol as mobile phase indicates that enantioseparation of LLLL/DDDD-**1** may be achieved because of the significant differences in binding patterns of each enantiomer. Two amino moieties present in tetrapeptide Tyr and Lys residues may participate in complexation for the stronger retained enantiomer, whereas only the amino moiety in Lys may be responsible for the retention of the opposite enantiomer. This hypothesis was supported by HRMS experiments, where adducts corresponding to 1:1, 1:2, and 1:3 complexes between tetrapeptide **1** enantiomers and crown ether selectors were observed. Further study by NMR revealed complexation induced chemical shifts in Lys protons for both tetrapeptide **1** enantiomers, whereas enantioselective proton shifts were observed in Tyr and Phe exclusively for LLLL-**1** upon binding with **CR (+)** selector (and DDDD-**1** with **CR (−)** selector), indicating that these two amino acid residues contribute to chiral recognition. These data are in agreement with the obtained LC data and suggest deviation from the generally acceptable 1:1 stoichiometry in chiral chromatography.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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