UNIVERSITY OF LATVIA

Summary of Doctoral Thesis

Toms Upmanis

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

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FACULTY OF MEDICINE AND LIFE SCIENCES

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

SUMMARY OF THE DOCTORAL THESIS

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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

CONTENTS

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].

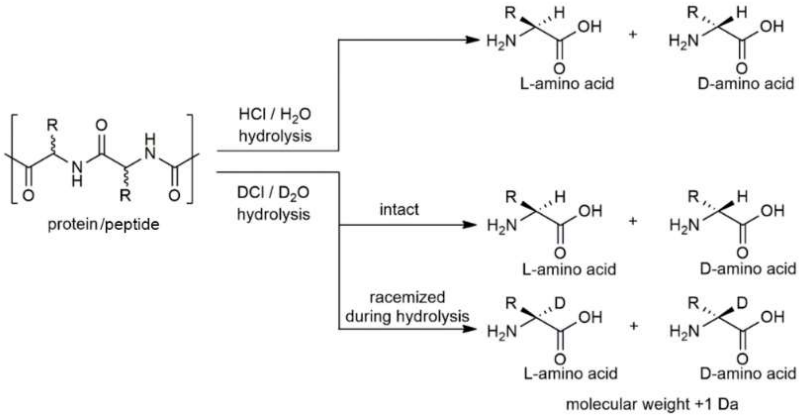

Fig. 1.1. **Schematic representation of DCl / D_2O peptide hydrolysis** [16].

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].

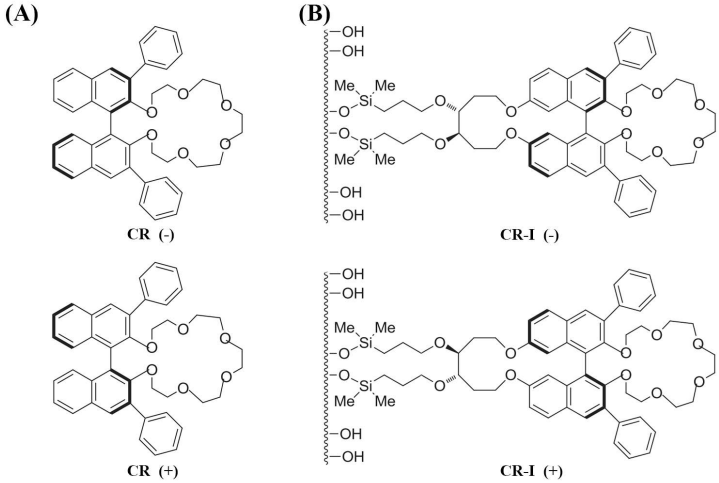

Fig. 1.2. **Chemical structures of:** (*R*)- and (*S*)-(3,3'-diphenyl-1,1-binaphthyl)-20-crown-16 chiral selectors found in CRONWPAK **CR (-)** and **(+)** CSPs - **(A)**; immobilized chiral selectors found in CROWNPAK **CR-I (-)** and **(+)** CSPs – **(B).**

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].

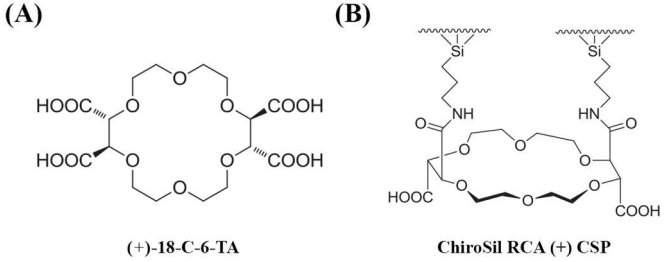

Fig. 1.3. **Chemical structures of:** (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid – **(A)**; immobilized **(+)-18-C-6-TA** chiral selector found in ChiroSil **RCA (+)** CSP – **(B)**.

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**.

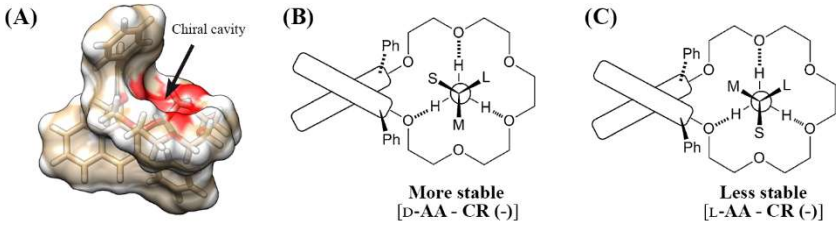

Fig. 1.5. **The proposed chiral recognition mode CR (-) selector:** Electrostatic potential surface of **CR (-)** chiral selector obtained from crystal structure [59], visualized by *Chimera 1.16* software – **(A)**; The proposed chiral recognition mode showing the more – **(B)**; and less stable complexes – **(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**.

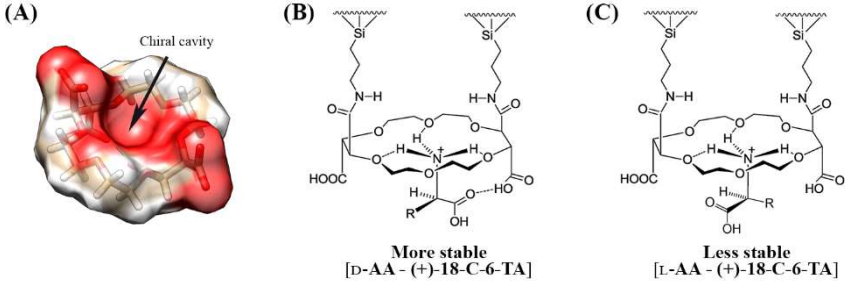

Fig. 1.6. **The proposed chiral recognition mode of (+)-18-C-6-TA selector:** Electrostatic potential surface of **(+)-18-C-6-TA** selector, obtained from crystal structure [61], visualized by *Chimera 1.16* software – **(A)**; The proposed chiral recognition mode showing the more stable **(B)** and 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.

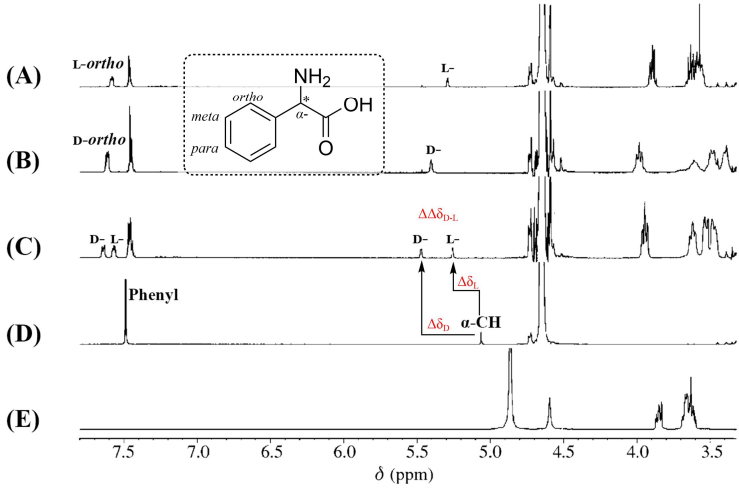

Fig. 1.7. **^{1}H-NMR spectra of phenylglycine and phenylglycine/(+)-18-C-6-TA complex with equimolar mixtures (2 mM each in MeOH-*d*$_4$):** L-phenylglycine with (+)-**18-C-6-TA - (A)**; D-phenylglycine with (+)-**18-C-6-TA - (B)**; racemic phenylglycine with (+)-**18-C-6-TA - (C)**, racemic phenylglycine - **(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.).

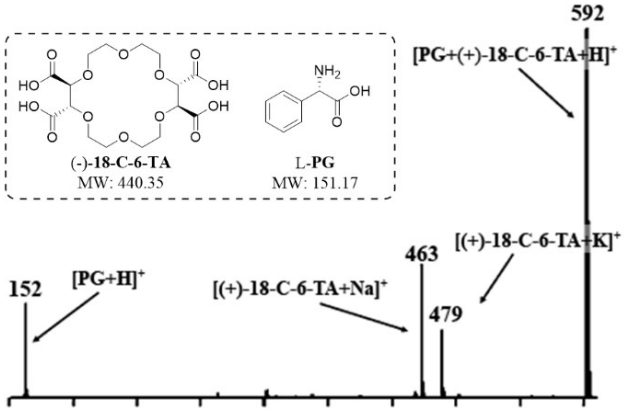

Fig. 1.8. **Partial ESI-TOF mass spectra of an equimolar solution (10^{-5} M in MeOH) of L-phenylglycine (PG) with (-)-18-C-6-TA** [51]**.**

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.

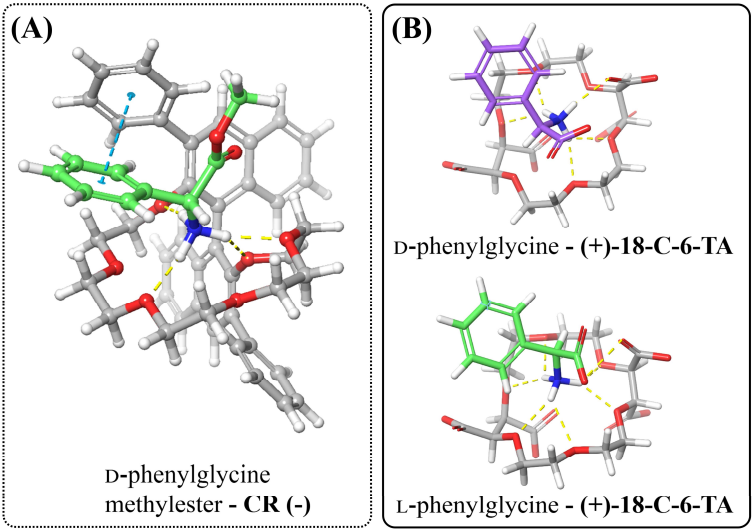

Fig. 1.9. **Stereoview of the intermolecular interactions in complex crystals obtained by X-ray diffraction:** D-phenylglycine methylester and **CR (-)** selector [59] – **(A)**; between phenylglycine enantiomers and **(+)-18-C-6-TCA** selector [74] – **(B)**. Interactions were visualized with *Maestro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

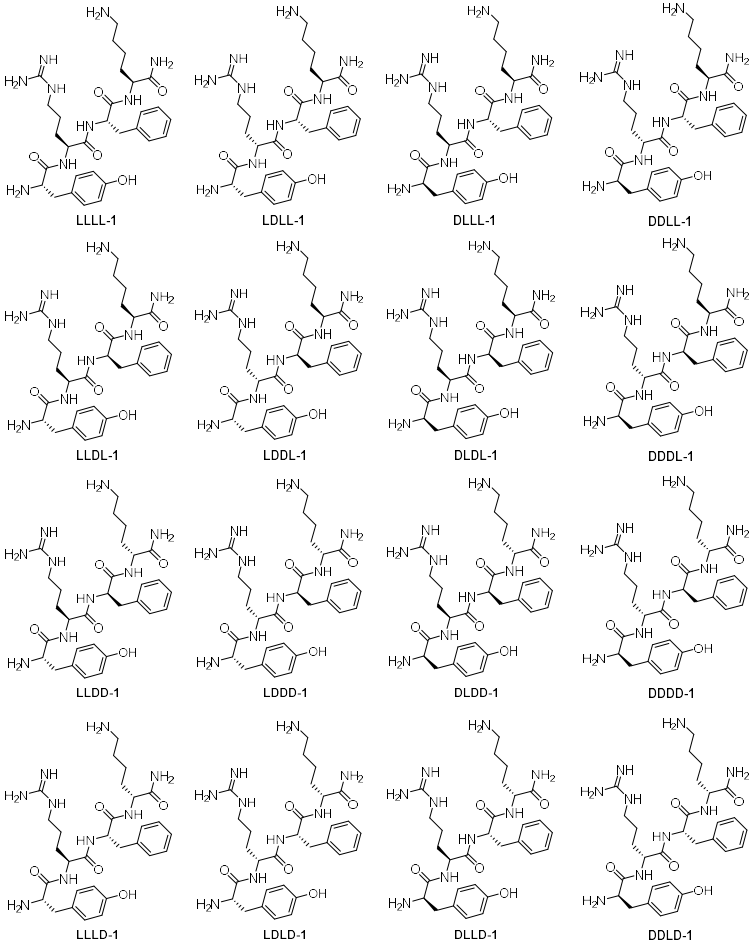

Fig. 1.10. **Structures of Tyr-Arg-Phe-Lys-NH_2 stereoisomers.**

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*-

[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).

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 ($R\text{-}NH_3^+$) of the analyte. In order to confirm this hypothesis, further in-depth studies have been carried out.

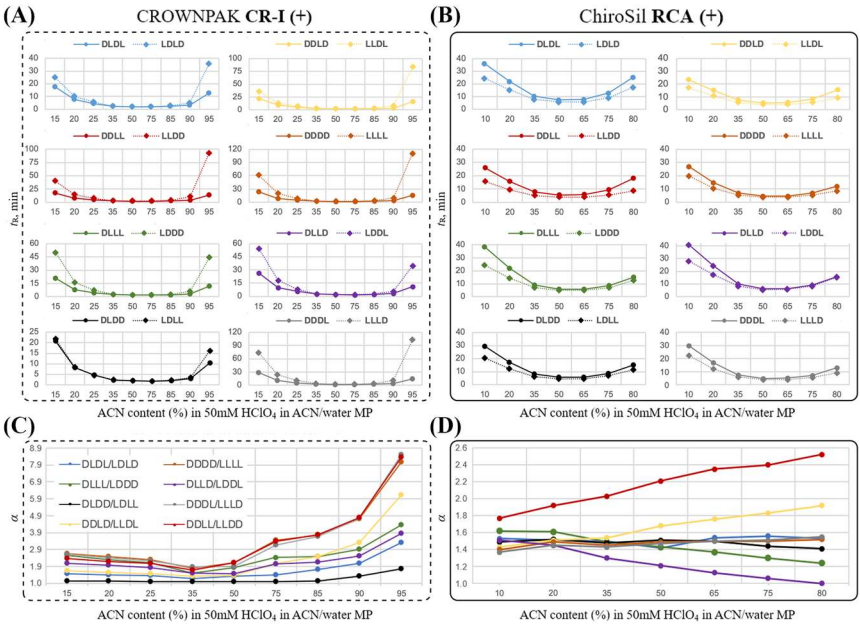

Fig. 2.1. **Effect of ACN content in mobile phase on Tyr-Arg-Phe-Lys-NH_2 stereoisomer retention times (t_R) and enantiomer separation (α):** on CROWNPAK **CR-I (+)** – (**A**) and (**C**); on ChiroSil **RCA (+)** – (**B**) and (**D**).

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, $\alpha > 1.2$ is considered sufficient for chromatographic separation between two compounds (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.

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.

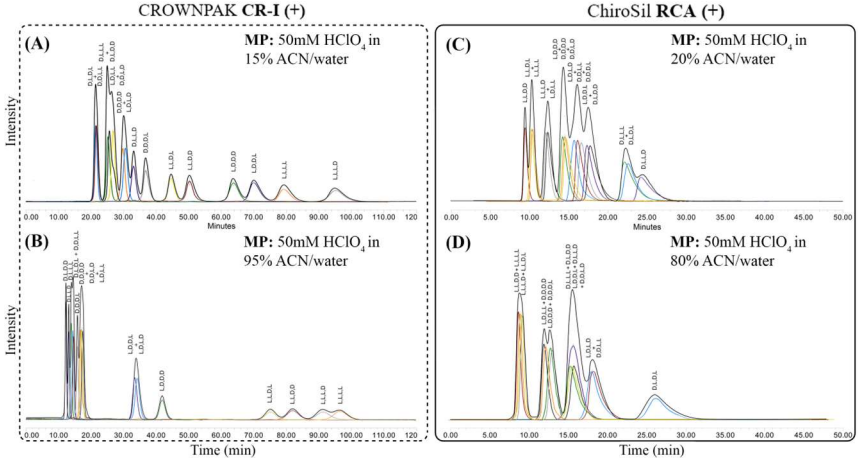

Fig. 2.2. **Overlay of chromatograms of all 16 Tyr-Arg-Phe-Lys-NH_2 stereoisomer (*in black*) and enantiomer (*in colour*) separations on CR-I (+) CSP - (A) and (B); RCA (+) CSP – (C) and (D).** Columns: CROWNPAK **CR-I (+)** (3.0 ×150 mm, 5µm); ChiroSil **RCA (+)** (4.6 ×150 mm, 5µm); Flow rate: F = 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.

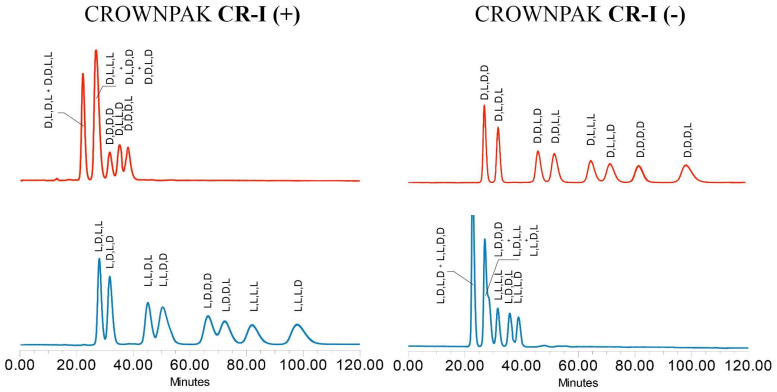

Fig. 2.3. **Separation chromatograms of mixed stereoisomer standard solutions:** of eight DXXX (***in red***) and LXXX (***in blue***) Tyr-Arg-Phe-Lys-NH_2 stereoisomers on CROWNPAK **CR-I (+)** and CROWNPAK **CR-I (-)** columns. Mobile phase: 50 mM $HClO_4$ in ACN/water 15/85 (v/v).

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.

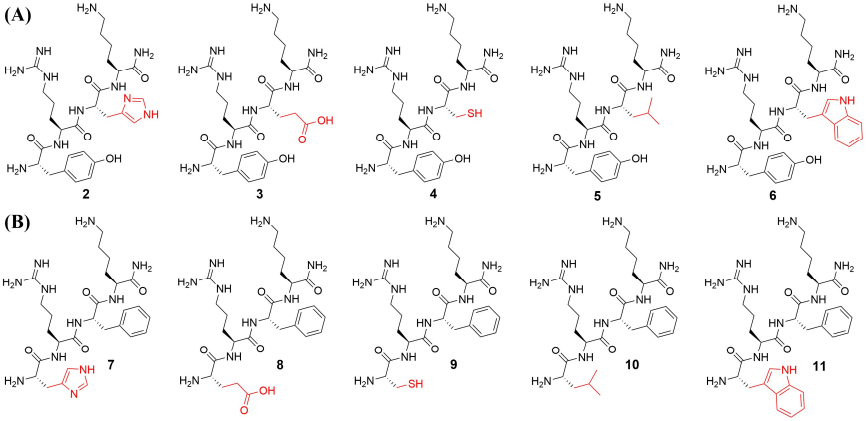

Fig. 2.4. **Structures of tetrapeptide 1 modified analogues:** tetrapeptides **2** – **6** modified at Phe position – (**A**); tetrapeptides **7** – **11** modified at N-terminus – (**B**).

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

[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)

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 >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.

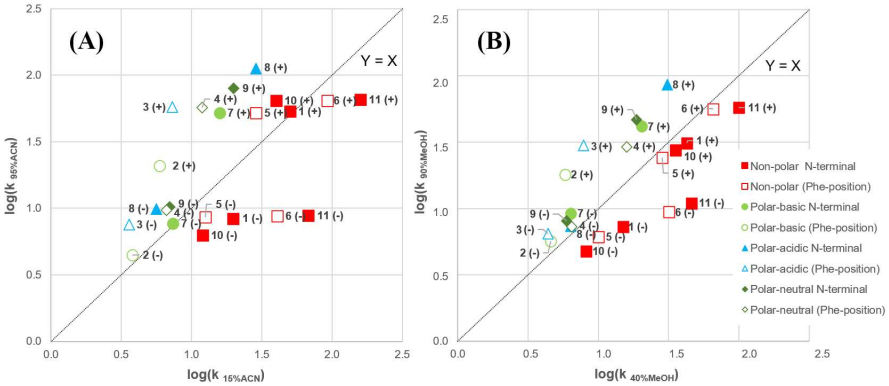

Fig. 2.5. **The effect of introduced amino acid residue on tetrapeptide 1 – 11 retention depending on the mobile phase composition:** plots of log (k 15%ACN) *vs.* log (k 95%ACN) – for 50 mM $HClO_4$ in ACN/water containing MPs – **(A)**; log (k 40%MeOH) *vs.* log (k 90%MeOH) – for 50 mM $HClO_4$ in 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.).

$$\alpha = \frac{k_2}{k_1}, \tag{2.1.}$$

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.).

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

Where α* - "apparent" enantioselectivity;

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

$k_{(-)}$ – retention observed 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.

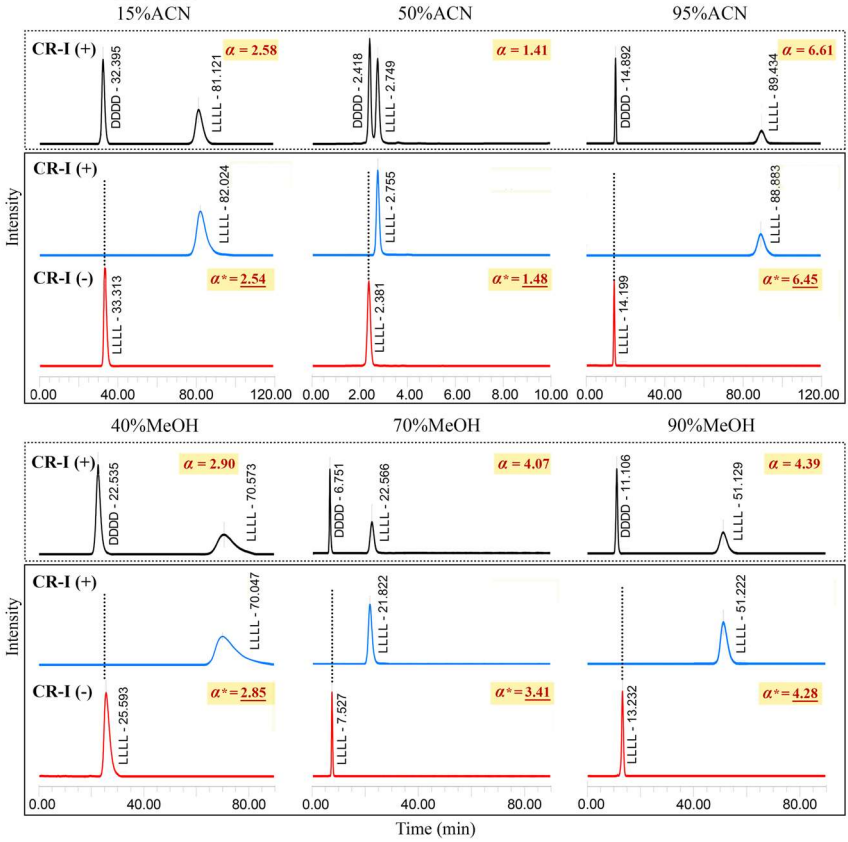

Fig. 2.6. **Chromatograms characterizing tetrapeptide 1 LLLL and DDDD enantiomer chromatographic behaviour on CROWNPAK CR-I CSPs:** tetrapeptide **1** LLLL/DDDD enantiomer separation on **CR-I (+)** (*represented in black*); retention of LLLL enantiomer on **CR-I (+)** (*in blue*); retention of LLLL 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** ($\alpha^*(1)$) 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.).

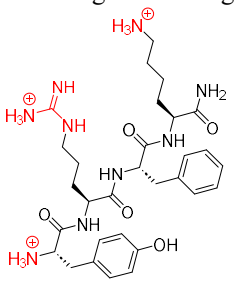

Fig. 2.7. **Tetrapeptide 1 functional groups capable of interacting.** Potential H-bond donors are highlighted in red.

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

[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)

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.

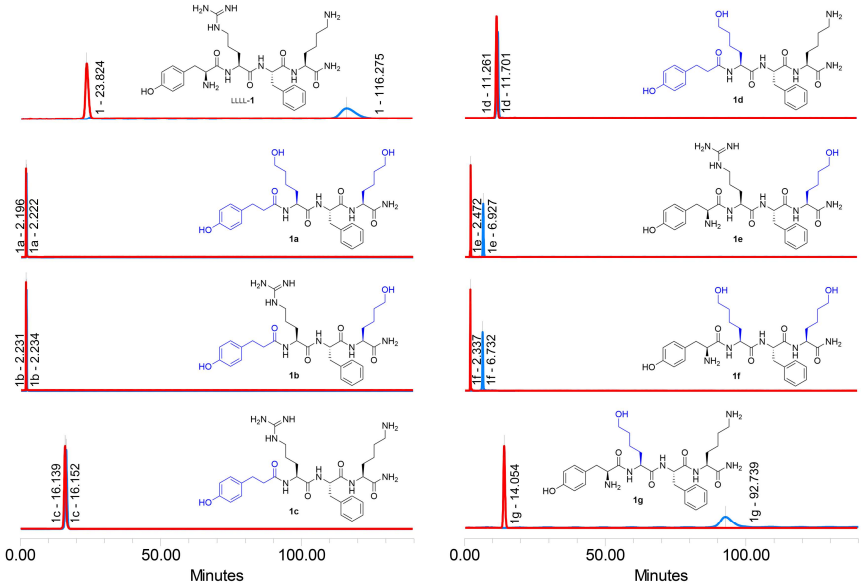

Fig. 2.8. **Overlay of chromatograms representing retention of LLLL-1 and structural analogues 1a – 1g on CR-I (+) (*represented in blue*) and CR-I (-) (*in red*) CSPs.** Mobile phase: 50mM $HClO_4$ in MeOH.

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 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.

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. 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.

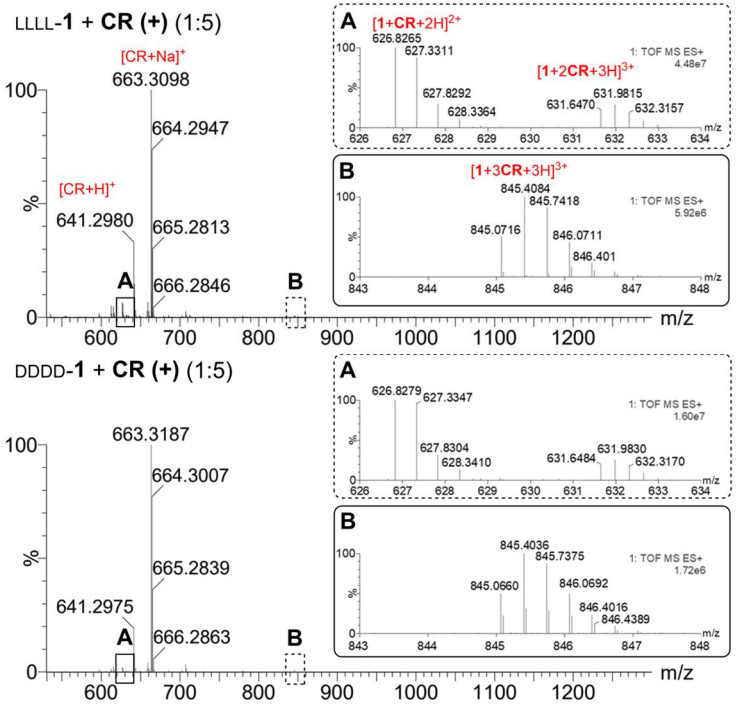

Fig. 2.9. **Partial ESI-TOF mass spectra of tetrapeptide 1 upon complexation:** LLLL-**1** with **CR (+)** chiral selector (in fivefold excess; *represented on top*); DDDD-**1** with **CR (+)** chiral selector (in fivefold excess; *bottom*). Mass signals corresponding to 1:1 and 1:2 complex adduct are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

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).

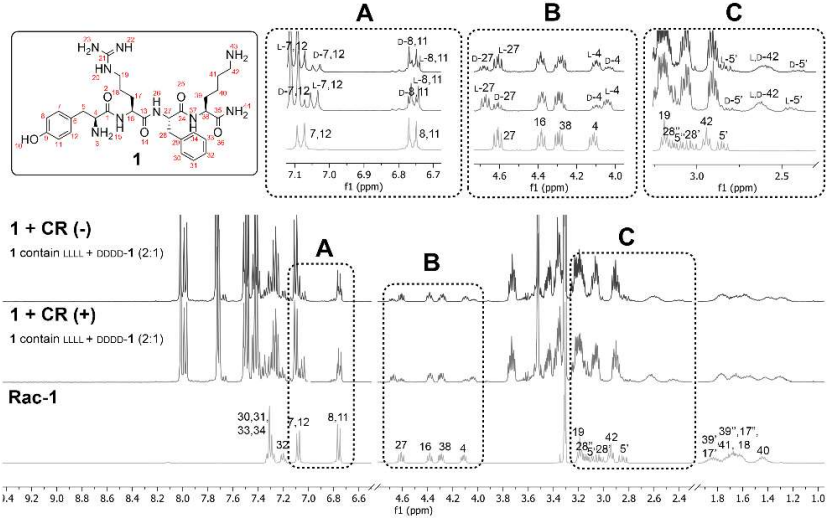

Fig. 2.10. **^{1}H-NMR (400 MHz) spectra:** of free 5 mM pseudoracemic tetrapeptide **1** (Rac-**1;** LLLL:DDDD = 2:1; *represented in bottom*); complexes with 10 mM **CR (+)** (*middle*) and 10mM **CR (-)** selectors (*top*). All spectra are obtained in methanol-d_4 containing 50 mM $HClO_4$. Signal 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-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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