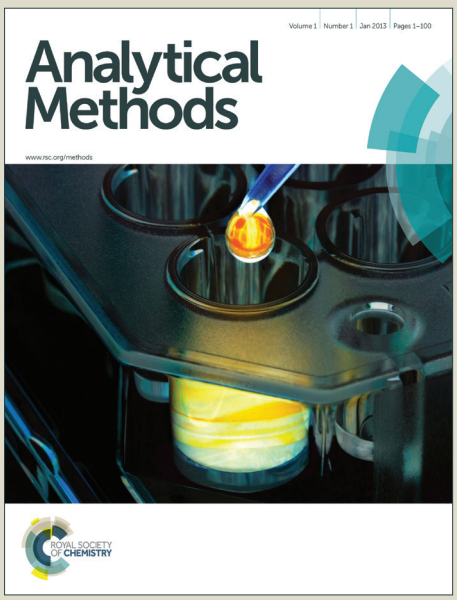
Analytical Methods

Accepted Manuscript



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Preparation and characterization of tripeptide chiral stationary phases with varying amino acid sequences and terminal groups

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ABSTRACT

In this study, two tripeptide bonded chiral stationary phases (CSPs) on silica have been prepared and evaluated in order to investigate the effect of amino acid sequence and 5-Nitronicotinoyl as the terminal group on chiral separation in HPLC and to study chiral recognition mechanism. Phenylalanine, proline, and valine were selected as the chiral moiety of CSPs, and the resultant two CSPs (CSP 1 and CSP 2) provide effective recognition and separation of adrenoceptor agonists and analytes containing amide or naphthalene ring with different selectivity profiles. CSP 1 typically affords higher enantioselectivity for those analytes facilitating stronger π-π interaction, whereas CSP 2 is more pronounced for separation of enantiomers bearing more hydrophobic groups. This work has demonstrated the importance of amino acid sequences which strongly impacted the chiral separation selectivities of these two CSPs. 5-Nitronicotinoyl as analogue of 3,5- Dinitrobenzoyl(DNB) exhibited similar enantioseparation ability and could form π-π interaction with analytes by combining with phenylalanine.

Keywords: chiral stationary phase; peptide; chiral separation; 5-Nitronicotinoyl chloride

1. Introduction

Since it was found that some enantiomers exhibited quite different biological properties in protein binding, pharmacodynamics, pharmacokinetics and toxicity, the importance of marketing chiral drugs as pure enantiomers has been realized [1,2]. Therefore, the field of enantioseparation gets paid a lot of attention. Especially, fast, efficient and direct enantioseparations on chiral stationary phases (CSPs) by high-performance liquid chromatography (HPLC) have been studied extansively in the last few decades [3,4].

"Three-point rule" is considered as the most suitable mechanism to explain enantioseparation on amino acid-derived CSPs, because the amino acids are small molecules [5-7]. In the case of CSPs with proteins as chiral selectors which have shown excellent enantioselectivity, the mechanism can be much more complex [8,9]. It was found that hydrophobic, electrostatic and hydrogen bonding interactions were responsible for chiral recognition on protein-based CSPs, and the good enantioselectivity might be due to tertiary structure of proteins, especially the hydrophobic pocket or cleft [10]. The structures of proteins are closely related to the

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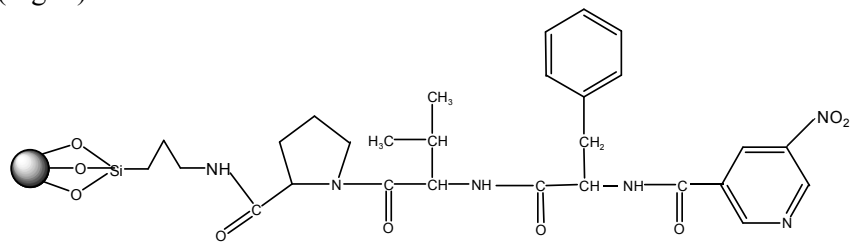
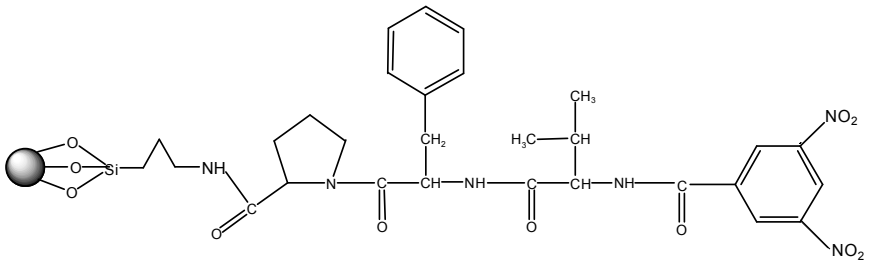
three-dimensional structures of peptides. Peptides as chiral selectors would offer similar promise [11] if not the same as proteins. Many studies related to peptide chiral stationary phases were done to invested the effect of peptide length or linker to the enantioselectivity. We have been interested in developing chiral columns using short oligopeptides consisting of different amino acids with varied sequences to investigate their combined effects and help us to understand the enantioselectivity of proteins. In this study, we developed two novel CSPs based on tripeptides based on proline(Pro), valine(Val) and phenylalanine(Phe), which are all hydrophobic amino acids and had shown broad applicability as chiral selectors of CSPs in the past. For a peptide chiral selector, one way to achieve conformational rigidity is chemically bonded it to aminopropyl silica (APS) through a cyclic structure (like proline), which holds a favorable structure of the interaction sites for chiral recognition [12,13]. Thus, the proline with a cyclic structure is designed to attach to APS first to introduce conformational rigidity into the chiral selector and enhance its enantioselectivity. Pirkle and coworkers have replaced phenyl ring with group that could only cause sterical hindrance to develop a novel chiral stationary phase, Consequently, different chiral recognition and good chromatographic performance can be achieved [14,15]. Thus, we switched the positions of Val (sterical hindrance) and Phe (phenyl ring) to develop two CSPs (Fig.1) and believed that it should lead to a qualitative change. The novel CSPs were prepared with 3,5- Dinitrobenzoyl or 5-Nitronicotinoyl as the end-capping group[12,16]. The molecular structures of 3,5- Dinitrobenzoyl and 5-Nitronicotinoyl are similar, so 5-Nitronicotinoyl was supposed to provide effect similar to 3,5-Dinitrobenzoyl and we decided to investigate the role of the NNC endcapping group.

For HPLC evaluation of the novel CSPs under normal phase conditions, a comprehensive set of chiral analytes, mainly common drug enantiomers, were used (Fig. 2).



CSP1 APS-Pro-Val-Phe-NNC



CSP2 APS-Pro-Phe -Val-DNB

Fig.1. Structures of chiral stationary phases prepared in this work

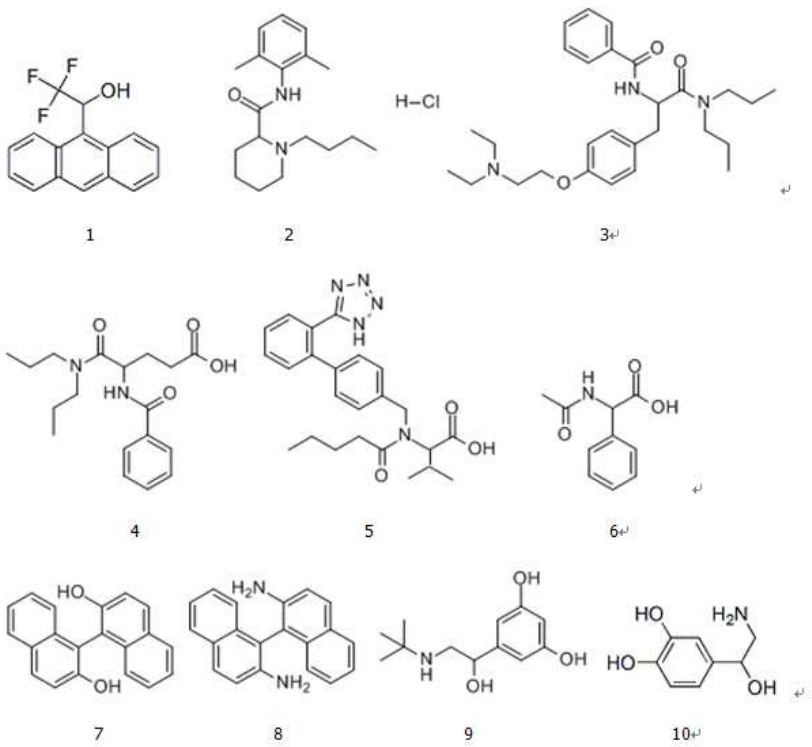


Fig.2. The structures of chiral analytes used in this study 2,2,2-trifluoro-1-(9-anthryl)ethanol (1) , Bupivacaine Hydrochloride (2) , Tiropramide (3) , Proglumide (4) , D L – Valsartan (5), DL-N-Acetyl-2-phenylglycine (6) , 1,1'-Bi-2-naphthol (7) , 1,1′-Binaphthyl-2,2′-diamine (8) , Terbutaline (9) , (±)Norepinephrine (10) .

2. Experimental

2.1. Chemicals and reagents

3,5-Dinitrobenzoyl (DNB) chloride, 5-Nitronicotinoyl (NNC) chloride, 9-fluorenylmethoxycarbonyl (Fmoc)-L-Pro-OH, Fmoc-L-Val-OH, Fmoc-L-Phe-OH , 1-Hydroxybenzotriazole (HOBt), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), and N,N-Diisopropylethylamine (DIEA) were purchased from Aladdin Chemicals (Shanghai, China). HPLC-grade spherical silica gel (particle size, 5 μm; pore size, 90 Å and surface area 300 m^2/g) was obtained from Zhengzhou Innosep Biosciences (China). HPLC-grade solvents were purchased from Merck (Germany).

2.2. Preparation of CSPs

2.2.1. Preparation of APS In a three-neck round flask with Dean-stark trap, a certain amount of silica in 80 ml of dry toluene was heated to reflux under nitrogen for 6h, and residual water was azeotropically removed. An appropriate amount of of 3-aminopropyltriethoxysilane and pyridine were added slowly and the mixture was heated at 110 ℃ to reflux for 14 h. Modified silica (APS) was isolated by filtration

and washed by toluene, N,N –dimethylformamide (DMF), methanol, acetone, and dried under reduced pressure (60 ℃,0.1 mbar, 12 h).

2.2.2. Preparation of APS-Pro-Val-Phe-NNC(CSP 1) (Fig.3.) A mixture of Fmoc-Pro-OH (0.4 g), HOBt (0.5 g), EDCI (0.5 g), DIEA (0.5 ml) in 50 ml dry DMF was then added to a certain amount of APS. After agitating the mixture for 24 h at 35℃, the silica gel was drained and washed with DMF, methanol. The Fmoc group was then removed by treatment with 50% (v/v) ethanolamine in dichloromethane for 3 h at 35 ℃. The deprotected silica, APS-Pro-H, was collected by filtration and washed with DMF, methanol, toluene, acetone. Then the next 2 modules, Fmoc-Val-OH (first) and Fmoc-Phe –OH (second), were coupled to above product following exactly the same procedures as described above to yield the desired APS-Pro-Val-Phe-H.

5-Nitronicotinoyl chloride (0.3 g) in 40 ml dichloromethane was reacted with a certain amount of APS-Pro-Val-Phe-H for 3 h at 35 ℃ to end-cap amino group of Phe. The stationary phase was collected and washed with dichloromethane, DMF, methanol, toluene and acetone to give CSP 1.

2.2.3. Preparation of APS-Pro-Phe -Val –DNB (CSP 2) This stationary phase was prepared similarly to CSP 1 , following the scheme of CSP 2 in Fig.3. The difference from CSP 1 is that the positions of Val and Phe are switched and DNB as the terminal group.

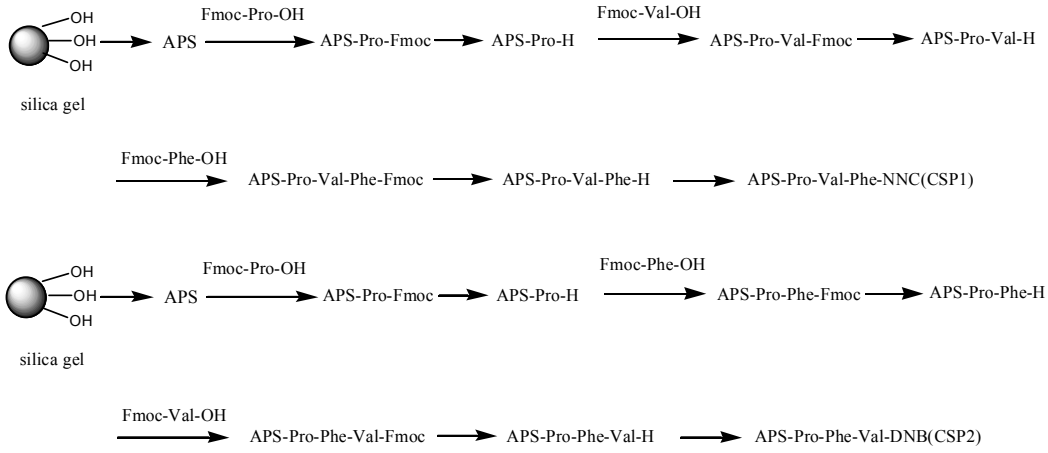


Fig.3. Synthetic scheme for the preparation of CSP1 and CSP2

2.3. Packing of the column

CSPs 1 and 2 were packed into a stainless steel column (150mm×4.6mm I.D.) under a pressure of 40 MPa using a slurry method with methanol as solvent.

2.4. Characterization of CSPs

The thermogravimetric analysis (TGA) was measured on a Q500 TGA instrument (Waters) at a heating rate of 10 ℃/min starting from room temperature to 850 ℃ under a flow of nitrogen. The FT-IR spectra were collected with a Frontier (Perkin Elmer Co.). The determination of the particle size distribution was performed with a LS particle size analyzer (Mastersizer 2000, Malvern) at room temperature with an equilibrium time of 120s.

2.5. Enantioseparation by HPLC

All analytes were dissolved in mobile phases at a concentration of about 80 μg mL^{-1}. Chromatographic separation was performed on a SHIMADZU HPLC system (JP) equipped with a LC-20AT quaternary HPLC pump, a SIL-20A auto-injector and a SPD-M20A diode array detector from 200 nm to 400 nm. Data collection and analysis were performed with the Labsolutions (SHIMADZU Corporation) software. The analysis was performed at 25 ℃, and 20 μL of each sample was injected into the LC system. The hold-up time (t_0) was measured with 1,3,5-tri-*t*-butylbenzene. All experiments were carried out at a flow-rate of 1.0 ml min^{-1} and mobile phases consisting of 2-propanol, methanol and n-hexane (contatining trifluoroacetic acid as additives in some cases).

3. Results and discussion

3.1 TGA and particle size distribution

The curves of TGA confirmed that Fmoc-Pro-OH, Fmoc-Val -OH or Fmoc-Phe-OH, was coupled to the resulting silicas following an identical reaction sequence and yielded CSP 1 and CSP 2 (see Supplementary Fig.S1). The investigation of the size distribution confirmed the size of CSP 1 and CSP 2 remain after the functionalization process, with diameters ranging from3.088 um to 8.646 um for CSP 1 and from 3.087 um to 8.679 um for CSP 2. The material is stable and no breakdown of the particles takes place (see Supplementary Fig.S2).

3.2 IR spectroscopy

The IR spectroscopy was recorded and used to confirm the synthesis of CSP 1 and CSP 2. The overlaid FT-IR spectra for silica gel, APS, CSP 1 and CSP 2 are shown in Fig.4. The presence of new peak at 3000-2800 cm^{-1} of APS can be attributed to the $–CH_2–$, which indirectly confirms the successful addition of 3-aminopropyltriethoxysilane. the CSPs IR spectra provided an amide I absorption band at 1659 cm^{-1} ,an amide II absorption band at 1547 cm^{-1} [11], $-NO_2$ symmetric stretching vibration at 1351 cm^{-1}, Out-of-plane bend vibration of benzene or pyridine ring at 922 cm^{-1}, 735 cm^{-1}, 703 cm^{-1}, frame vibration of benzene or pyridine ring at 1512 cm^{-1}(CSP 1) and 1455 cm^{-1} (CSP 1, CSP 2). The absorption bands are characteristic of amide , $-NO_2$ and benzene or pyridine ring, and their presence confirmed that the peptides with the end-capped group of NNC or DNB are immobilized on CSP 1 and CSP 2 successfully.

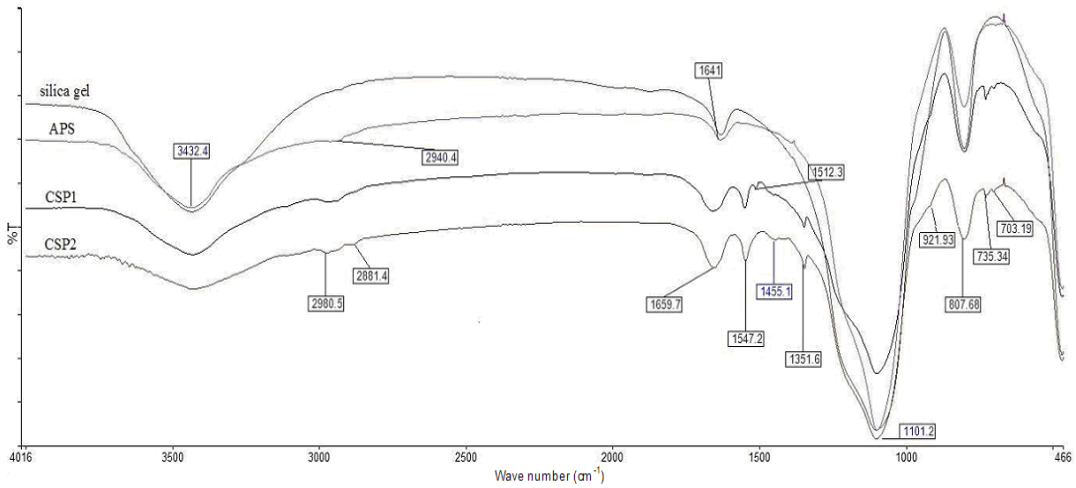


Fig.4. FT-IR spectra of silica gel, APS, CSP1 and CSP2.

3.3 Chromatographic evaluation of CSPs

Comparing the structure of these two types of stationary phases, three extra amide protons exist in CSP 1 and CSP 2, respectively. Such amide protons could introduce hydrogen-bonding interaction with the analytes [9]. The peptides in the CSPs could not exist in an α-helical formation because proline contains secondary amine in a stable cyclic structure [17] and the peptide is short. In the CSPs, three hydrophobic amino acids may form a hydrophobic active center which is similar to hydrophobic pockets of globular proteins, thus the hydrophobic interaction is likely to be the main driving force for chiral separation in CSPs 1 and 2. DNB is a much stronger electron-withdrawing group than NNC, if electronic properties are important in enantioselective separation, CSP1 is going to behave very differently from CSP2. The chiral stationary phases CSP 1 and CSP 2 were evaluated by HPLC for enantiomer separations of a set of chiral analytes.

Analytes (2,3,4,5,6) were chosen as they contain amide groups that can form hydrogen-bonding interaction with the amide protons in the CSPs. Analytes (7,8) were chosen as they contain π-basic groups (two naphthalene rings). Analytes (9,10) are adrenoceptor agonists and both possess phenylmethanolamine structure with a chiral center.

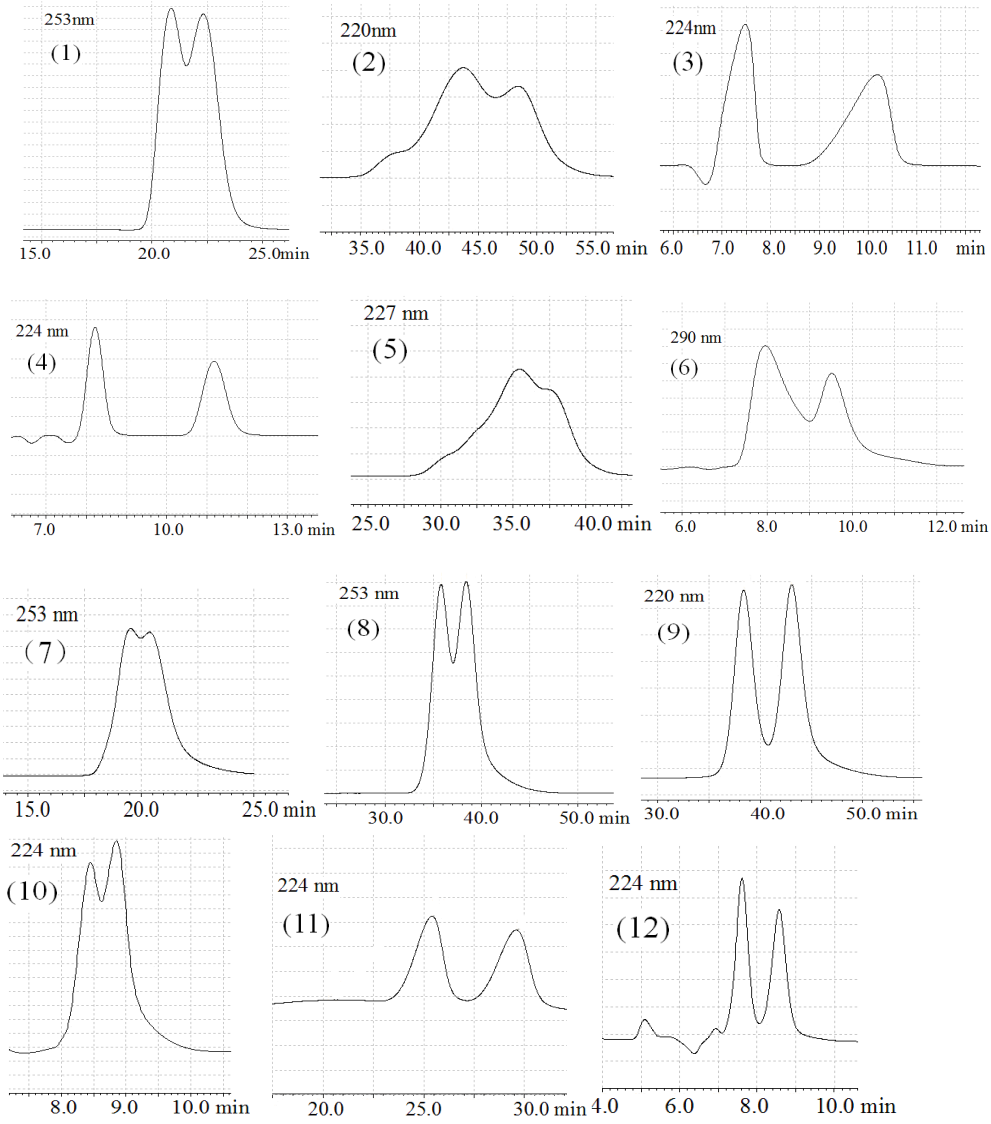
Flexibility in the choice of mobile phases is not only beneficial for analytes with solubility issues, but is also important in providing valuable selectivity switches. Although all miscible solvents can be utilized as the mobile phase, hexane/2-propanol and hexane/2-propanol/methanol were selected for valuable selectivity. Resolution results and representative chromatograms of these chiral analytes are listed in Table 1 and Fig.5.

Table 1 Comparison of the resolution of analytes on CSP1 and CSP2

chiral analytes	CSP1				CSP2			
	k_1	α	Rs	mp	k_1	α	Rs	mp
1	7.54	1.08	0.7	A	7.00	1.08	0.5	A
					14.06	1.08	0.6	B

2	16.91	1.08	0.9	A	14.70	1.13	1.4	A
3	2.06	1.54	1.9	C	2.46	1.07	0.3	C
					9.40	1.18	1.5	A
4	2.36	1.51	3.2	C	2.12	1.18	1.2	C
					3.64	1.32	2.6	A
5	3.13	1.00	0.0	C	2.82	1.00	0.0	C
6	3.01	1.00	0.0	C	2.70	1.00	0.0	C
7	13.52	1.05	0.6	D	14.86	1.11	1.1	D
8	9.70	1.00	0.0	D	9.34	1.00	0.0	D
9	8.30	1.00	0.0	E	6.41	1.82	3.3	E
10	2.26	1.28	1.0	E	2.95	1.00	0.0	E

mp: mobile phases; Rs: resolution.
A, n-hexane / isopropanol = 90: 10; B, n-hexane / isopropanol = 95:5; C, n-hexane / isopropanol = 60: 40; D, n-hexane / isopropanol / methanol = 50: 25: 25; E, n-hexane / isopropanol / methanol / trifluoroacetic acid = 50: 25: 25: 0.1.
flow rate: 1.0 ml min^{-1}



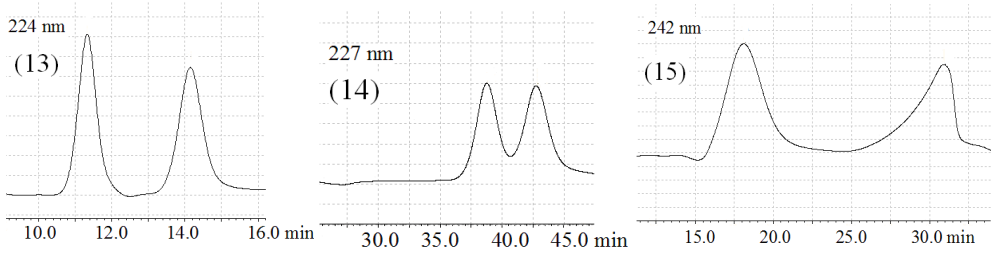
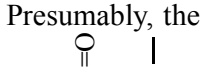


Fig.5a/b. Representative chromatograms of analytes 1-6 (7-15) on CSP 1 (CSP2). Separation conditions see Table 1
(1)analyte 1, mp: A ; (2) analyte 2, mp: A ; (3) analyte 3, mp: C ; (4) analyte 4, mp: C ; (5) analyte 7, mp: D ; (6) analyte 10, mp: E; (7) analyte 1, mp: A ; (8) analyte 1, mp: B ; (9) analyte 2, mp: A ; (10) analyte 3, mp: C ; (11) analyte 3, mp: A ; (12) analyte 4, mp: C ; (13) analyte 4, mp: A ; (14) analyte 7, mp: D ; (15) analyte 9, mp: E

As shown in Table 3, 2,2,2-trifluoro-1-(9-anthryl)ethanol (π -basic, analyte 1) was separated on CSP 1 and CSP 2 with the equal α-values (α=1.08), although the peptides and terminal groups are both different from each other.

CSP 1 separated analytes (3, 4, 10) better (α>1.08), while CSP 2 separated analytes (2, 7, 9) better with the same mobile phase as for CSP 1. For structurally analogous analytes, CSPs 1 and 2 even showed different separation trends. For example, analytes (3,4) were resolved comparably on CSP 1 (α=1.54 *vs.* 1.51), but analyte 4 was separated better than analyte 3 by CSP 2 (α=1.18 *vs.* 1.07) ; CSP 2 seems to work better for analyte 7 than CSP 1, and it can be clearly seen that analyte 8 cannot be separated either on CSP 1 or on CSP 2. Besides, we observed that analogous analytes (9,10) were recognized by CSP 1 and CSP 2 selectively. Analyte 9 was only separated by CSP 2 while analye 10 was only resolved by CSP 1. The different performances of CSP 1 and CSP 2 for analogous analytes indicate that chiral recognition processes may differ much on chiral selectors of varying amino acid sequence and endcapping groups. Presumably this is why only a limited number of proteins have been developed to chiral stationary phases though all proteins are inherently chiral: the amino acid sequence strongly impacts the chiral recognition.

3.4 chiral recognition mechanism

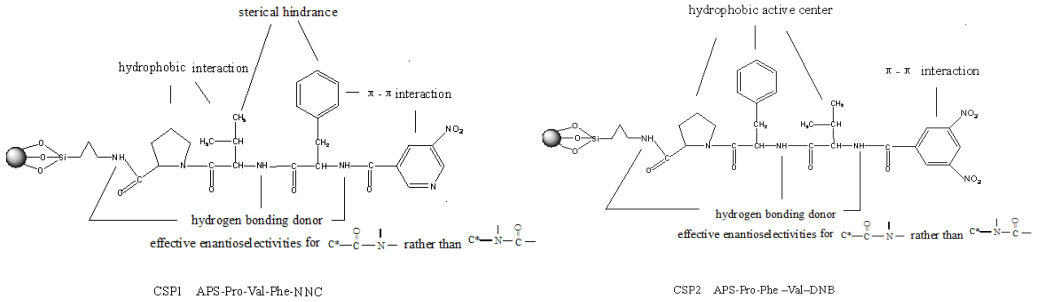
3.4.1 hydrogen bonding In contrast to better enantioseparation of analyte 7 on the two CSPs, the amino groups of analyte 8 do not have as strong hydrogen bonding ability as hydroxyl groups, consequently, effective chiral recognition for analyte 8 could not be obtained on the two CSPs. For analytes (2,3,4) with the  unit (C*: chiral center), two CSPs could provide effective enantioselectivities, However, analytes(5,6) with the  unit were not resolved at all on the two CSPs, very possibly due to the different hydrogen bonding interactions. The unit attached to the chiral center in analytes (5,6) is amide N-R instead of amide C=O which is a hydrogen bonding acceptor. Presumably, the two CSPs could afford better enantioselectivity for the analytes with  unit.

3.4.2 hydrophobicity The better chiral recognitions in CSP 2 than in CSP 1 for analyte 7 containing hydrophobic naphthalene rings, analyte 9 containing hydrophobic tertiary butyl, indicates that CSP 2 tended to show higher chiral recognition for analytes with hydrophobic groups due to the hydrophobic active center in CSP 2.

3.4.3 π - π interaction The equal enantioseparation of analyte 1 (π -basic) on CSP 1 and CSP 2 indicates the sequences are not important in influencing the enantioselectivity of 1 and NNC as analogue of DNB exhibited similar enantioseparation ability. The phenyl ring in analyte 10 could participate in π - π interaction and the lower enantioselectivity of CSP 2 than that of CSP 1 for analyte 10 clearly indicates that the π - π interaction should be the major interaction for CSP 1. Presumably, it was achieved by the combination of Phe and NNC. For the same reason, CSP 1 was more effective than CSP 2 for analyte 3 and 4.

3.4.4 sterical hindrance For CSP 1, analyte 3 has larger molecular size and greater separation factor but lower retention than analyte 4. This might suggest that the sterical hindrance in CSP 1 resulted in a suppression of superfluous interaction and contributed to the chiral recognition for CSP 1. However, in some cases the sterical hindrance may block the enantiomer molecule into the asymmetric space. Analytes 9 and 10 have similar structures but the former has tertiary butyl, which contributed to the failure in enantioseparation on CSP 1.

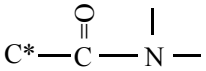
From the comparison of CSP 1 and CSP 2, we observed that they exhibited notable difference in chiral recognition (Fig.8). CSP 1 typically affords higher enantioselectivity for those analytes facilitating π - π interaction, whereas CSP 2 is more pronounced for separation of enantiomers bearing more hydrophobic groups.



CSP1 APS-Pro-Val-Phe-NNC CSP2 APS-Pro-Phe –Val–DNB

Fig.8. chiral recognition mechanism

4. Conclusions

Two new tripeptide CSPs , L-Pro-L-Phe-L-Val , with different amino acid sequences and endcapping groups have been immobilized on APS treated silica and investigated for chiral separation in HPLC. Good enantioseparations have been achieved by the combination of the advantages of Pro, Phe, and Val, such as conformational rigidity, π - π interaction, sterical hindrance, hydrophobic interaction and hydrogen bonding. CSP 1 and CSP 2 are very successful in the resolution of adrenoceptor agonists and racemic compounds containing  unit, naphthalene ring. From the comparison of CSP 1 and CSP 2, we observed that they exhibited notable difference in chiral recognition. NNC as analogue of DNB exhibited similar enantioseparation

ability and could form π - π interaction with analytes by combining with Phe. This work has demonstrated the importance of amino acid sequences, which strongly impacted the chiral recognition for different enantiomers, and these results can give us a hint to understand the chiral recognition mechanism on proteins-based stationary phase.

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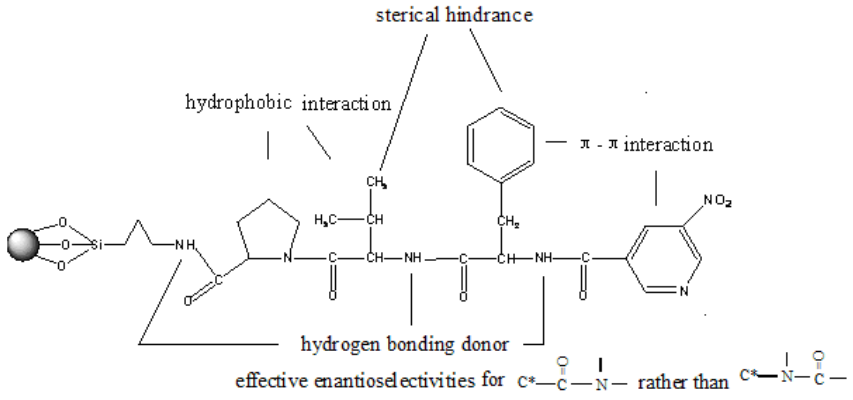
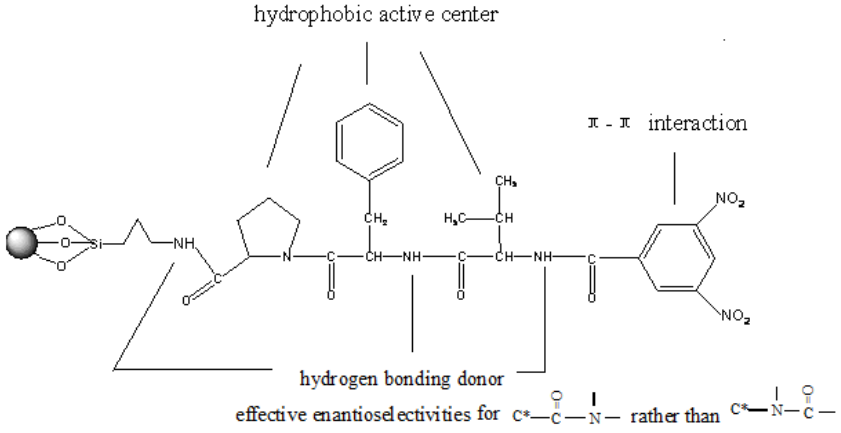


CSP1 APS-Pro-Val-Phe-NNC



CSP2 APS-Pro-Phe –Val–DNB

CSP 1 typically affords higher enantioselectivity for those analytes facilitating stronger π-π interaction, whereas CSP 2 is more pronounced for separation of enantiomers bearing more hydrophobic groups. 5-Nitronicotinoyl as analogue of 3,5- Dinitrobenzoyl(DNB) exhibited similar enantioseparation ability and could form π-π interaction with analytes by combining with phenylalanine.