Journal of Chromatography B, 875 (2008) 93–101

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Polyproline derivatives as chiral selectors in high performance liquid chromatography: Chromatographic and conformational studies☆

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ARTICLE INFO

Article history:
Received 31 March 2008
Accepted 24 May 2008
Available online 3 June 2008

Keywords:
Poly-L-proline oligomers
Chiral selectors
Enantioseparation
Chiral stationary phases

ABSTRACT

A proline oligopeptide-derived chiral selector (CS), containing 3,5-dimethylphenylcarbamate residues on the 4 position of the pyrrolidine rings, was bonded to a silica gel chromatographic matrix by the N-terminal group. The chromatographic behaviour of the resulting chiral stationary phase (CSP) was compared with that of a CSP containing the analogous monomeric CS and that resulting from bonding of the polyproline-derived CS by the carboxy-terminal group using several solvents as mobile phase. The CSs were also studied from the conformational point of view in solution using circular dichroism and ^{13}C NMR. A relationship was found between the presence of an ordered conformation in the particular conditions used and increased enantioselectivity.

1. Introduction

As a result of the increasing demand for enantiomerically pure drugs, intensive research into direct liquid chromatographic procedures has led to the production of improved selective chiral stationary phases (CSPs). However, given that chromatographic enantioseparations are used not only in process monitoring and quality control but also in the improvement of the enantiopurity of products prepared by asymmetric synthesis, new CSPs with broad applicability and high loadability are required to separate the increasing number of racemic compounds to be resolved. Diverse chiral materials have been tested as chiral selectors (CSs) in CSPs. Among these, proteins have shown enantioselectivity towards a broad range of chiral chemical structures [1]. Nevertheless, in recent years the labile character of this kind of CSPs to chemical and biochemical degradation has relegated them in favour of polysaccharide-derived CSPs, mainly because of the broad application domain and high loadability of the latter [2]. In the case of polysaccharides, a helical secondary structure, which determines the presence of diverse multiple stereoselective microenvironments, has been associated with these two characteristic features [3]. Polyamino acids also adopt helical conformations. In particular, PPI and PPII conformations have been described for polyprolines, which are unable to adopt the most common α-helix [4,5]. Nevertheless, the possibility to use oligopolyproline chains as CSs in enantioselective HPLC has been considered only recently [6,7]. The capacity for these chains to adopt helical conformations has attracted our attention. On the basis of our previous experience in the field of polysaccharide-derived CSPs, the introduction of a 3,5-dimethylphenylcarbamoyloxy group in the 4*R* position on the pyrrolidine ring was chosen. This feature provides a certain analogy to the polyproline structure with the polysaccharide derivatives most used for enantioseparation purposes, 3,5-dimethylphenylcarbamates of cellulose and amylose [3].

In our previous study in this context [8], single proline- and polyproline-derived CSs anchored through their C-terminal free carboxylate functions were used. The application domain and the loading capacity of a polyproline CSP were significantly enhanced in comparison with those of an analogous monomeric proline CSP. These observations could be explained by the presence of a polyproline helical structure. The hypothesis was supported by the changes in enantioselectivity observed for several types of solvents used as mobile phase. In the present study proline- and polyproline-derived CSs are anchored through their N-terminal amino function (Fig. 1). Although the central moiety of the CS is the same in the two cases, the final more accessible unit differs. This unit may have a directing role in the orientation of CS–analyte for recognition thereby affecting enantioselectivity [9]. The chromatographic behaviour for the new CSPs has been explored in several solvents and the CSs have been studied in solution using circular dichroism

☆ This paper is part of the Special Issue 'Enantioseparations', dedicated to W. Lindner, edited by B. Chankvetadze and E. Francotte.

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doi:10.1016/j.jchromb.2008.05.041

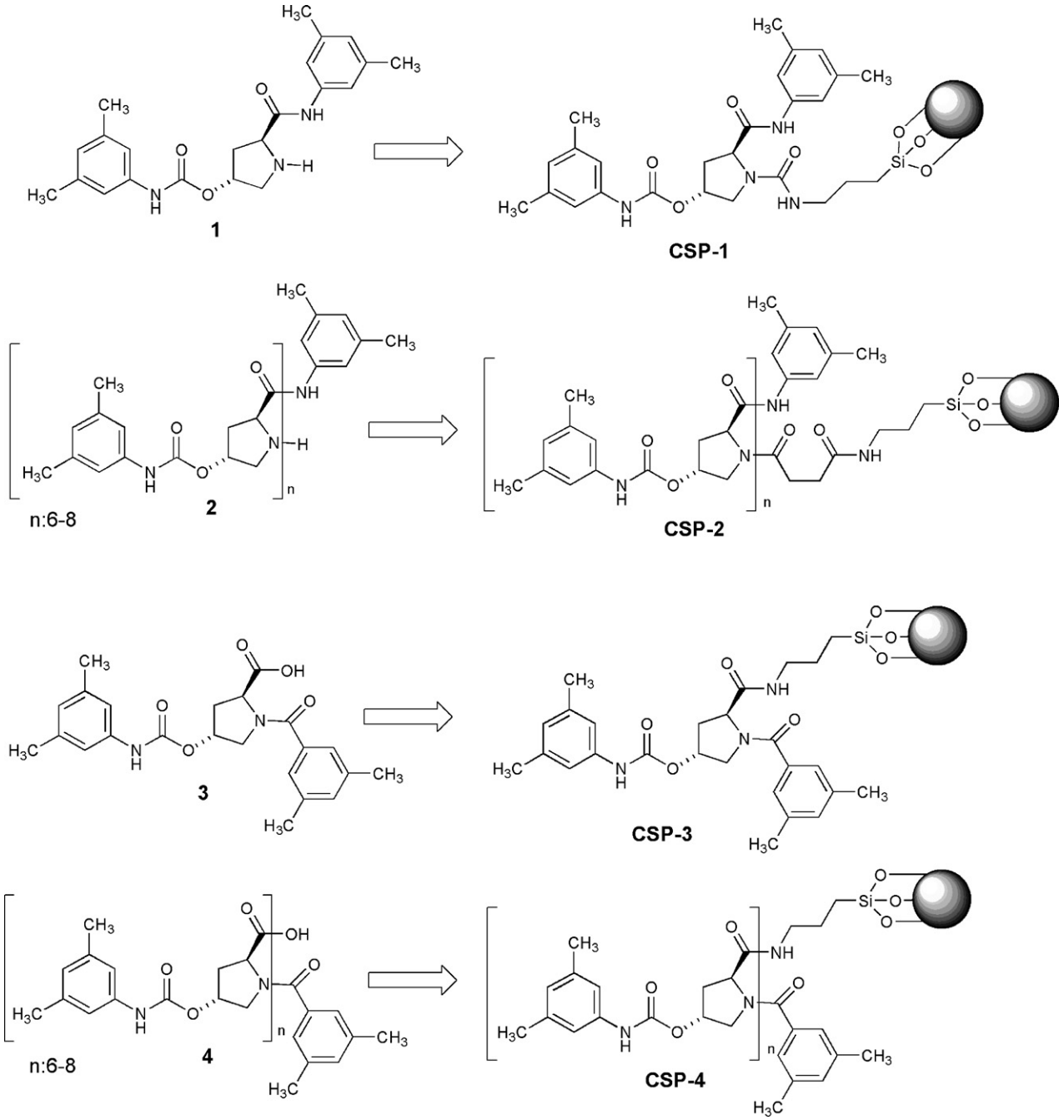

Fig. 1. CSPs and CSs used in this study. **CSP-3** and **CSP-4** were described in Ref. [8].

(CD) and nuclear magnetic resonance (NMR). The data obtained indicate the presence of distinct conformations in equilibrium depending on the solvent used and contribute to our understanding of the recognition behaviour for this kind of CS.

2. Experimental

2.1. Abbreviations

EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; DIC, diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid.

2.2. General supplies and equipment

Aminomethylated polystyrene resin (AmPS) (100–200 mesh, 1.2 mmol/g, NovaBiochem, Läufeldingen, Switzerland) was used as a solid phase matrix in the peptide synthesis. All chemicals and solvents were purchased from Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland) or Panreac (Barcelona, Spain). Organic solutions were dried over sodium sulphate. HPLC analyses of peptides were performed on an Agilent HPLC system HP 1100 (pump, autosampler, UV detector, and chromatographic data station software) (Agilent Technologies, Palo Alto, CA, USA). A C_{18} column (5 μm, 15 cm × 0.46 cm) was used as a stationary phase. Gradients of H_2O + 0.1% TFA/ACN + 0.1% TFA were used as solvent system. MS MALDI-TOF spectra were obtained from a 4700 Proteomics analyzer

(Applied Biosystems). ^{13}C NMR spectra were recorded on a Varian Mercury 400. Chemical shifts are quoted in δ values downfield of TMS. Elemental analyses were performed by the *Serveis Científico-Tècnics* of the *Universitat de Barcelona* (Spain) on a Thermo Electron apparatus EA 1108 using standard conditions.

CD measurements were made on a JASCO J810 apparatus at room temperature, with resolution set at 1 nm and sensitivity at 100 mdeg. The spectra were recorded at a scan speed of 50 nm/min and a time constant of 0.5 s. A quartz cell with a path length of 2 mm was used and sample concentration was of 0.05 mg/mL.

The chromatographic enantioselective experiments were performed on a Waters HPLC system (Milford, MA, USA) consisting of a 600E pump, a 717 autosampler and equipped with a 996 photodiode-array detector. The CSPs were packed into stainless-steel tubes (150 mm × 4.6 mm ID) by the slurry method. The volume of the injected sample was 10 μL and the flow rate was set at 1 mL/min. The void volume was determined using tri-*tert*-butylbenzene. The elution order was determined when possible using samples enriched in one of the enantiomers of known absolute configuration or by using a PerkinElmer 241LC polarimetric detector (PerkinElmer, Uberlingen, Germany).

2.3. *N*-(*t*-Butoxycarbonyl)-(4*R*)-(3,5-dimethylphenylaminocarbonyloxy)-L-proline (**5**)

The modified *N*-Boc-protected (4*R*)-hydroxy-L-proline derivative **5** used in the peptide synthesis was obtained by the procedure already described [10]. Thus, 3,5-dimethylphenyl isocyanate (970 mg, 6.5 mmol) was added to *N*-(*t*-butoxycarbonyl)-(4*R*)-hydroxy-L-proline (1 g, 4.2 mmol) in anhydrous pyridine (30 mL) and the solution was refluxed for 22 h. After crystallization from EtOH–H_2O the title compound was obtained in 90% yield.

2.4. *N*′-(3,5-Dimethylphenyl)-(4*R*)-(3,5-dimethylphenylaminocarbonyloxy)-L-prolinamide (**1**)

The (4*R*)-substituted-L-proline derivative **1** used as a CS in **CSP-1** was obtained from **5** by the procedure already described [10]. 3,5-Dimethylaniline (3.3 mmol) was added to a stirred solution of **5** (1.3 mmol) and EEDQ (3.3 mmol) in 20 mL of DCM. After being stirred for 60 h at room temperature, the solution was washed with HCl 2N solution, Na_2CO_3 5% solution and water. The solid product obtained from the organic layer was then dissolved in a TFA/DCM (30/70, v/v) mixture and stirred at room temperature for 30 min. The reaction mixture was neutralized with concentrated NH_4OH solution. Compound **1** was obtained in a global yield of 60%.

2.5. Synthesis of the peptide mixture **6** (Fig. 2)

After attachment of the linker, *N*-(9-hydroxymethyl-2-fluorenyl)succinamic acid (HMFS) [11], to an AmPS, eight cycles of coupling with **5**, DIC and HOBt in DMF, and seven *N*-Boc deprotection treatments (TFA in DCM, 40:60 (v/v)) were performed. The modified resin was treated with a solution of morpholine in DMF (1:4, v/v) to cleave the peptidic material from the resin. The resulting solution was evaporated and the residue dissolved in DCM and neutralized with 2N HCl. The organic solution was dried and evaporated. From 200 mg of starting AmPS (1.3 mmol/g) 66 mg of peptidic mixture **6** were obtained. The HPLC analysis revealed that the mixture comprised three peptides corresponding to 8, 7 and 6 monomer units in an approximated relative ratio 63%, 30% and 7%, respectively, assuming similar extinction coefficients for the three components. For $n = 8$: MALDI calcd. [M+H] 2200.47; found [M+Na] 2222.18. For $n = 7$: MALDI calcd. [M+H] 1940.17; found [M+Na] 1961.98. For $n = 6$: MALDI calcd. [M+H] 1679.88; found [M+Na] 1702.92.

2.6. Synthesis of CS **2**

EEDQ (106 mg, 0.428 mmol) and 3,5-dimethylaniline (54 μL, 0.428 mmol) were added to the *N*-Boc-protected peptide mixture **6** (234 mg, 0.12 mmol, considering a MW average corresponding to 7 monomer units) in DCM (15 mL). After being stirred for 24 h at room temperature, the resulting solution was washed with 2N HCl and water. The peptide mixture obtained (207 mg) was analyzed by HPLC. Compounds corresponding to 8, 7 and 6 monomer units were identified in a relative ratio of 62%, 31% and 7%, respectively. For $n = 8$: MALDI calcd. [M+H] 2303.64; found [M+Na] 2325.26. For $n = 7$: MALDI calcd. [M+H] 2043.34; found [M+Na] 2065.15. For $n = 6$: MALDI calcd. [M+H] 1783.05; found [M+Na] 1805.00.

This material was dissolved in 10 mL of a TFA/DCM mixture (3:7) and stirred at room temperature for 40 min. After neutralization with NH_4OH, the solution was washed with water and dried. The peptidic mixture obtained (**2**) (195 mg) was analyzed by HPLC. The components corresponding to 8, 7 and 6 monomer units were also identified in this mixture (relative ratio: 62%, 31% and 7%, respectively). For $n = 8$: MALDI calcd. [M+H] 2204.54; found [M+Na] 2225.28. For $n = 7$: MALDI calcd. [M+H] 1944.24; found [M+Na] 1965.16. For $n = 6$: MALDI calcd. [M+H] 1683.95; found [M+Na] 1705.04.

2.7. Coupling of the succinyl linker (**7**)

Succinic anhydride (139 mg, 1.39 mmol) was added to the peptide mixture **2** (270 mg, 0.14 mmol considering a MW average corresponding to 7 monomer units) in pyridine (8 mL). After being stirred for 24 h at room temperature, the resulting solution was diluted with DCM, washed with 2N HCl and water, and dried. The final peptide mixture **7** (260 mg) was analyzed by HPLC. The three components corresponding to 8, 7, and 6 monomer units were also identified in a relative ratio: 60%, 32% and 8%, respectively. For $n = 8$: MALDI calcd. [M+H] 2303.59; found [M+Na] 2324.80. For $n = 7$: MALDI calcd. [M+H] 2043.30; found [M+Na] 2065.72. For $n = 6$: MALDI calcd. [M+H] 1783.00; found [M+Na] 1804.63.

The peptide containing 8 monomeric units was purified from the crude product by preparative HPLC on a 19 mm × 100 mm C18 column (Waters Symmetry, 5 μm) using a gradient elution starting from 80% to 96% ACN in 15 min at a flow rate of 15 mL/min. The purity of the eluted fractions was then analyzed by HPLC and characterized by MALDI-TOF. An amount of 50 mg of this compound (purity >99.5%) was obtained to be used in the NMR experiments.

2.8. Preparation of **CSP-1**

The fixation of **1** onto silica gel was performed following the general procedure already described [12,13]. Thus, CS **1** (3 mmol) was dissolved in anhydrous pyridine (9 mL) and 3-isocyanatepropyltriethoxysilane (0.95 mL, 3.6 mmol) in pyridine (6 mL) was added. The solution was refluxed for 90 min. At this point the excess of reagent and the solvent was removed by distillation at reduced pressure. The residue was dissolved in anhydrous pyridine (10 mL) and the resulting solution was added to the suspension of spherical silica gel (3 g, 5 μm, 100 Å, Kromasil), previously dried by azeotropy with toluene, in anhydrous pyridine (14 mL). The mixture was stirred at reflux temperature for 90 min. Hexamethyldisilazane (1 mL, 5.7 mmol) was then added and the reaction mixture was allowed to react for one additional hour at reflux temperature. The resulting bonded silica was collected

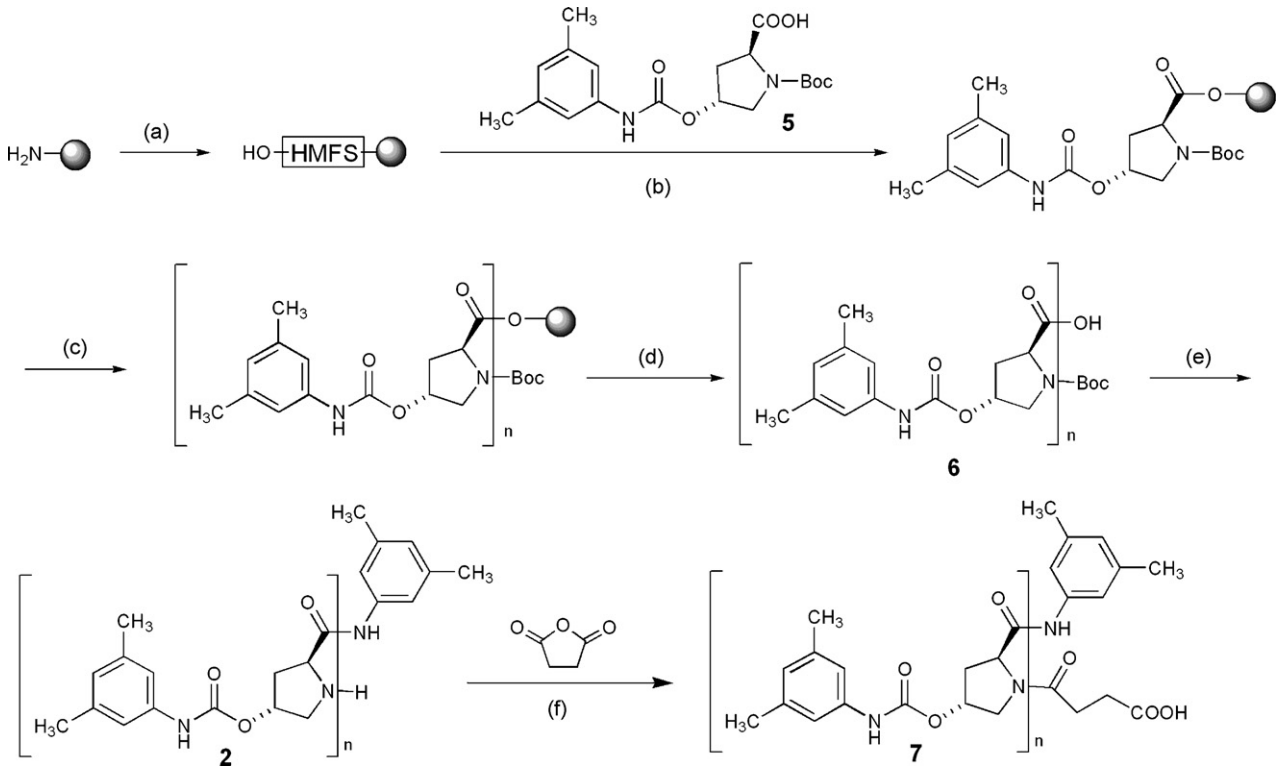

Fig. 2. Synthesis of CS **7**. (a) HMFS, DIC, HOBt, DMF; (b) DIC, DMAP, DMF; (c) (1) TFA/DCM 40%, (2) **5**, DIC, HOBt, DMF (7 cycles); (d) morpholine/DMF 20%; (e) (1) 3,5-$(CH_3)_2C_6H_3NH_2$, EEDQ, DCM, r.t., (2) TFA/DCM (3:7) r.t.; (f) pyridine, 24 h, r.t.

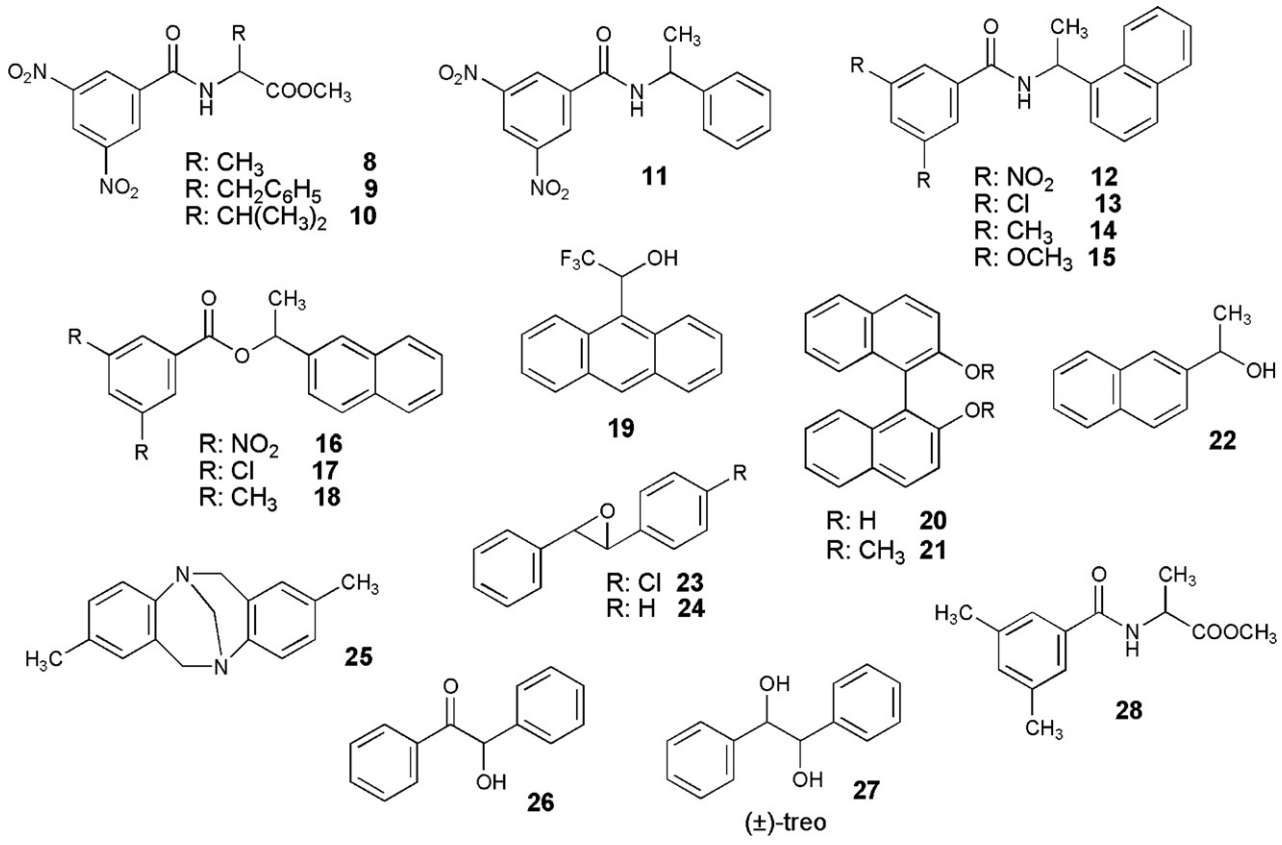

Fig. 3. Racemic compounds used in the test of the CSPs.

by filtration, washed exhaustively with pyridine, ethanol, water (until neutral washing liquors were obtained), ethanol, acetone and diethyl ether, and dried in vacuum at room temperature. The resulting **CSP-1** was characterized by elemental analysis. On the basis of the results obtained (11.44% C, 1.93% H and 1.64% N) a content of 0.29 mmol of chiral entities per gram of CSP can be calculated.

2.9. Preparation of *CSP-2*

EEDQ (110 mg. 0.44 mmol) was added to a solution of the peptide mixture **7** (420 mg, 0.2 mmol, considering a MW average corresponding to 7 proline derived units) in THF (40 mL). γ-aminopropylated silica gel (2 g), obtained previously from spherical silica gel (5 μm, 100 Å, Kromasil) following the conventional procedure [14] (analysis: 3.18% C, 0.92% H; 0.91% N), was added to the solution and the mixture was allowed to react overnight at room temperature. The resulting material was collected by filtration and washed with THF. The solid product was then suspended in toluene (10 mL) and hexamethyldisilazane (1 mL) was added and the resulting suspension was allowed to react for 1 h at reflux temperature. The modified silica gel obtained was collected by filtration, washed exhaustively with toluene, acetone, ethanol, water until neutral washing liquors, acetone and ethyl ether, and then dried in vacuum at room temperature. The resulting **CSP-2** was characterized by elemental analysis (4.59% C, 1.01% H and 1.12% N). A content of 0.17 mmol of proline units per gram of CSP was calculated on the basis of the percentage of nitrogen.

3. Results and discussion

Monomeric proline **CSP-1** is a typical π-donor Pirkle-type CSP. This type of CSP is designed to exploit π–π stacking interactions between electron-donor and electron-acceptor aromatic systems as one of the main attractive interaction forces. The polar carbamate motifs introduced into the proline scaffold provide this CSP with an electron-rich aromatic ring capable of inducing charge transfer interactions with analytes bearing electron-deficient rings in their structure. Also, the O=C–NH group of the polar carbamate motif has the capacity to act as a hydrogen bonding donor/acceptor group, thereby allowing the analytes containing hydrogen bonding donor/acceptor groups to interact through this kind of dipolar bonding interactions. The racemic analytes used in this study were chosen on the basis of these characteristics. Among the racemic analytes considered, molecules with dinitrobenzoyl groups (**8**–**12**, **16**) as well as hydrogen bond donor groups (**8**–**15**, **19, 20, 22**, **26–28**) or both (**8**–**12**) were tested (Fig. 3).

In a previous study [8], with the aim to induce multivalency in the CS, we studied the impact of the introduction of several substituted proline units on enantioselectivity. Enantioselectivity values as well as application domain and loading capacity were enhanced when compared to an analogous monomeric proline CSP. The increased enantioselectivity and loadability, in spite of the lower content of proline units regarding the monomeric CSP, and extended applicability to compounds containing electron-donor groups, in spite of the electron-donor character of the CSP, suggest the presence of a helical conformation for the CS. Moreover, some unexpected higher retention for certain analytes and loss of enantioselectivity was observed when chloroform was used in the mobile phase. This effect on enantioselectivity could be the result of a change in the helical conformation. We sought to explore these effects in greater dept and thereby contribute to our understanding on how this new kind of CS acts.

In a first step, **CSP-1** and **CSP-2** were tested using heptane/2-PrOH as a mobile phase (Table 1). In these conditions, monoproline-

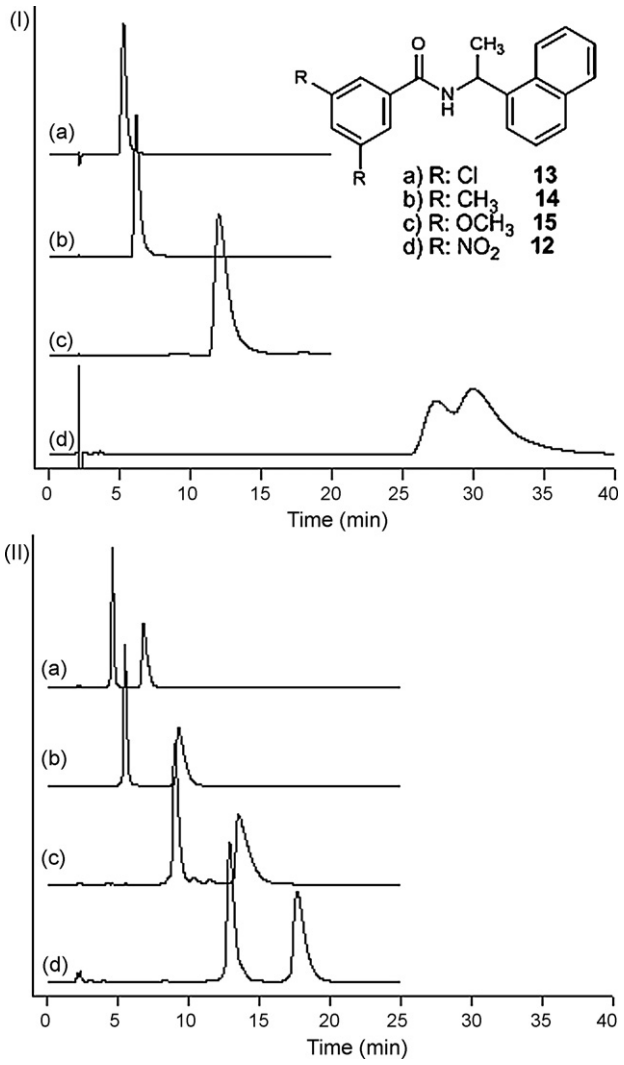

Fig. 4. Chromatograms corresponding to the separation of racemic compounds **12**–**15** on (I) **CSP-1** and (II) **CSP-2**. Conditions: heptane/2-PrOH (90:10), 1 mL/min.

derived **CSP-1** showed good enantioselectivity, which was found to be almost exclusive to the resolution of the racemic 3,5-dinitrobenzoyl amino acid derivatives **8**, **9** and **10**. Other racemates bearing dinitrobenzoyl aromatic rings, such as **11** and **12**, were found to be only partially resolved. Compound **16**, with a dinitrobenzoyl group but without any hydrogen bond donor group, was not resolved and no resolution was observed for compounds with only hydrogen bond donor character, such as **19**, **22**, **26** and **27**, with the exception of compound **20**.

When tested in these mobile phase conditions, although polyproline **CSP-2** showed a more modest enantioselectivity for dinitrobenzoyl amino acid compounds (**8**–**10**), it partially resolved these compounds. In contrast, baseline resolution was achieved for compounds **11** and **12** in **CSP-2** while only partial or lack of resolution was observed for the same compounds in **CSP-1**. These compounds show a common feature of having a second aromatic system, which may be involved in the association with the CS. This observation points to a distinct recognition mechanism for **CSP-2**, as does the complete resolution of compounds **14** and **15**, which contain only π-donor groups (Fig. 4). The highest separation factor and the best resolution among the racemic mixtures tested were attained in these conditions for the former. **CSP-2** also resolved **27**, a hydro-

Table 1
Chromatographic results using heptane/2-PrOH mixtures as mobile phase

Racemic compounds	CSP-1			CSP-2		
	k'_1 [a]	α	R_s	k'_1 [a]	α	R_s
8	14.61[b] (*R*)	1.59	2.37	4.14[b] (*R*)	1.11	1.00
9	13.22[b] (*R*)	1.37	1.60	4.64[b] (*R*)	1.08	0.75
10	7.69[b] (*R*)	1.39	1.83	2.40[b] (*R*)	1.06	0.42
11	12.03[b]	sh.	–	3.94[b] (*R*)	1.36	3.06
12	13.02[b]	1.10	0.38	4.58[b] (*R*)	1.45	3.74
13	5.21	sh.	–	0.99[b]	1.96	4.21
14	2.12[b]	1.00	–	1.37[b]	2.20	5.24
15	5.08[b]	1.00	–	2.90[b]	1.68	3.76
16	3.82	1.00	–	1.87 (*S*)	1.13	1.15
17	0.14	1.00	–	0.16	1.00	–
18	0.19	1.00	–	0.25	1.00	–
19	4.04	1.00	–	6.00 (*R*)	1.04	0.36
20	2.53[b] (–)	1.49	1.71	3.43[c]	1.00	–
21	0.87	1.00	–	0.87	1.00	–
22	3.07	1.00	–	3.84	1.00	–
23	0.23	1.00	–	0.21	1.00	–
24	0.21	1.00	–	0.22	1.00	–
25	2.05	1.00	–	1.35	1.00	–
26	2.55	1.00	–	3.16	1.00	–
27	9.65	1.00	–	2.58[b]	1.14	1.33
28	9.05	1.00	–	5.47 (*R*)	1.17	1.65
Mobile phase		Hep/2-PrOH (98:2)			Hep/2-PrOH (98:2)	

[a] The absolute configuration (or optical rotation sign) of the first eluting enantiomer is indicated.
[b] Heptane/2-PrOH (90:10).
[c] Heptane/2-PrOH (70:30).

gen bond donor compound, and **28**, the π-donor analogue of **8**.

When compared to the polyproline-derived CSP described in our previous study (**CSP-4**) (Fig. 1) [8], which contains a peptidic CS bonded to the chromatographic matrix by the carboxylate end, retention time for most of the analytes tested was significantly reduced and selectivity substantially increased for **8**, **9**, **13**, **14** and **15** when the selector was anchored by the N-terminal proline end, in spite of a slightly lower content of organic material in the corresponding CSP. Moreover, the application domain of **CSP-2** was extended to compounds **16**, **19**, **27** and **28**, which were not resolved previously in **CSP-4**. **CSP-2** was also enantioselective for the drug warfarin (k'_1: 24.06, α: 1.09; R_s: 1.08, hept/2-PrOH/TFA (98:2:0.5)).

At this point it is worth noting that a density of 0.17 mmol of proline units per gram of CSP was estimated for **CSP-4**, while 0.12 mmol/g of CSP was estimated for **CSP-2**, the main difference being the distribution of peptide length in the mixture used in the preparation of the CSPs, with a higher content of the peptide containing 7 proline units in the former (50%, only 34% of 8 units) and a higher content of that containing 8 proline units in **CSP-2** (60%, only 32% of 7 units) and the terminal and more accessible proline unit in the peptidic selector.

Although the use of heptane/chloroform mixtures as mobile phase is common practice when testing Pirkle-type CSPs, some unexpected behaviour was observed when using these conditions to test polyproline CSP [8]. Thus, in contrast to the conventional

Table 2
Chromatographic results obtained using heptane/MTBE mixtures as mobile phase

Racemic compounds	CSP-1			CSP-2			CSP-3			CSP-4		
	k'_1 [a]	α	R_s	k'_1 [a]	α	R_s	k'_1 [a]	α	R_s	k'_1 [a]	α	R_s
8	4.34[b] (*R*)	1.70	1.22	15.72 (*R*)	1.07	0.47	10.17	1.09	0.44	3.61[b]	1.00	–
9	3.45[b] (*R*)	1.57	1.53	7.00[c]	1.00	–	7.47 (*R*)	1.14	0.75	3.03[b]	1.00	–
10	2.18[b] (*R*)	1.78	1.97	12.03 (*R*)	1.06	0.28	4.58 (*R*)	1.17	0.95	12.19	1.00	–
11	26.90	sh.		10.89 (*R*)	1.41	1.70	4.47 (*R*)	1.13	0.67	10.39 (*R*)	1.45	1.57
12	20.70 (*R*)	1.11	0.35	8.81 (*R*)	1.54	2.10	3.50 (*R*)	1.45	1.71	8.45 (*R*)	2.11	2.63
13	16.25[d]	sh.		0.78[c]	2.51	3.32	0.64	1.00	–	1.19	1.57	1.50
14	6.22	1.00	–	3.42[c]	2.21	3.76	2.42	1.00	–	4.12	1.48	1.77
15	20.13	1.00	–	6.10[c]	1.45	1.71	5.30	1.00	–	9.67	1.24	0.93
16	12.62[d]	1.00	–	6.05[d] (*S*)	1.18	1.38	9.99[e]	1.00	–	13.35[e]	1.00	–
20	4.35	1.38	1.35	5.97	sh.	–	2.17	1.00	–	10.89	1.00	–
21	14.85[e]	sh.	–	5.87[e]	1.00	–	7.01[e]	1.00	–	8.89[e]	1.00	–
27	3.80	1.00	–	4.94 (+)	1.06	0.33	2.20	1.00	–	7.27	1.00	–
28	14.91	1.00	–	11.93	1.08	0.46	5.27	1.00	–	8.40	sh.	–
Mobile phase		Hept/MTBE (60:40)			Hept/MTBE (60:40)			Hept/MTBE (60:40)			Hept/MTBE (60:40)	

[a] The absolute configuration (or optical rotation sign) of the first eluting enantiomer is indicated.
[b] MTBE 100%.
[c] Heptane/MTBE (40:60).
[d] Heptane/MTBE (90:10).
[e] Heptane/MTBE (98:2).

monomeric proline-derived **CSP-3**, long retention times and loss of enantioselectivity were observed for polyproline-derived **CSP-4** after a long stabilization time. **CSP-1** and **CSP-2** were also tested using chloroform in the mobile phase in the absence of any hydrogen bond donor solvent. **CSP-1** showed the expected behaviour for a conventional Pirkle-type CSP, with enantioselectivity restricted to those analytes containing 3,5-dinitrobenzoyl groups (**8**: k'_1, 32.73; α, 1.11; **9**: k'_1, 7.99; α, 1.21 and **10**: k'_1, 7.72; α, 1.15 in chloroform 100%, for instance). When **CSP-2** was tested using 100% chloroform as mobile phase compounds **16**, **21**, **23** and **24**, with hydrogen bond acceptor character, were not retained while compounds **9**, **10**, **19**, **20** and **28** showed k' values around 10, being $k'_1 > 18$ for compound **8**. The addition of a small amount of 2-PrOH to the mobile phase (0.2%) produced k' values between 2 and 6 for the same compounds although no resolution was observed for any of the compounds tested.

To test the involvement of hydrogen bonding in the behaviour observed for the polyproline-derived CSPs in chloroform, methyl *t*-butyl ether (MTBE) was used as a mobile phase without any hydrogen bond donor co-solvent. Only heptane was used to control retention time. Using these conditions **CSP-2** and **CSP-4** showed an enantioselective pattern similar to that obtained in heptane/2-PrOH mobile phases (Table 2). Although resolution was lower than when heptane/2-PrOH was used, sensibly the same separation factors were obtained with MTBE (Fig. 5). Therefore, although hydrogen bonding has an indisputable role in enantiorecognition, this does not appear to be the main factor in determining enantioselectivity of these polyproline CSPs.

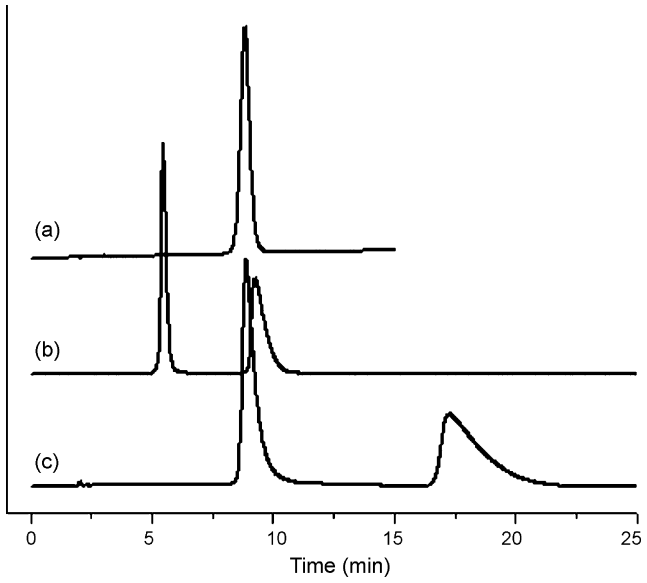

Fig. 5. Separation of **14** into its enantiomers on **CSP-2**. Mobile phase: (a) Chloroform (100%), (b) heptane/2-PrOH (90:10) and (c) heptane/MTBE (40:60). Flow rate: 1 mL/min.

The changes observed in enantioselectivity were proven to be reversible. When polyproline-derived columns were fluxed again with heptane/2-PrOH mixtures after the use of MTBE or chloroform mobile phases, retention times and selectivity values reached those

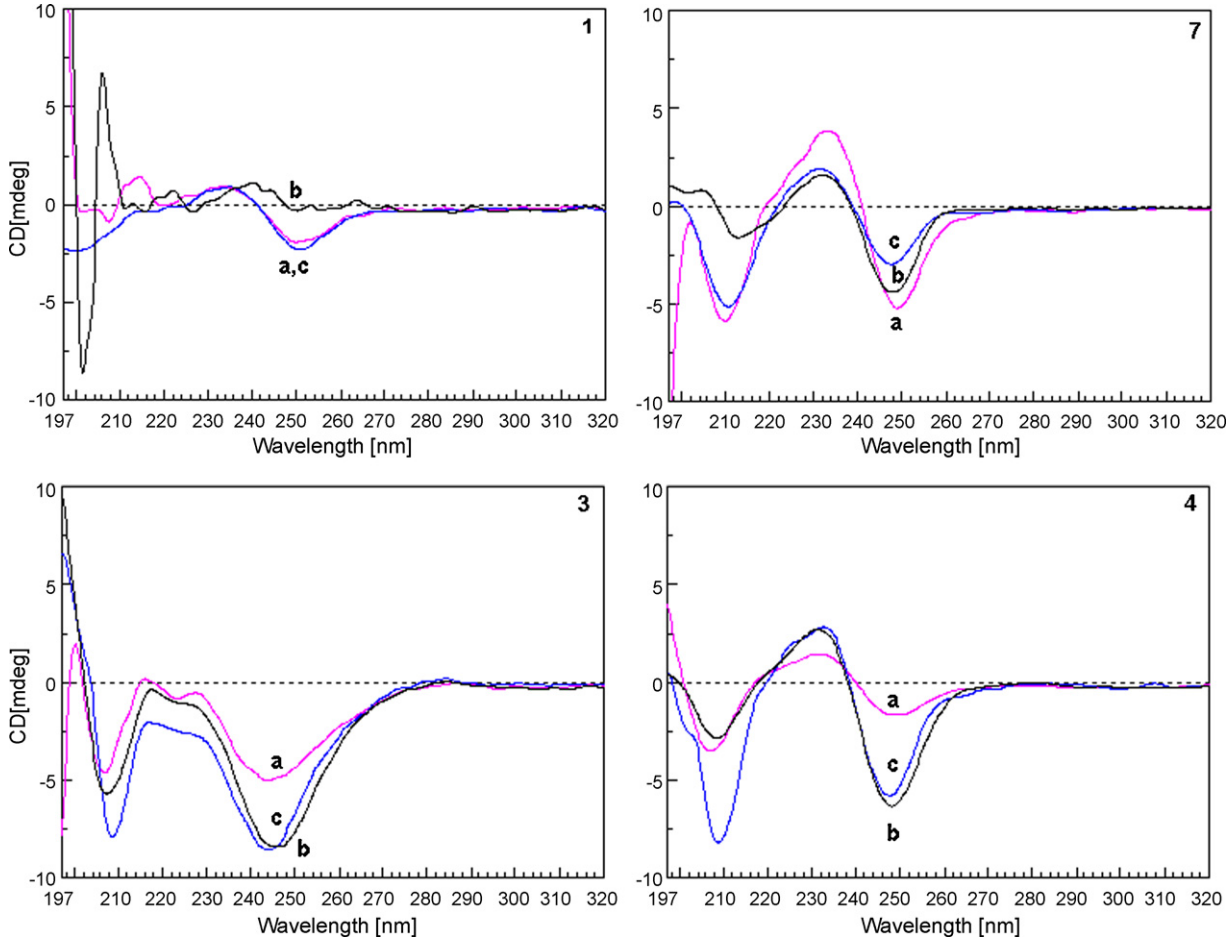

Fig. 6. Circular dichroism spectra of monoproline (**1**,**3**) and polyproline (**7**,**4**) CSs using (a) MTBE; (b) heptane–2-PrOH (90:10) and (c) ACN as solvents (0.05 mg/mL, cell 2 mm, 21 °C).

obtained previously. In addition, heating of columns reduced the stabilization time required to attain starting enantioselective values. These observations point to the involvement of conformational changes between mobile phases in the changes in chromatographic behaviour in these polyproline CSs.

3.1. Circular dichroism conformational studies on polyproline CSs

Two main helical conformations have been characterized for poly-L-proline peptides, the so-called PPI and PPII helices. The right-handed helical form PPI has all amide bonds in *cis* conformation while the PPII helix is a left-handed helix with all peptide bonds in *trans* conformation. Solvents can favour the stability of one conformation over the other. Thus, PPI conformation is favoured in aliphatic alcohols, and PPII is favoured in water, trifluoroethanol, benzyl alcohol and organic acids [4,5].

These two conformations can be distinguished using several spectroscopic techniques. Circular dichroism is one of the most useful in this regard. Thus, PPI conformation is characterized by a CD spectrum with a medium intensity negative band at 199 nm, a strong positive band at 215 nm, and a weak negative band at 231 nm. The CD spectrum of a PPII conformation is characterized by a strong negative band at 204 nm and a weak positive band at 227 nm [15].

CD spectra for the monomeric (**1** and **3**) as well as for the polyproline CSs (**7** and **4**) were registered using several solvents chosen on the basis of those used in the chromatographic tests (Fig. 6). Thus, MTBE 100%, ACN 100% and heptane/2-PrOH (90:10) were considered. The strong absorption of chloroform at wavelengths below 220 nm, taking into account the path length of the cell used (2 mm), prevented its use in this technique. All CD spectra, including the monomers **1** and **3**, showed a strong negative band at 248 nm, which was attributed to the intrinsic structural nature of the monomers. The two polyproline CSs exhibited analogous CD spectra in the conditions used. Thus, a strong negative band appeared in the range of 201–218 nm followed by a medium positive band in the range of 225–240 nm in the three conditions considered. These spectra indicate a PPII like conformation for the polyproline CSs in the solvents tested. This conclusion was confirmed by the observation of a change in conformation to the more stable PPII in ACN/water (40:60). The CD spectrum of the sample, a solution obtained using a peptide mixture coming from the evaporation of chloroform, was registered at 9 and 48 h (Fig. 7).

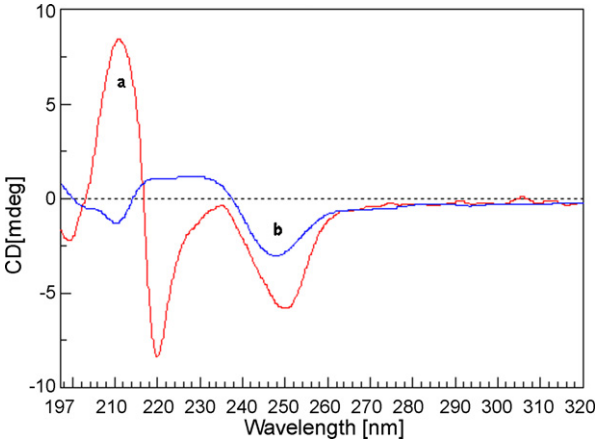

Fig. 7. Circular dichroism spectra of **4** using ACN/water (40:60) (a) 9 h and (b) 48 h after preparing the solution (0.1 mg/mL, cell 2 mm, 21 °C).

3.2. ^{13}C nuclear magnetic resonance studies

Chloroform is a common solvent in NMR, a technique also used in the conformational study of molecules in solution. In particular, a number of studies on the conformation of polyprolines have been published using this technique [16–19]. Therefore, additional NMR experiments were carried out for the polyproline CS structure using several solvents. To this purpose a purified octaproline peptide **7** was used. The solubility characteristics of the octamer **7** in

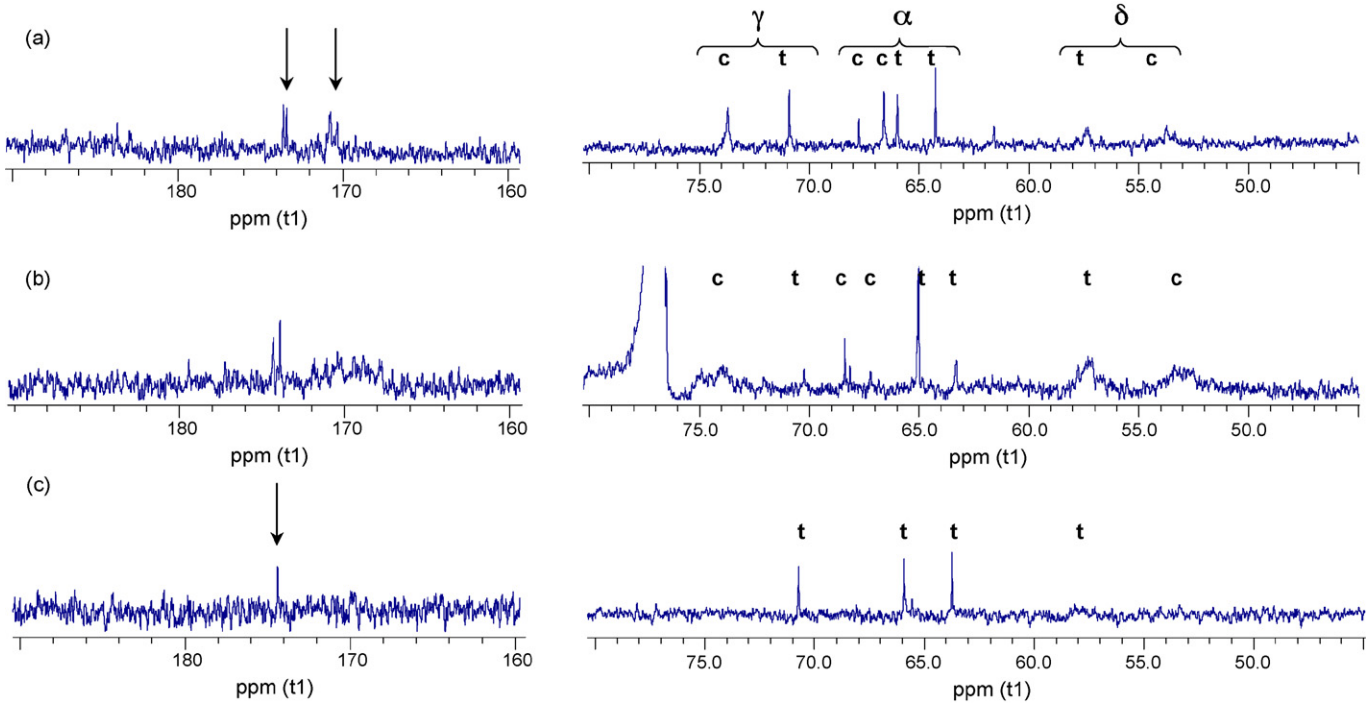

Fig. 8. ^{13}C NMR spectra of the regions 45–80 ppm and 160–180 ppm. (a) Pyridine-d_5, 25 °C; (b) $CDCl_3$, 25 °C and (c) CD_3CN, 25 °C (the assignment of the amide bond disposition is indicated as (***c***) *cis* and (***t***) *trans*).

the deuterated solvents considered limited the amount of 7 used to only 2 mg/0.7 mL solved in CD_3CN, while amounts of 20 and 15 mg were used in $CDCl_3$ and pyridine-d_5, respectively. The lack of strict magnetic equivalence for the proton signals of the different proline units, and the short relaxation time that originates wide signals, limit the usefulness of ^1H NMR. ^{13}C NMR spectra of the octamer **7** at 25 °C were registered instead (Fig. 8).

The signals were assigned on the basis of the data in the literature [18,19], and by comparison with the spectra of the monomers **1** and **3** and related synthetic intermediates [10]. In spite of the *a priori* non-equivalence of proline units, the ^{13}C NMR spectrum recorded using CD_3CN was quite simple. Thus, the signal observed at 70.7 ppm was assigned to the γ carbon of pyrrolidine rings. The two signals located at 65.9 and 63.7 ppm were assigned to magnetically non-equivalent α carbon atoms and the broad signal observed at 57.8 ppm was attributed to the δ carbon atoms of the different pyrrolidine rings in the molecule. Finally, a single absorption is observed at 174.4 ppm in the region of carbonyl groups. As pointed out by CD measurements and the simplicity of the ^{13}C NMR spectrum, a PPII conformation, with an all-*trans* disposition for peptide bonds, can be assumed for the polyproline molecule in ACN/CD_3CN.

The splitting of all signals was observed in the ^{13}C NMR spectra in $CDCl_3$, which suggests a random distribution of *cis* and *trans* disposition for the amide bonds within the molecules. In order to assess a possible change in the distribution of *cis*/*trans* amide bonds, the spectra was also recorded in pyridine-d_5 at 25 and 60 °C. The spectra in this solvent resulted to be similar to that obtained in $CDCl_3$. Nevertheless, no significant change in intensity of *cis*/*trans* signals was observed within the considered range of temperatures.

The splitting of signals observed in $CDCl_3$ and pyridine-d_5 in contrast to the single resonances observed in CD_3CN, implies a higher flexibility of the CS in the former and a decrease in the ratio of ordered structure. This characteristic is in agreement with the lack of enantioselectivity of this kind of CSs when chloroform is used as a mobile phase in chromatography.

4. Conclusions

The polymeric nature of CS **2** strongly affects the chromatographic behaviour of **CSP-2** regarding what is common for a Pirkle-type CSP. The application domain is enlarged and not exclusively focused on the complementarity of π–π interactions. Hydrogen bonding has an indisputable role in enantiorecognition. However, enantioselectivity is affected by the conformation that a particular mobile phase induces to the CS. The lack of enantioselectivity observed for polyproline CSs when using chloroform in the mobile phase could be explained by the higher conformational flexibility observed in this solvent by NMR.

Acknowledgements

Financial support from the *Ministerio de Educación y Ciencia* of Spain and from the European Regional Development Fund (ERDF) (project number CTQ2006-003378/PPQ) is gratefully acknowledged. The study has been partially supported by the *Comissionat per a Universitats i Recerca* of the *Generalitat de Catalunya* (project 2005-SGR00180). R.S. thanks the *Generalitat de Catalunya* for a doctoral fellowship.

References

[1] J. Haginaka, J. Chromatogr. A 906 (2001) 253, doi:10.1016/S0021-9673(00)00504-5.
[2] T.J. Ward, Anal Chem. 78 (2006) 3947, doi:10.1021/ac060622o.
[3] E. Yashima, J. Chromatogr. A 906 (2001) 105, doi:10.1016/S0021-9673(00)00501-X.
[4] P.M. Cowan, S. McGravin, Nature 176 (1955) 501.
[5] W. Traub, U. Shmueli, Nature 198 (1963) 1165.
[6] J. Huang, P. Zhang, H. Chen, T. Li, Anal. Chem. 77 (2005) 3301, doi:10.1021/ac050050s.
[7] J. Huang, H. Chen, T. Li, J. Chromatogr. A 1113 (2006) 109, doi:10.1016/j.chroma.2006.01.128.
[8] R. Sancho, A.M. Pérez, C. Minguillón, J. Sep. Sci. 29 (2006) 905, doi:10.1002/jssc.200500484.
[9] J. Huang, H. Chen, P. Zhang, T. Li, J. Chromatogr. A 1109 (2006) 307, doi:10.1016/j.chroma.2006.01.172.
[10] T. González, O. Abad, M.C. Santano, C. Minguillón, Synthesis (2004) 1171, doi:10.1055/s-2004-822384.
[11] A. Albericio, M. Cruz, L. Debéthune, R. Eritja, E. Giralt, A. Grandas, V. Marchán, J.J. Pastor, E. Pedroso, F. Rabanal, M. Royo, Synth. Commun. 31 (2001) 225, doi:10.1081/SCC-100000203.
[12] L. Oliveros, C. Minguillón, B. Desmazières, P.-L. Dèsbene, J. Chromatogr. 606 (1992) 9, doi:10.1016/0021-9673(92)85252-O.
[13] L. Oliveros, C. Minguillón, T. González, J. Chromatogr. A. 672 (1994) 59.
[14] W.H. Pirkle, D.W. House, J.M. Finn, J. Chromatogr. 192 (1980) 143, doi:10.1016/S0021-9673(00)81849-X.
[15] S. Kakinoki, Y. Hirano, M. Oka, Polym. Bull. 53 (2005) 109.
[16] D.A. Torchia, Macromolecules 5 (1972) 566.
[17] S.K. Sarkar, D.A. Torchia, K.D. Kopple, D.L. VanderHart, J. Am. Chem. Soc. 106 (1984) 3328.
[18] J.J. Dechter, S.D. Clark, L. Mandelkern, Macromolecules 11 (1974) 274.
[19] D.A. Torchia, J.R. Lyerla, Biopolymers 13 (1974) 97.