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Liquid chromatographic separation of amino acid enantiomers on a silica-bonded chiral *s*-triazine column

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Abstract

A chiral derivatizing reagent (CDR) was synthesized by consecutive nucleophilic replacement of two chlorine atoms in 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) by L-valinamide and L-phenylalaninamide, yielding N-[4-((*S*)-1-carbamoyl-2-methyl-propylamino)-6-chloro-[1,3,5]triazin-2-yl]-L-phenylalanine. This CDR was used for the derivatization of free DL-amino acids, followed by the liquid chromatographic separation of the diastereomers thus formed, and for the synthesis of a chiral stationary phase (CSP). The CSP was synthesized by substitution of the remaining chlorine atom with aminopropylsilica, yielding {3-[4-((*S*)-1-carbamoyl-2-methyl-propylamino)-6-((*S*)-1-carbamoyl-2-phenyl-ethylamino)-[1,3,5]triazin-2-ylamino]-propyl}-functionalized silica gel. This new CSP was found to effect, in part very high, resolutions for enantiomers of dansylamino acids when mixtures of acetonitrile and 0.01 *M* sodium acetate buffer (pH 4) were used as eluents.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Diastereomer separation; Derivatization, LC; Amino acids; Triazines

1. Introduction

As a result of its high reactivity, trifunctionality and economic large-scale production, cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) (CC) is a versatile starting material for the bulk synthesis of reactive dyes, herbicides, polymers and pharmaceuticals [1,2].

In separation technology, *s*-triazine-derivatized stationary phases are widely used for affinity chromatography [3] and applications of chiral *s*-triazinyl phases in gas–liquid chromatography [4] and high-performance liquid chromatography (HPLC) [5–7] have also been reported.

We have previously shown that cyanuric halides are also suitable starting materials for the synthesis of various tailor-made chiral reagents for the derivatization and indirect liquid chromatographic separation of amino acid enantiomers as diastereomers [8–10].

Application of the opposite approach, i.e., reaction of amino acid-derived chiral *s*-triazines with aminopropylsilica (APS), yielded chiral stationary phases (CSPs) suitable for the direct HPLC resolution of N-(2,4-dinitrobenzoyl) (Dnb)-DL-amino acids or their esters [5,6].

In this paper, we describe the synthesis of a CSP by reaction of APS with a bisubstituted, chiral monochloro-*s*-triazine, obtained from CC by consecutive nucleophilic replacement of two

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chlorine atoms by L-valinamide and L-phenylalaninamide. This mixed (with respect to chiral moieties) CSP is particularly suitable for resolving N-(dansyl)-DL-amino acids.

2. Experimental

2.1. Instrumentation

The HPLC instrument consisted of a Model 880 PU reciprocating pump with active damper, a Model 880-902 low-pressure gradient former, a Model 801-SC controller and a Model 875-UV variable-wavelength UV detector (Jasco, Kyoto, Japan). A D-2500 chromato-integrator (Merck–Hitachi, Darmstadt, Germany) was used for recording the chromatograms and for data processing. Samples were injected manually on to the column by means of a Model 7125 injector (Rheodyne, Cotati, CA, USA).

2.2. Solvents and chemicals

Methanol (MeOH) and acetonitrile (MeCN) (chromatography grade), acetone and ethyl acetate (synthetic grade), acetic anhydride (pro analysi, p.a.) sodium carbonate (p.a.), sodium hydrogencarbonate (p.a.) and sodium acetate (NaOAc) trihydrate (p.a.) were purchased from Merck, dimethyl sulfoxide (DMSO) from Fluka (Buchs, Switzerland) and CC from Aldrich (Steinheim, Germany). Free L- and D-amino acids were obtained from Fluka or Sigma (St. Louis, MO, USA). N-Substituted DL-Phe [substituents dansyl (Dns), methyl (Me), benzoyl (Bz), benzyloxycarbonyl (Z), 2,4-dinitrophenyl (Dnp), 9-fluorenylmethyloxycarbonyl (Fmoc)], Dns-DL-Trp, Z-DL-Trp, Trp-OBz, L-phenylalaninamide (L-Phe-NH_2) and L-valinamide hydrochloride (L-Val-NH_2·HCl) were purchased from Bachem (Bubendorf, Switzerland) or Sigma. C-Substitution was carried out in the laboratory by esterification of free or N-protected DL-Phe with acetyl chloride in 1-propanol [11], yielding the 1-propyl (Prp) esters, viz., DL-Phe-Prp, Dns-DL-Phe-Prp, Bz-DL-Phe-Prp and Z-DL-Phe-Prp.

APS [Nucleosil 100-5 NH_2, particle size 5 μm, pore size 100 Å (0.1 nm) was obtained from Macherey–Nagel (Düren, Germany). Aliquots (1–5 μl) of Dns-amino acids (1% in MeOH) were injected manually on to the column and UV absorption was measured at 254 nm.

2.3. Syntheses of the chiral derivatizing reagent and the chiral stationary phase

Characterization

For thin-layer chromatography (TLC), precoated plates (Kieselgel 60 F_{254}, size 20 × 20 cm; Merck), were used; R_F values were determined at room temperature (ca. 21°C) in glass chambers (Desaga, Heidelberg, Germany) coated with filter-paper; the distance from the start to the solvent front of the TLC plates was ca. 10 cm. The solvent systems were as follows (v/v): (A) *n*-hexane–acetone (55:45); (B) *n*-hexane–acetone–dichloromethane (50:30:20); and (C) *n*-hexane–acetone–acetic acid (57.5:40:2.5). Analytes were detected by inspection under UV light at 254 nm.

Electron impact mass spectra were measured at an ionizing energy of 70 eV using a Varian Model 311 A mass spectrometer.

Elemental analyses were carried out with a Model 1106 CHN elemental analyser (Carlo Erba, Milan, Italy); optical rotations were determined at 25°C using a Perkin-Elmer (Überlingen, Germany) Series 241 polarimeter; a cuvette of 100 mm × 3.6 mm I.D. was used. Melting points were determined in open capillaries using a Model 520 apparatus (Büchi, Flawil, Switzerland) and were not corrected.

Exhaustion of L-Val-NH_2 and L-Phe-NH_2 in the course of the synthesis of the dichlorotriazine and the chiral derivatizing reagent (CDR), respectively, was monitored by TLC and spraying with ninhydrin reagent (1% in 70% ethanol, w/v).

Derivatization of the amino groups of APS with the CDR and end-capping of CSP was tested with ninhydrin: to 50-mg aliquots of the stationary phase, 500 μl of ninhydrin reagent were added and the suspension was heated for several minutes at 100°C. Formation of a blue colour indicated the presence of amino groups.

N-(4,6-Dichloro-[1,3,5]triazin-2-yl)-L-valine amide (DTVA)

To CC (2.58 g, 14 m*M*) in acetone (35 ml) at 0–5°C, H-Val-NH_2·HCl (2.14 g, 14 m*M*) in 10 ml of water was added with stirring and the temperature was kept at 5–10°C. After addition of 2 *M* Na_2CO_3 (8 ml), ca. pH 8 was measured. The mixture was stirred for 1 h at 20°C, evaporated to dryness in vacuo and ethyl acetate (350 ml) was added. The organic phase was washed with water (2 × 175 ml) and evaporated to dryness after the addition of toluene (10 ml). Yield, 3.0 g (81%) of white solid, m.p. 151–152°C; MS, *m/z* 219, 221 ($M - CONH_2$), intense; TLC, R_F (A) 0.45, R_F (B) 0.35, R_F (C) 0.59.

N-[4-((S)-1-Carbamoyl-2-methyl-propylamino)-6-chloro-[1,3,5]triazin-2-yl]-L-phenylalanine (chiral derivatizing reagent)

To DTVA (2.64 g, 10 m*M*) in acetone (35 ml), H-Phe-NH_2 (1.64 g, 10 m*M*) in 10 ml of water was added at room temperature with stirring and the pH was adjusted to 8 by addition of 1 *M* $NaHCO_3$ (10 ml). After 3 h at 20°C, the mixture was evaporated to dryness in vacuo and the residue treated as described above for DTVA. Yield, 3.64 g (93%); m.p. 128–130°C.

For elemental analysis, aliquots of CDR (12 mg) in 50% MeCN (0.5 ml) were purified by HPLC using a 250 mm × 16 mm I.D. preparative column packed with Nucleosil 100-C_{18}, particle size 5 μm; the eluent was MeCN–water (25:75, v/v) at a flow-rate of 5 ml min^{-1}. Suitable fractions were combined, evaporated to dryness, dried over P_4O_{10} and analysed. MS, *m/z* 347, 349 ($M - CONH_2$), 302, 304 ($M - 2\ CONH_2$); $[\alpha]_D^{25} = -32.14$ ($c = 1$, dioxane); elemental analysis for $C_{17}H_{22}ClN_7O_2$ (391.86), calc. (found) C 52.11 (51.81), H 5.62 (5.63), Cl 9.07 (9.19), N 25.03% (24.77%); TLC, R_F (A) 0.08, R_F (B) 0.03, R_F (C) 0.21.

{3-[4-((S)-1-Carbamoyl-2-methyl-propylamino)-6-((S)-1-carbamoyl-2-phenyl-ethylamino)-[1,3,5]triazin-2-ylamino]-propyl}-functionalized silica gel (chiral stationary phase)

To a suspension of APS (3.25 g) in DMSO (15 ml), CDR (1.3 g, 3.32 m*M*) in DMSO (30 ml) was added with stirring and the pH was adjusted to 8 by addition of 1 *M* $NaHCO_3$ (13 ml). The suspension was heated at 100°C in a closed vessel with occasional manual shaking. After 24 h at 100°C, further CDR (1.3 g) in DMSO (20 ml) was added and heating continued for 24 h at 100°C. CSP was collected on a glass sinter and washed with water (3 × 50 ml), DMSO and acetone. Yield, 3.58 g; elemental analysis gave 3.49% N (unmodified APS 1.10% N), corresponding to a 41% derivatization yield. A sample of the *s*-triazine-modified APS was still positive in the ninhydrin test (although the colour yield was much lower in comparison with the unmodified APS). For end-capping of the underivatized amino groups, CSP was suspended in ethyl acetate (40 ml) and acetic anhydride (5 ml) was added dropwise at 0°C with gentle manual shaking. After 75 min at room temperature, water (10 ml) was added dropwise with shaking and the CSP was collected and washed as described above. The ninhydrin test on a sample was negative after heating.

2.4. Derivatization of DL-amino acids with CDR

Standard solutions (100 m*M* in 1 *M* HCl) of selected DL-amino acids (Glu, Pro, Phe) were prepared. Aliquots of 30 μl (3 μmol) were mixed with 1 *M* $NaHCO_3$ (45 μl) and 500 μl (5 μmol) CDR (10 m*M* in DMSO). The mixture was heated for 1 h at 100°C. DMSO (1425 μl) was added and aliquots (1–5 μl) were analysed by HPLC. For chromatographic conditions, see Fig. 2.

2.5. Packing of stationary phase

A slurry of CSP (3 g) in a mixture of toluene (20 ml) and cyclohexanol (25 ml) was sonicated and packed at ca. 450 bar in a 250 mm × 4 mm I.D. stainless-steel column by Bischoff Analysentechnik (Leonberg, Germany). The phase was thoroughly washed with 1-propanol and aqueous MeCN and finally equilibrated with the eluents used for analysis.

Plate numbers (N) were calculated according to the equation $N = 5.545(t_R/w_h)^2$, where t_R is the total retention time and w_h the peak width at

half-height. They were determined using a standard mixture composed of toluene (1), ethyl benzoate (2) and diethyl phthalate (3), with *n*-hexane–2-propanol (1.5%, v/v) as eluent at a flow-rate of 1.2 ml min^{-1} and ambient temperature. The N/m values were 85 470, 68 493 and 56 179 for components 1, 2 and 3, respectively.

3. Results and discussion

3.1. Synthesis of the chiral derivatizing reagent and its use for the indirect chiral resolution of DL*-amino acids*

In previous investigations, we had synthesized and investigated a number of monohalo-*s*-triazine-based reagents obtained by substitution of two of the three chlorine atoms in CC by a chromophore or fluorophore and by L-Ala-NH_2 serving as the chiral moiety [9,10]. These CDRs were used for the derivatization of DL-amino acids and the liquid chromatographic resolution of the diastereomers thus formed [8–10].

In continuation of that work, we now describe the synthesis of an *s*-triazine-based CDR containing two different chiral amino acids, viz., L-Val-NH_2 and L-Phe-NH_2 (Fig. 1). These amino acids are known to exert good chiral recognition in GC [4,12] and LC [13]. The phenyl ring of L-Phe-NH_2, in addition, serves as a UV-absorbing chromophore. This CDR, when reacted with free DL-amino acids, yields diastereoisomers which are resolvable by reversed-phase HPLC with good resolution (Fig. 2). Consequently, this reagent was also chosen for the synthesis of a CSP by reaction with APS.

3.2. Synthesis of the chiral stationary phase and its use for the direct resolution of dansyl amino acids

The CDR described above was used as a reagent for preparing a CSP by reaction with APS in a mixture of DMSO and aqueous $NaHCO_3$ at pH 8 and 100°C for 48 h. An aliquot of the CSP was isolated, washed with solvents and tested for the presence of unreacted amino

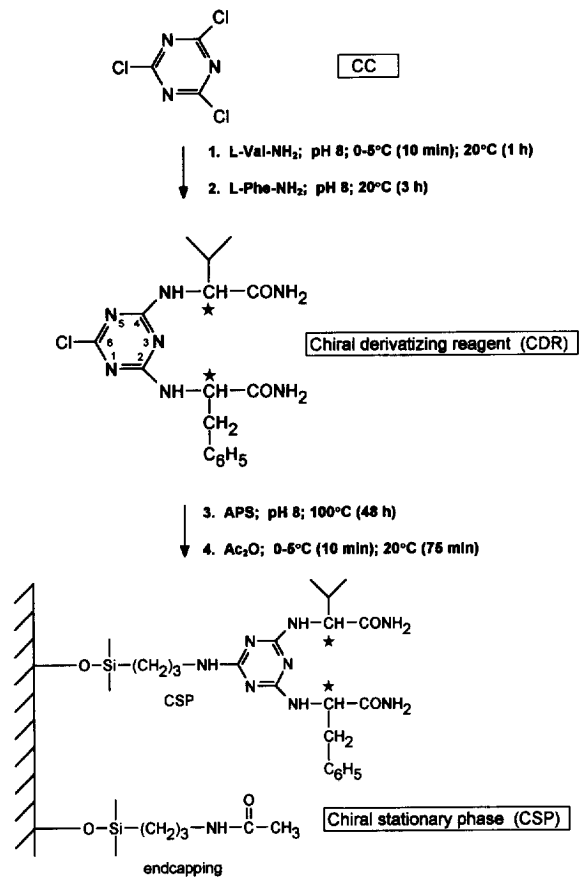

Fig. 1. Scheme of the synthesis of the chiral derivatizing reagent (CDR) from cyanuric chloride (CC) and L-amino acid amides, reaction of CDR with aminopropylsilica (APS) and end-capping with acetic anhydride (Ac_2O) yielding the chiral stationary phase (CSP); asterisks indicate chiral centres.

groups by the ninhydrin test (see Experimental). Although giving a much lower colour yield in comparison with the underivatized APS, the test was still positive. From the elemental analysis data, a substitution of 41% of the amino groups in APS by the chiral moiety was calculated.

The amino groups of APS which had not reacted with CDR were end-capped by reaction with acetic anhydride. Application of the ninhydrin test gave a negative result. The CSP was slurry-packed in a stainless-steel column (see Experimental).

The enantioselectivity of the CSP was tested for the hydrophobic, free DL-amino acids Trp,

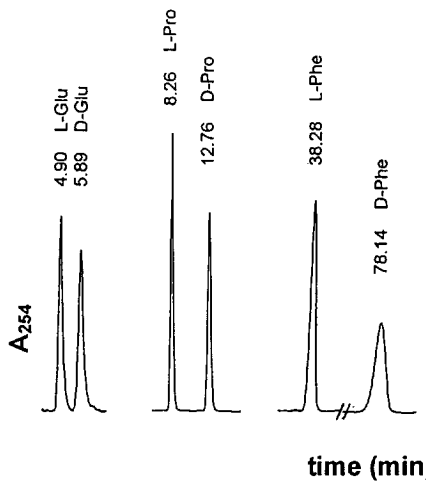

Fig. 2. Resolution of diastereomers formed by derivatization of DL-Glu, DL-Pro and DL-Phe with CDR. Chromatographic conditions: column, 250 × 4 mm I.D.; Nucleosil 100 C_{18}, 5 μm; eluent, 20% MeCN in 0.01 *M* NaOAc (pH 4); flow-rate, 1 ml min^{-1}; A_{254}, absorbance at 254 nm; for derivatization conditions see Experimental.

Phe, Val and Leu. Further, DL-Phe, which was either N- or C-terminal derivatized or fully protected, was investigated. Also studied were racemic Dns-Phe, N-Me-Phe, Bz-Phe, Z-Phe, Fmoc-Phe, Dnp-Phe, Phe-Prp, Dns-Phe-Prp and Bz-Phe-Prp. In addition, free DL-Trp and racemic Dns-Trp, Z-Trp and Trp-OBzl were examined (for abbreviations, see Experimental).

Although the composition and pH of the eluents were systematically varied (tested 20–30% MeCN in 0.01 *M* NaOAc adjusted to pH 4 or 7; 20–30% MeCN in 0.1% TFA adjusted to pH 2.0) it was found that, among the free DL-amino acids, only DL-Trp showed sufficient retention for chiral resolution (Fig. 3b). Less hydrophobic amino acids such as DL-Phe, DL-Val and DL-Leu were not retained on the CSP even when using pure water as eluent. The enantiomers of Dns-DL-Phe with a free carboxyl group, however, were resolved when the above eluents were used.

Among the other derivatized amino acids, only Bz-DL-Phe was partly resolved using 20% MeCN in 0.1% TFA (pH 2) as eluent (Fig. 3c); none of the other derivatives of DL-Phe described above were resolved with either of the eluents.

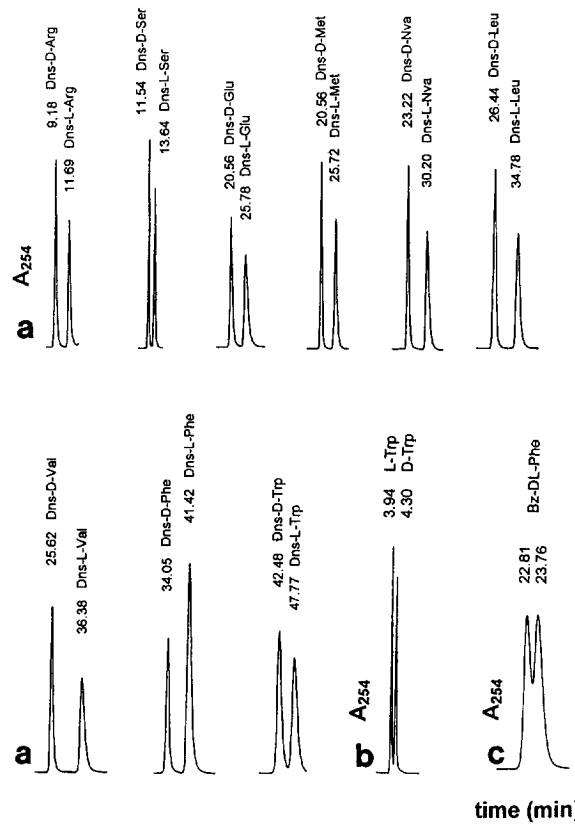

Fig. 3. Sections from chromatograms of the resolution of (a) racemic Dns-DL-amino acids (exception, Dns-DL-Phe to which Dns-L-Phe has been added), (b) Bz-DL-Phe and (c) DL-Trp on a CSP. Eluent: (a) 30% MeCN in 0.01 *M* NaOAc adjusted to pH 4 by addition of 2 *M* H_2SO_4; (b) 20% MeCN in 0.1% TFA (pH 2); (c) 20% MeCN in 0.01% ammonium acetate (pH 7); flow-rate, 1 ml min^{-1}.

Therefore, for a systematic evaluation of resolution, several Dns-DL-amino acids were selected according to their characteristic side-chains, viz., Dns-Arg (basic), Dns-Glu (acidic), Dns-Leu, Dns-Val, Dns-Nva, Dns-Met and Dns-Ser (aliphatic and neutral, with heteroatoms for Met and Ser), Dns-Phe and Dns-Trp (aromatic).

The pH dependence was investigated for resolution (R_s), separation factor (α) and capacity factors (k') using 30% MeCN in 0.01 *M* NaOAc as eluent and a maximum of pH 4 was determined (Fig. 4).

Chromatograms of selected Dns-amino acids are shown in Fig. 3a and data are given in Table

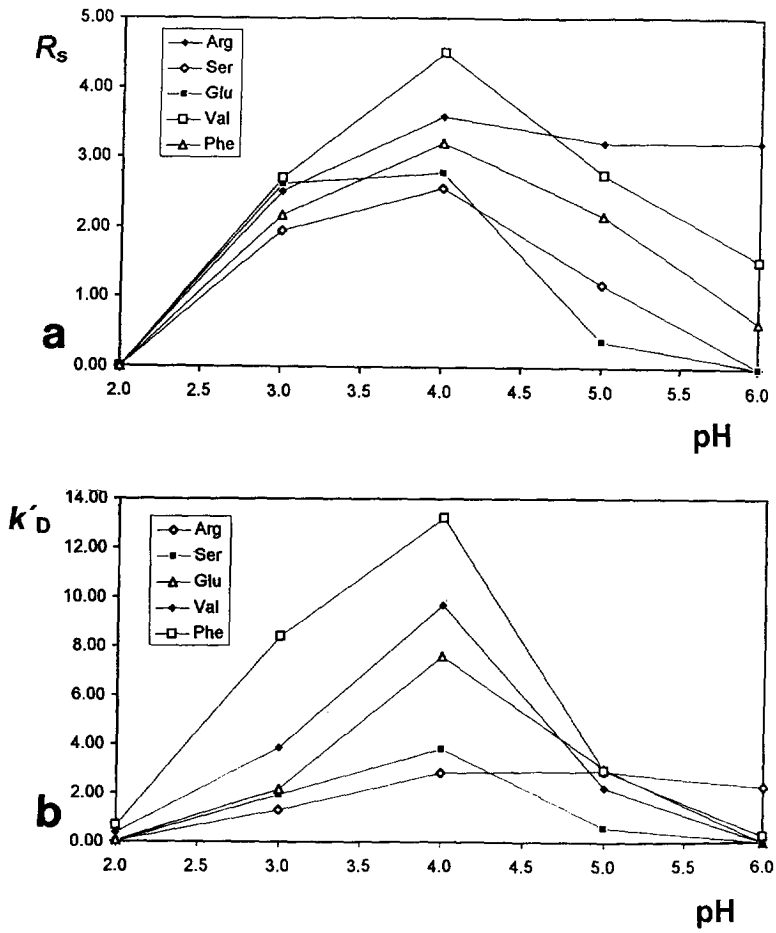

Fig. 4. Dependence of (a) resolution (R_s) of Dns-DL-amino acids and (b) capacity factor (k'_D) of Dns-D-amino acids on pH of eluent (30% MeCN in 0.01 *M* NaOAc; pH was adjusted to 2–6 by addition of 2 *M* H_2SO_4).

1. The D-enantiomers eluted before the L-enantiomers in all cases.

These results are remarkable since the enantioseparations of Dnb-amino acids, Dnb-amino acid methyl esters and Dnb-amino alcohols have been reported on structurally related CSPs [5–7], whereas those of free or dansylated DL-amino acids were previously unknown.

Although the spatial arrangements and $\pi-\pi$ interactions between the dansyl groups of the chiral selectands and the *s*-triazinyl and phenyl groups of CSP are assumed to be responsible for the chiral discrimination, it has not yet been rationalized why under almost identical chromatographic conditions (20–30% MeCN in 0.01

Table 1
Capacity factors (k'_D and k'_L), separation factors (α) and resolution (R_s) of Dns-DL-amino acids on a CSP

Dns-DL-amino acid	k'_D	k'_L	α	R_s
Dns-DL-Arg	2.84	3.89	1.37	3.59
Dns-DL-Ser	3.83	4.71	1.23	2.56
Dns-DL-Pro	6.11	6.11	1.00	0.00
Dns-DL-Glu	7.60	9.79	1.29	2.78
Dns-DL-Met	7.60	9.76	1.28	3.71
Dns-DL-Nva	8.72	11.64	1.33	3.77
Dns-DL-Leu	10.06	13.55	1.35	3.97
Dns-DL-Val	9.72	14.22	1.46	4.50
Dns-DL-Phe	13.25	16.33	1.23	3.20
Dns-DL-Trp	16.77	18.99	1.13	1.60

Eluent: 30% (v/v) MeCN in 0.01 *M* NaOAc, adjusted to pH 4 by addition of 2 *M* H_2SO_4; flow-rate, 1 ml min^{-1}.

M NaOAc adjusted to pH 4), for example, Dns-DL-Phe, Dns-DL-Trp and DL-Trp are resolved whereas the enantiomers of Fmoc-DL-Phe, Z-DL-Phe, Bz-DL-Phe, Bz-DL-Phe-Prp, DL-Phe-Prp, Z-DL-Trp and DL-Trp-OBz are not resolved.

4. Conclusion

It has been shown that an APS-bonded *s*-triazine with mixed (with regard to amino acids) chiral selectors is capable of resolving enantiomers of dansylated amino acids with free carboxyl groups and of an underivatized, heteroaromatic amino acid, viz., DL-Trp; Bz-DL-Phe is partly resolved. Syntheses of the mono- and disubstituted triazines, required for the syntheses of the target CSPs, are straightforward and proceed in good yields [5–9], in contrast to their attachment to APS, which is moderate with a 41% yield. A higher efficiency of the CSP described would be expected if derivatization could be forced to a higher degree of completion. The yield might be increased if cyanuric fluoride (CF) [9] is used as the starting material. Alternatively, CF or CC might first be attached to APS with subsequent replacement of the halogens by chiral selectors. Although in this work end-capping of the remaining amino group of APS with acetic anhydride was performed, it might also be worthwhile to investigate the non-end-capped CSP as a mixed retention mode (e.g., ion pairing similar to that described in Ref. [7]) might be possible). Alternatively, end-capping with a higher homologue of acetic anhydride, which would increase the hydrophobicity of the CSP, might be advantageous, since most free and several of the derivatized DL-amino acids show very low retentions on the CSP described. The CSP was found to be stable under the experimental conditions described in this work for ca. 250 analyses.

An interesting aspect of the present work is that the approach described will allow, as supplements to the CDRs already described [5–7], the design of a large variety of well defined CDRs. Thus, for example, various protein or non-protein, free or suitably derivatized amino acid enantiomers, or even certain peptides [4,5], might be used as chiral selectors. Furthermore, owing to the trifunctionality of CC and the easy substitution of its halogen atoms, it might be possible to synthesize CSPs using other nucleophiles such as amines, phenols, alcohols and thiols. Finally, replacement of APS by differently derivatized or free silicas, materials such as linear or cyclic oligosaccharides or polysaccharides such as cellulose [14], or activated carbon [15] could greatly extend the scope of the chiral approach described here.

Note added in proof

The *Chemical Abstracts* name for the CSP used in this study is [*S*-(*R**,*R**)]-[3-[[4-[[1-(Aminocarbonyl)-2-methylpropyl]amino]-6-[[2-amino-2-oxo-1-(phenylmethyl)ethyl]amino]-1,3,5-triazin-2-yl]amino]propyl]-functionalized silica gel.

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