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New synthetic strategies for the preparation of novel chiral stationary phases for high-performance liquid chromatography containing natural pool selectors☆

Ilaria D'Acquarica *

Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Faculty of Pharmacy, University 'La Sapienza' of Rome, P. le Aldo Moro 5, 00185 Rome, Italy

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Abstract

Twelve new chiral stationary phases (CSPs) for high-performance liquid chromatography (HPLC) were generally prepared starting from the macrocyclic glycopeptide antibiotic teicoplanin, according to novel and efficient 'one-pot' synthetic strategies. Their chiral recognition abilities were evaluated under polar-organic mode HPLC, towards a variety of biopharmacological interesting racemates, such as β-amino acids and quaternary ammonium salts (e.g. carnitine and its derivatives). All materials were prepared by two different synthetic strategies, both leading to the formation of one or two stable ureidic functions on the CSP structure. The influence of the different spacers and of the silica matrix nature on the chiral performances was investigated. The obtained results suggested that the optimal synthetic strategy was that leading to the formation of two ureidic functions on the CSP structure, spaced-out by a six-carbon atoms aliphatic chain; the best chromatographic results were reached with the use of the spherical LiChrospher silica gel. Enantioselectivity factors (α) particularly high and short-time analyses characterised the analytical procedures; in addition, analytes lacking in chromophore groups were easily detected by evaporative light scattering (ELS) with no need of preliminary derivatization.

Keywords: Enantioselective HPLC; Glycopeptides containing chiral stationary phases; Teicoplanin; Evaporative light scattering detection; Polar-organic mode HPLC

1. Introduction

The increasing demands for the production of enantiomerically pure compounds in the field of pharmacology, chemistry, biotechnology, chemical engineering, etc. have led to enantioselective separations becoming one of the most important analytical tasks. In this context, enantioselective HPLC is one of the most powerful and widely

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* Fax: +39-6-49912780.

E-mail address: dacquari@caspur.it (I. D'Acquarica)

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employed separation techniques, both for analytical and preparative purposes. In particular, the use of chiral stationary phases (CSPs) allows the direct separation of the chiral analytes without any preliminary derivatization with chiral reagents.

Very recently, glycopeptide antibiotics have been successfully used as chiral selectors to resolve the enantiomers of a variety of racemic compounds by means of TLC, HPLC and HPCE techniques [1]. Armstrong et al., at the Pittsburgh Conference in 1994 [2] first introduced the concept of utilising glycopeptide antibiotics as chiral selectors. These natural compounds contain multiple stereogenic centres and a variety of functional groups. Much of their selectivity towards numerous compounds (underivatized amino acids, N-blocked amino acids, small peptides, α-hydroxycarboxylic acids, cyclic amides and neutral cyclic amines) can be attributed to their ability to form multiple interactions, e.g. hydrogen-bonding, hydrophobic and π–π interactions, etc.

For about 3 years in our laboratories, we have been preparing CSPs containing teicoplanin (TE) and related structures as chiral selectors. TE is a macrocyclic glycopeptide antibiotic, produced by the growth of certain strains of *Actinoplanes teichomyceticus* [3]. It is applied in the treatment of severe hospital-acquired infections caused by Gram-positive bacteria [4]. It binds stereospecifically to the carboxy-terminal of D-Ala-D-Ala sequences of the muramylpentapeptide formed during the biosynthesis of peptidoglycan, a key component of the bacterial cell wall. Structurally, TE contains a heptapeptide aglycone that bears three sugar units. It is noteworthy that the peptide backbone contains a *cis* peptide bond, which is essential to keep the structure in its rigid macrocyclic form. The aglycone moiety consists of four fused macrocyclic rings, which form a 'semi-rigid basket'. The basket contains seven aromatic rings, two of which have chloro-substituents and four have ionisable phenolic moieties. In the aglycone moiety, there are also a primary amine (the 'cationic site') and a carboxylic acid group (the 'anionic site'). The three sugar units are monosaccharides, namely α-D-mannose, β-D-*N*-acetyl-glucosamine, β-D-*N*-acyl-glucosamine. Five main components of TE have been identified, differing from each other only in the nature of the hydrocarbon chain of the *N*-acyl-glucosamine moiety. The chemical structure of the prevalent component of TE glycopeptide complex (A_2-2, $>85\%$) is reported in Fig. 1.

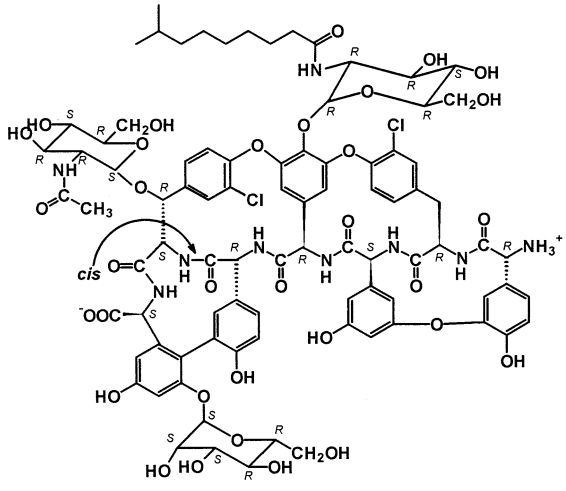


Fig. 1. Chemical structure of the prevalent teicoplanin component, A_2-2.

The direct chromatographic resolution of carnitine and O-acyl carnitines enantiomers on a teicoplanin-bonded chiral stationary phase has been recently reported, without any pre- or post-column derivatization [5].

The twelve chiral stationary phases reported in the present work were generally prepared by covalently linking the glycopeptide antibiotic TE to an aminopropyl-functionalized silica gel, via different bifunctional aliphatic and aromatic spacers, according to novel and efficient 'one-pot' synthetic strategies [6]. The influence of the silica matrix nature on the chiral performances was also briefly discussed. The separations of a variety of racemates were carried out in hydro-organic eluent systems with or without the addition of ammonium acetate buffers.

2. Experimental

2.1. Apparatus

Analytical liquid chromatography was performed on a Waters chromatograph equipped with a Rheodyne Model 7725i 20-μl injector and two Model M510 solvent-delivery systems. Different detectors were used, including a Model M490 programmable multi-wavelength detector (Waters Chromatography, Milford, MA), a Model Sedex-55 evaporative light scattering detector (Se.de.re., France) and a Model OR-990 chiroptical detector (Jasco Europe, Italy). Chromatographic data were collected and processed using the Millennium 2010 Chromatography Manager software (Waters Chromatography). IR spectra were recorded as potassium bromide pellets on a Nicolet (Madison, WI) Model 5DX Fourier transform (FT) IR spectrometer.

2.2. Chemicals and reagents

Irregular LiChrosorb Si 100 silica gel (5 μm particle size, 300 m^2/g), spherical LiChrospher Si 100 silica gel (5 μm particle size, 400 m^2/g) and HPLC-grade solvents were purchased from Merck (Darmstadt, Germany); spherical Kromasil Si 100 silica gel (5 μm particle size, 340 m^2/g) was from Eka Nobel (Bohus, Sweden); spherical Hypersil Si 120 silica gel (5 μm particle size, 170 m^2/g) was purchased from Shandon (Astmoor, UK); spherical Spherisorb Si 80 silica gel (3 μm particle size, 220 m^2/g) was from Waters (Milford, MA); spherical Nucleosil Si 500 silica gel (5 μm particle size, 35 m^2/g) was from Macherey–Nagel (Düren, Germany); spherical Prodigy Si 100 silica gel (5 μm particle size, 450 m^2/g) was from Phenomenex (Torrance, CA); (3-aminopropyl)triethoxysilane, dry toluene, dry pyridine, dry acetonitrile, 1,6-diisocyanatohexane (spacer A), 1,12-diisocyanatododecane (spacer B), di(*N*-succinimidyl) carbonate (spacer C), 1,3-phenylene diisocyanate (spacer D), 1,4-phenylene diisocyanate (spacer E), (3-isocyanatopropyl)triethoxysilane (spacer F) and water for HPLC were from Fluka (Sigma–Aldrich Company, Buchs, Switzerland); ammonium acetate was purchased from J.T. Baker (Division of Mallinckrodt Baker, Inc., Phillipsburg, NJ); teicoplanin was kindly provided by the Lepetit Research Centre (Gerenzano, Italy). Racemic and enantiomerically pure carnitine and derivatives were supplied from Sigma–Tau S.p.A. (Pomezia, Italy) and used as received, without further purification.

2.3. Preparation of the (3-aminopropyl)-functionalized silica gels

A slurry of 5.0 g of the proper silica gel (LiChrosorb Si 100, 5 μm; LiChrospher Si 100, 5 μm; Kromasil Si 100, 5 μm; Hypersil Si 120, 5 μm; Spherisorb Si 80, 3 μm; Nucleosil Si 500, 5 μm; Prodigy Si 100, 5 μm) in 120 ml of toluene was heated to reflux, and residual water was azeotropically removed. After cooling to room temperature, (3-aminopropyl)triethoxysilane was added (2.5 ml, 11 mmol) and the mixture was heated to reflux for 4 h. After cooling to room temperature, modified silica was isolated by filtration, washed with 50 ml portions of toluene, methanol and dichloromethane and dried at reduced pressure (90°C, 0.1 mbar, 1 h). Anal. found: for LiChrosorb, C 3.96, H 0.84, N 1.01, corresponding to 795 μmol of aminopropyl groups per gram of unmodified silica (2.65 $\mu mol/m^2$) (based on nitrogen); for LiChrospher Si 100, C 4.18, H 1.81, N 1.14, corresponding to 909 μmol of aminopropyl groups per gram of unmodified silica (2.27 $\mu mol/m^2$) (based on nitrogen); for Kromasil Si 100, C 3.79, H 1.03, N 1.31, corresponding to 1063 μmol of aminopropyl groups per gram of unmodified silica (3.13 $\mu mol/m^2$) (based on nitrogen); for Hypersil Si 120, C 1.96, H 0.58, N 0.55, corresponding to 414 μmol of aminopropyl groups per gram of unmodified silica (2.43 $\mu mol/m^2$) (based on nitrogen); for Spherisorb Si 80, C 1.79; H 0.57, N 0.67, corresponding to 510 μmol of aminopropyl groups per gram of unmodified silica (2.32 $\mu mol/m^2$) (based on nitrogen); for Nucleosil Si 500, C 0.47, H 0.14, N 0.15, corresponding to 108 μmol of aminopropyl groups per gram of unmodified silica (3.10 $\mu mol/m^2$) (based on nitrogen); for Prodigy Si 100, C 5.28, H 1.53, N 1.71, corresponding to 1450 μmol of aminopropyl groups per gram of unmodified silica (3.22 $\mu mol/m^2$) (based on nitrogen).

Table 1
Characterisation of the teicoplanin containing CSPs prepared by different synthetic strategies[a]

CSP[b]	Spacer	C (%)	H (%)	N (%)	μmol/g[c] silica	μmol/m^2 silica
TE-CSP-I-1A	A	16.43	1.51	3.80	~163	0.54
TE-CSP-I-1B	B	15.60	2.42	3.61	~139	0.46
TE-CSP-I-1C	C	6.54	1.28	2.16	~26	0.09
TE-CSP-I-1D	D	19.36	2.27	5.41	~218	0.73
TE-CSP-I-1E	E	22.5	2.52	5.10	~289	0.96
TE-CSP-I-2F	F	3.25	0.81	0.42	~31	0.10

[a] Spacers: A, 1,6-diisocyanatohexane; B, 1,12-diisocyanatododecane; C, di(*N*-succinimidyl)carbonate; D, 1,3-phenylene diisocyanate; E, 1,4-phenylene diisocyanate; F, (3-isocyanatopropyl)triethoxysilane.
[b] On LiChrosorb Si 100, 5 μm, 300 m^2/g.
[c] Based on carbon (C).

2.4. Preparation of the TE-CSPs according to different synthetic strategies

All reactions were carried out in a modified Model ROTAVAPOR-M rotary-evaporator apparatus (Büchi, Labortecnik, Flawil, Switzerland), in which the reaction flask is fitted with solvent condenser, solvent collector, argon inlet and allows syringe addition of reactant solutions and isolation of the CSPs by filtration under an inert atmosphere. Stirring was obtained by spinning the flask around its axis.

Silica gels and teicoplanin were vacuum-dried before use (0.1 mbar, 1 h, at 150 and 70°C, respectively). Chemical purity of teicoplanin samples was checked by reversed-phase HPLC on a 250 × 4 mm i.d. ODS Hypersil column (mobile phase, A: ammonium acetate (0.1 M); B: acetonitrile–ammonium acetate (0.1 M) (80:20, v/v); linear gradient from 10% B to 50% B in 20 min, to 75% B in 15 min, to 100% B in 5 min. Flow rate 1.00 ml/min, $T = 25$°C, UV detection at 254 nm): the main teicoplanin peak area ($A_2 - 2$) was always greater than 85% of the total by relative area. All reactions were carried out under an argon atmosphere.

2.4.1. Method 1

To an ice-bath cooled slurry of (3-aminopropyl)silica gel (LiChrosorb Si 100, 5 μm) in dry toluene (3.0 g in 50 ml) were added 15 mmol of the proper spacer (A, B, C, D, E) with a syringe. Spacers A and B (both liquids) were used pure; in the case of spacer C, the reaction was carried out in dry acetonitrile, the spacer being previously dissolved in the same solvent (40 mg/ml). For spacers D and E, both solid, a solution in dry toluene (80 and 40 mg/ml, respectively) was previously prepared. The ice-bath was removed and the mixture was heated to 70°C for 2 h; after cooling to room temperature, the liquid phase was removed by suction filtration through an immersion sintered teflon filter under an argon atmosphere. The intermediate silica was freed from excess spacer by addition of 10 ml of dry toluene (dry acetonitrile, in case of spacer C) and removal of the liquid phase by suction filtration (two times). A suspension of teicoplanin in dry pyridine (1.0 g, 0.53 mmol in 100 ml) was added to the activated silica and the mixture was heated to 70°C for 12 h, with continuous stirring. Disappearance of the main teicoplanin peak from the reaction liquid phase was checked by reversed-phase HPLC, as described above. After cooling to room temperature, the different TE-CSPs were isolated by filtration and washed with 50 ml portions of pyridine, water, methanol, acetonitrile and dichloromethane, and dried under reduced pressure (70°C, 0.1 mbar, 2 h). Disappearance of the isocyanate groups, –N=C=O (2200–2300 cm^{-1}) and of the carbonate group, –O – COO (1820–1750 cm^{-1}), and appearance of the ureidic groups, –NH–CO–NH– (1655; 1570 cm^{-1}) were monitored by FT-IR spectroscopy. The different amount of bonded selector was determined by elemental analysis, for all the TE-CSPs prepared. The obtained results are reported in Table 1.

2.4.2. Method 2

To a suspension of teicoplanin in dry pyridine (1.0 g, 0.53 mmol in 100 ml) was added a small amount of spacer F (1 mmol) as a solution in dry pyridine (30 μl/ml), under an argon atmosphere, and the mixture was heated to 70°C for 1 h, with continuous stirring. After cooling to room temperature, a slurry of 3.0 g of unmodified silica (LiChrosorb Si 100, 5 μm) in 50 ml of dry pyridine, treated ultrasonically for 5 min., was added to the modified selector, and the mixture was heated to 70°C for 12 h, with continuous stirring.

Disappearance of the main teicoplanin peak from the reaction liquid phase was checked by reversed-phase HPLC, as described for Method 1. After cooling to room temperature, the TE-CSP in question was isolated by filtration and washed with 50 ml portions of pyridine, *N*,*N*-dimethylformamide, water, methanol, acetonitrile and dichloromethane, and dried under reduced pressure (70°C, 0.1 mbar, 2 h). The obtained amount of bonded selector was determined by elemental analysis, and the corresponding value is reported in Table 1.

2.5. Preparation of the TE-CSPs with different silica supports

The seven TE-CSPs that foresaw different silica matrices were prepared according to the first described synthetic strategy (Method 1), by the employment of spacer A (1,6-diisocyanatohexane), beginning from the corresponding (3-aminopropyl)-functionalized silica gels. The different amount of bonded selector was determined by elemental analysis, for all the differently supported TE-CSPs. The obtained results are reported in Table 2.

2.6. Columns packing and efficiency tests

The stainless-steel columns (250 × 4.5 mm i.d.) were packed with the above-mentioned TE-CSPs using a slurry packing procedure already described [5]. Columns efficiency towards achiral solutes was evaluated using n-hexane/chloroform stabilised with ~0.25% ethanol (90:10, v/v) as eluent, delivered at a flow rate of 1.00 ml/min at 25°C. The theoretical plates number (N/m) of columns ranged between 7 000 and 40 000 for acetophenone. The columns dead time (t_0) was determined from the elution time of an unretained marker (toluene, using methanol as eluent).

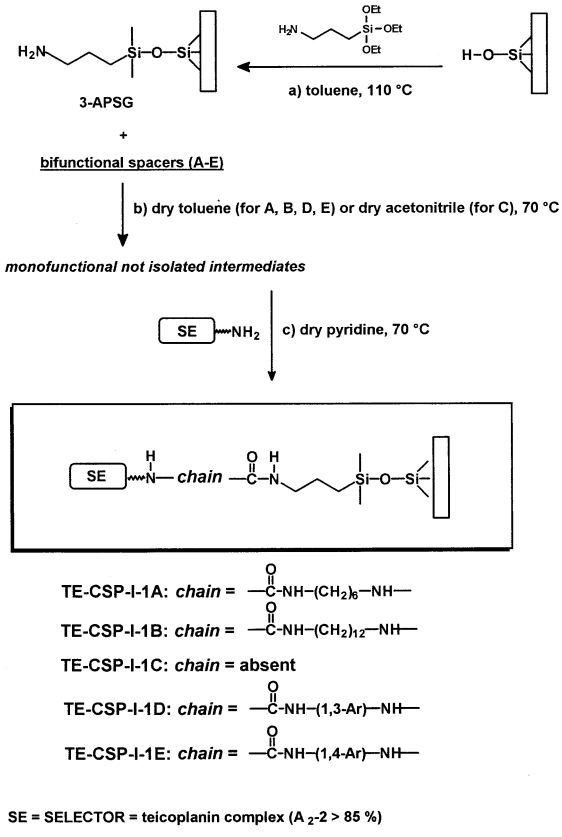
2.7. Chromatographic procedures

Racemic and enantiomerically pure samples were eluted with hydro-organic mobile phases consisting of an organic solvent (MeOH, EtOH, CH_3CN) and aqueous solutions of ammonium acetate (pH 6.5; 0.025–0.050 M) delivered at a flow rate of 1.00 ml/min at 25°C. The final pH of the mobile phase [the apparent pH (i.e. pH*) of

Table 2
Characterisation of the teicoplanin containing CSPs prepared by different silica supports

CSP	Material	Surface area (m^2/g)	C (%)	H (%)	N (%)	μmol/g[a] silica	μmol/m^2 silica
TE-CSP-I-1A	LiChrosorb Si 100, 5 μm	300	16.43	1.51	3.80	~163	0.54
TE-CSP-II-1A	LiChrospher Si 100, 5 μm	400	15.60	2.42	3.61	~144	0.36
TE-CSP-III-1A	Kromasil Si 100, 5 μm	340	12.30	2.19	3.73	~84	0.25
TE-CSP-IV-1A	Hypersil Si 120, 5 μm	170	9.79	1.47	2.24	~82	0.48
TE-CSP-V-1A	Spherisorb Si 80, 3 μm	220	9.29	1.44	2.24	~71	0.32
TE-CSP-VI-1A	Nucleosil Si 500, 5 μm	35	4.11	0.53	0.85	~33	0.94
TE-CSP-VII-1A	Prodigy Si 100, 5 μm	450	16.90	3.03	4.50	~141	0.31

[a] Based on carbon.



Scheme 1. Synthetic pathway for the preparation of the teicoplanin chiral stationary phases (TE-CSPs) obtained by the use of synthetic Method 1.

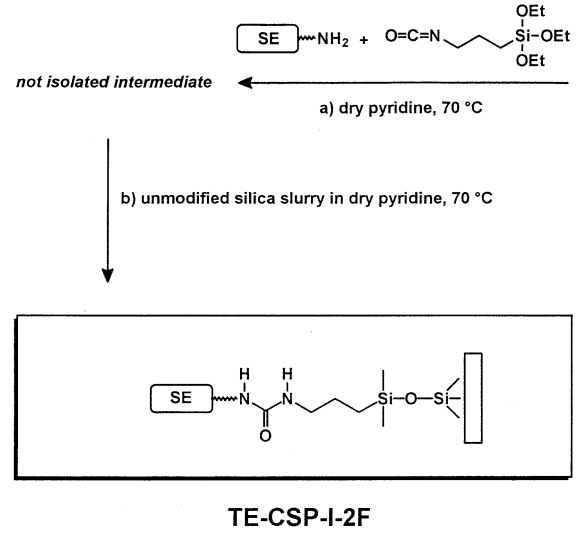
the mixed organic–aqueous eluents] was measured with a Metrohm Model 632 pH-meter (Metrohm Ltd., Heriscan, Switzerland). Samples were dissolved directly in the eluent or in water–organic mixtures and the resulting solutions were filtered through a 0.45 μm filter (injection volume: 10 μl). After the preparation, also mobile phases were filtered through 0.45 μm filter and degassed with helium. Evaporative light scattering detection was performed at 57°C, 2.0 bar (air) and gain = 7.

3. Results and discussion

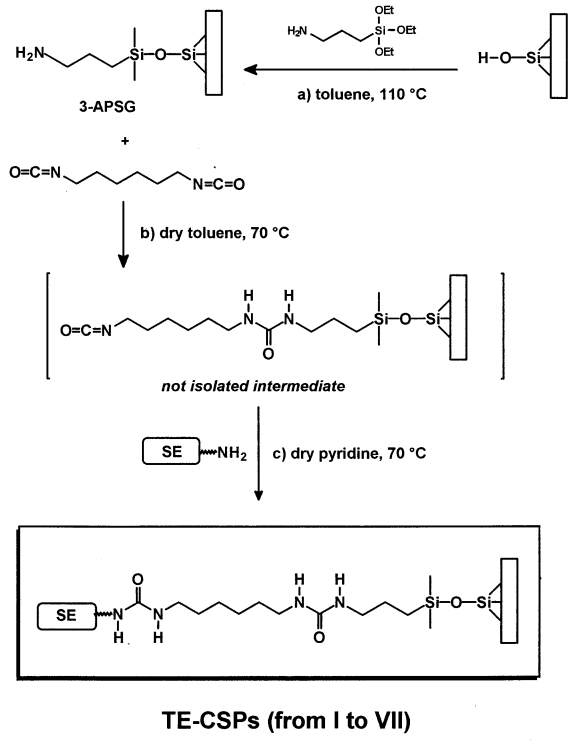
3.1. Synthesis of the chiral stationary phases

The structures of the TE-CSPs and the procedures for their preparation are reported in Schemes 1–3. The materials obtained were named according to the silica support used for the immobilisation, by using Roman numerals (from I to VII), and according to the spacer employed, by using alphabetical letters (from A to F). The Arab numerals between them indicate the different synthetic strategy applied (1 or 2). Six different bifunctional spacers (A, B, C, D, E and F), shown in Fig. 2, were used to immobilise the glycopeptide antibiotic to the same silica surface (LiChrosorb Si 100, 5 μm).

In synthetic Method 1 (Scheme 1), the macrocyclic antibiotic was covalently bonded to the silica matrix in three steps: (a) introduction of 3-aminopropyl groups on the silica surface by



Scheme 2. Synthetic pathway for the preparation of the teicoplanin chiral stationary phase (TE-CSP) obtained by the use of synthetic Method 2.



Scheme 3. Synthetic pathway for the preparation of teicoplanin chiral stationary phases (TE-CSPs) prepared by the use of different silica supports.

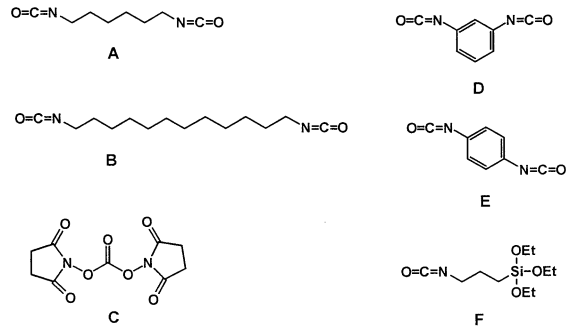


Fig. 2. Bifunctional spacers used for immobilisation of teicoplanin on silica particles.

standard silanization with (3-aminopropyl)-triethoxysilane; (b) activation of the aminopropylated silica with bifunctional spacers (A–E) to give a monofunctional intermediate. In this step, the large excess (7:1) of bifunctional spacer prevents the formation of bridged aminopropyl chains from a single spacer molecule reacting with two amino groups; (c) surface-linking of TE via addition of its free amino group (the better nucleophile in the molecule) to the pendant isocyanate or carbonate groups. Additional linkages, in which carbamate groups are formed between the glycopeptide alcoholic or phenolic hydroxyls and surface-linked isocyanate or carbonate groups, may however occur in the final material.

In synthetic Method 2 (Scheme 2), the macrocyclic antibiotic was covalently bonded to the silica matrix in two steps: (a) chemical modification of the selector via reaction between the primary amine group on the aglycone moiety of the antibiotic and the isocyanate groups of the spacer (F); (b) immobilization of the functionalized selector on 'one-pot' added unmodified silica particles. Elemental analyses of the final stationary phases gave a macrocycle loading ranging between ~ 289 and ~ 31 μmol per gram of unmodified silica.

3.2. Characterisation of the prepared materials

The teicoplanin containing chiral stationary phases prepared by the use of different synthetic strategies were firstly compared on the ground of the different amount of bonded selector, determined by elemental analysis. As it can be observed in Table 1, the highest calculated selector densities were obtained in the case of aromatic spacers (D and E), with a surface concentration of about 218 and about 289 μmol/g of silica, respectively. In particular, in the case of the 1,4-substitution of the two isocyanate groups on the benzene ring, the TE density was the highest of all the series of TE-CSPs considered. Smaller amounts of macrocycle loading were instead found for aliphatic spacers (A and B), with a surface concentration of ~ 163 and ~ 139 μmol/g of silica, respectively. It is worth noting that the longer the aliphatic chain (spacer B, twelve-carbon), the lower the amount of immobilised TE, probably due to steric difficulties with the macro-

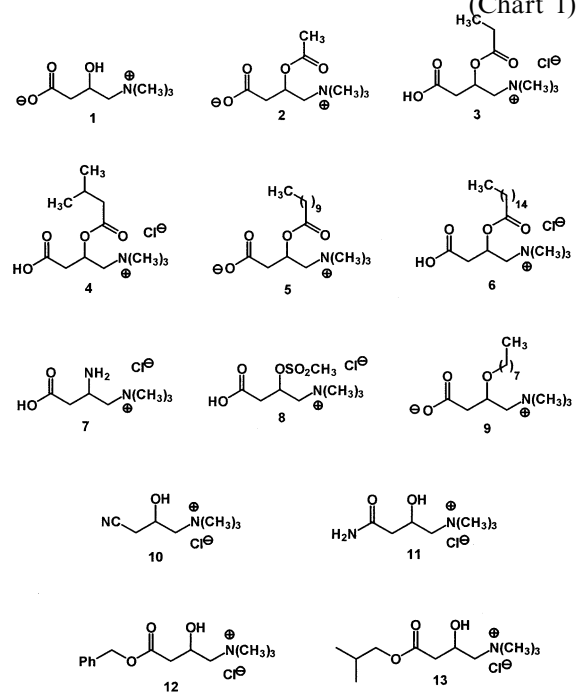
cycle reacting with the pendant isocyanate groups. The lowest values of bonded selector were finally obtained with spacers C and F, with a surface concentration of ~26 and ~31 μmol/g of silica, respectively. The first one, being an activated carbonate, represents a convenient reagent for the synthesis of ureas and carbamates [7]; the second one is the only spacer with different functionalities at the opposite terminals: on one side, it offers the same group as the rest of the spacers (isocyanate), and on the other it adds the behaviour of a trialkoxy–silane. This dual aspect made it a very helpful spacer in the preparation of CSPs containing 3,5-dimethylphenylcarbamate-functionalised β-cyclodextrins, chemically bonded to silica particles [8]. Although coming from different preparation methods and from the employment of different spacers, the phases prepared by reaction with spacers C and F contain identical linkages between the silica surface and the chiral selector (one ureidic group bonded to a three-carbon aliphatic chain) and are therefore comparable.

With regard to the macrocycle loading on the different silica matrices (see Table 2), it must be observed that the studied silica-gels present different chromatographic properties depending on their morphological and physico-chemical characteristics, such as specific surface areas (from 35 to 450 m^2/g), particle sizes (5 and 3 μm) and pore sizes (80, 100, 120 and 500 Å). The highest levels of bonded selector were reached with the use of irregular LiChrosorb Si 100, 5 μm silica-gel (~163 μmol/g of silica) and spherical LiChrospher Si 100, 5 μm and Prodigy Si 100, 5 μm silica-gels (~144 and 141 μmol/g of silica, respectively), followed by comparable values for Kromasil Si 100, 5 μm (about 84 μmol/g of silica), Hypersil Si 120, 5 μm (about 82 μmol/g of silica) and Spherisorb Si 80, 3 μm (about 71 μmol/g of silica) silica-gels. The lowest amount of immobilised TE was found in the case of macroporous Nucleosil Si 500, 5 μm silica-gel (about 33 μmol/g of silica), solely due to its low specific surface area (35 m^2/g); on the contrary, the corresponding calculated selector density per square meter of silica is the highest of all the series of TE-CSPs considered (0.94 $\mu mol/m^2$).

3.3. Chromatographic evaluation

After packing in stainless steel columns, the above-mentioned TE-CSPs were evaluated in the enantiomeric resolution of biopharmacological interesting racemates (Chart 1).

(Chart 1)



For comparison purposes, we choose a racemic sample of an O-acyl ester of carnitine (undecanoyl–carnitine, **5**) and we used, whenever possible, the same mobile phase composition (i.e. retention and enantioselectivity are not optimised). In Fig. 3 it is reported a comparison of enantioselectivity (α) between the various TE-CSPs prepared, under the same chromatographic conditions. As it can be clearly seen, TE-CSPs prepared according to synthetic Method 1 showed higher enantioselectivity values than that prepared according to Method 2 (TE-CSP-I-2F), which, together with the phase obtained by the employment of di(*N*-succinimidyl) carbonate (TE-CSP-I-1C), exhibited the lowest selectivity among all the materials synthesised. In the context of preparation Method 1, lower enantioselectivity was observed for the aromatic spacers D and E, more rigid, than for the aliphatic ones (A and B),

notwithstanding the particularly high macrocycle loading reached with them; no significant differences in enantioselectivity, depending on the type of substitution position of the two isocyanate groups on the benzene ring, could be observed between the two materials TE-CSP-I-1D (1,3) and TE-CSP-I-1E (1,4). A greater influence over the enantioselectivity of the materials was instead exerted by the different length of the spacer aliphatic chain: higher value was observed for the shorter one (spacer A, six-carbon) than for the longer one (spacer B, twelve-carbon). With regard to the influence of the silica matrix nature on the chiral performances of materials, it could be noted that the best results were obtained with LiChrosorb (irregular) and LiChrospher (spherical) silica-gels; in particular, the spherical support gave rise to better symmetrical peak shapes, thus revealing itself the best chromatographic siliceous support in terms of efficiency and selectivity.

The picture that emerges from these data can be summarised as follows: (1) a six-carbon atom aliphatic chain between the silica surface and the chiral selector TE strongly improves enantioselectivity; (2) shorter (three-carbon) or longer (twelve-carbon) aliphatic chains cause a drop in enantioselectivity; (3) aromatic spacers do not allow the required interactions between analytes and the proper sites of the teicoplanin binding pocket; and (4) the LiChrospher silica-gel is the best chromatographic support in terms of selectivity and efficiency.

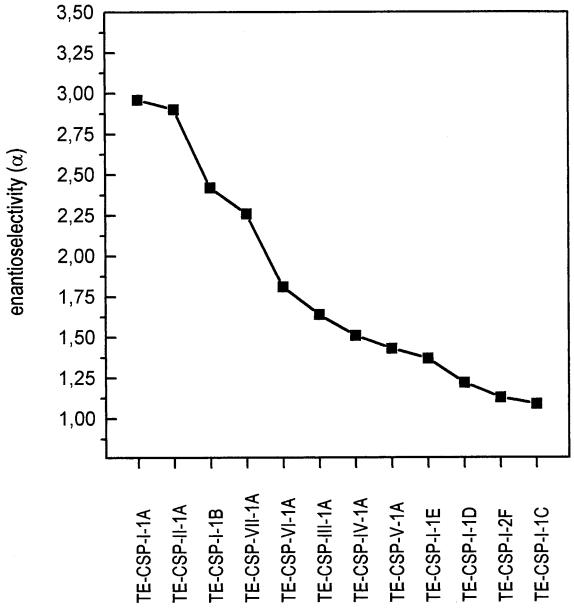


Fig. 3. Comparison of enantioselectivity (α) on the different TE-CSPs prepared. Sample: racemic undecanoyl-carnitine. Chromatographic conditions: columns geometry = 250 × 4.5 mm i.d.; eluent = ethanol–water (90:10, v/v) + ammonium acetate (0.050 M) (pH* 7.96); flow rate = 1 ml/min; temperature = 25°C; detection: ELSD; T = 57°C; P = 2.0 bar.

With regard to the molecular recognition mechanism towards carboxylate-containing compounds, it is consistent with the recently reported binding mode of the acetate anion [9] and of simple dipeptides to vancomycin and ristocetin A, two antibiotics structurally related to teicoplanin [10].

On the ground of the above chromatographic results, we recently performed, for the first time, the direct chromatographic resolution of carnitine and *O*-acyl carnitines enantiomers (Chart 1) on the TE-CSP-I-1A, without any pre- or post-column derivatization [5]. This novel and efficient chiral stationary phase showed high chemical inertness and effective passivation of the underlying silica by the two ureidic functions. In addition, shielding of polar or ionisable sites on the silica matrix lowered unselective retention and resulted in high levels of enantioselectivity, accompanied by symmetrical peak shapes for permanently charged carnitine and derivatives.

Some innovative chromatographic results obtained for linear and cyclic β-amino acids on the TE-CSP-I-1A are collected in a recently submitted paper [11], and compared to those performed on a totally new CSP containing another natural glycopeptide antibiotic, structurally related to teicoplanin, referred to its industrial designation as A-40,926.

3.4. Detection strategies

The analysis of carnitines (and of mostly amino acids) have always represented a problem in the choice of appropriate detection systems: chromophore groups being lacking, they show very weak UV absorption in the same low wavelength UV absorption region (210–220 nm) of com-

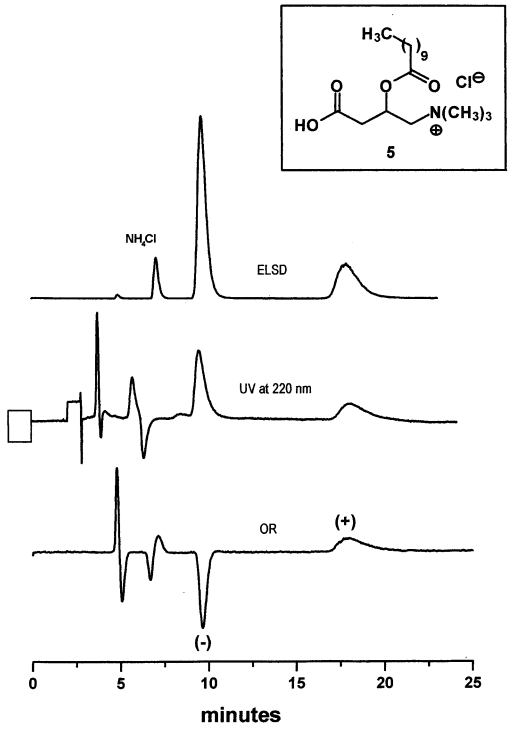


Fig. 4. Application of different detection systems in the enantioselective chromatography of racemic undecanoyl-carnitine: evaporative light scattering detection (top trace); ultra-violet at 220 nm (middle trace) and optical rotation (bottom trace). Experimental conditions: column = TE-CSP-I-1A (250 × 4.5 mm i.d.); eluent = ethanol–water (90:10, v/v) + ammonium acetate (0.050 M) (pH* 7.96); flow rate = 1 ml/min; pressure = 2000 p.s.i.; temperature = 25°C; detection: ELSD; $T = 57$°C; $P = 2.0$ bar; $\alpha = 2.96$.

monly used mobile phase additives, with consequent loss of detection sensitivity. On the other hand, the use of refracting index detection, aside from sensitivity issues, prevents the application of gradient elution for the analysis of complex mixtures containing species with large retention differences.

Compounds analysed on TE-CSPs were non-volatile solids and gave optimal ELSD response under a variety of experimental conditions (eluents with and without buffers, flow rates from 0.5 to 1.5 ml/min, different kind of organic modifiers and in variable proportions), with S/N ratios always much larger than UV detection. Since ELSD response is only marginally affected by changes in the eluent composition, we extensively used it during method development as well as in the analysis of multi-component samples with gradient elution. In addition to ELSD, polarimetric detection could be used to circumvent the low UV detectability of carnitine samples. In spite of the low optical rotation values of carnitine and its simple derivatives at the sodium D line ($[\alpha]^{20} = -31.5$ for R-(−)-carnitine HCl; $c = 1.00$, water) [12,13], multi-wavelength polarimetric detection (230–900 nm range) resulted in good S/N values (Fig. 4). While sensitivity of polarimetric detector was not sufficient to perform enantiomeric trace analysis, the stereochemical information contained in the bisignate polarimetric response was useful in establishing elution order for those compounds not available as single enantiomers of known configuration.

4. Conclusions

Two different synthetic strategies were developed for the grafting of the TE selector to the silica matrix, both leading to the formation of one or two stable ureidic functions on the CSP structure. The first one foresaw the use of bifunctional spacers, either aliphatic or aromatic, which were first bonded to aminopropyl-functionalized silica particles and then TE was immobilised by 'one-pot' reaction between the spacers and the primary amine group on the aglycone moiety of the antibiotic. In the second strategy, a small amount of spacer was first reacted with the primary amine group of TE, followed by immobilisation of the modified selector on 'one-pot' added unmodified silica particles. On the ground of the obtained chromatographic results towards a variety of racemic compounds, we were able to establish that the optimal synthetic strategy was that leading to the formation of two ureidic functions on the CSP structure, spaced out by a six-carbon aliphatic chain. The best chromatographic results were obtained with the LiChrospher silica matrix. Finally, evaporative light scattering (ELS) and optical rotation (OR) detections were used because of the low UV absorbing samples.

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References

[1] D.W. Armstrong, Y. Liu, K.H. Ekborgott, Chirality 7 (1995) 474 (and references cited within).
[2] D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J.R. Chen, Anal. Chem. 66 (1994) 1473.
[3] M.R. Bardone, M. Paternoster, C. Coronelli, J. Antibiotics 31 (1978) 170.
[4] A. Webster, A.P.R. Wilson, T. Treasure, R.N. Grüneberg, Int. J. Clin. Pharmacol. Res. 8 (1988) 95.
[5] A. Carotti, S. Cellamare, I. D'Acquarica, F. Gasparrini, D. Misiti, S. Muck, C. Villani, J. Chromatogr. A 857 (1999) 145.
[6] A. Carotti, S. Cellamare, I. D'Acquarica, F. Gasparrini, D. Misiti, S. Muck, C. Villani, presented at the 9th International Symposium on Chiral Discrimination, Nagoya, Japan, October 27–30, 1997.
[7] K. Takeda, H. Ogura, Synth. Commun. 12 (1982) 213.
[8] T. Hargitai, Y. Kaida, Y. Okamoto, J. Chromatogr. 628 (1993) 11.
[9] P.J. Loll, A.E. Bevivino, B.D. Korty, P.H. Axelsen, J. Am. Chem. Soc. 119 (1997) 1516.
[10] D.H. Williams, Natural Products Reports (1996) 469.
[11] A. Carotti, S. Cellamare, C. Cimarelli, I. D'Acquarica, F. Gasparrini, D. Misiti, G. Palmieri, C. Villani, G. Zappia, Tetrahedron: Asymmetry, submitted for publication.
[12] P. De Witt, R. Deias, S. Muck, B. Galletti, D. Meloni, P. Celletti, A. Marzo, J. Chromatogr. B 657 (1994) 67.
[13] W. Engewald, H. Engelhardt, W. Gotzinger, P. Klosser, H.P. Kleber, Pharmazie 45 (1990) 629.