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Review

High-performance liquid chromatography chiral stationary phases based on low-molecular-mass selectors

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Abstract

A review of HPLC chiral stationary phases (CSPs) based on low molecular mass selectors is given. The review is focused on brush- and monomeric-type CSPs obtained by covalent linkage of chiral selectors, with emphasis on those obtained by total synthesis. Emphasis is given to new, emerging aspects like enantioseparations on receptor-like chiral stationary phases and dynamic enantioselective chromatography of stereolabile compounds.

Keywords: Reviews; Chiral stationary phases, LC; Enantiomer separation; Brush-type chiral stationary phases; Receptor-like chiral stationary phases; Dynamic enantioselective chromatography; Chiral selectors

Contents

1. Introduction

The increased demand for enantiopure compounds has led to the development of a variety of stereoselective separation technologies. Among them, direct liquid chromatographic procedures are the focus of intensive research, leading to the rational design and production of highly selective and efficient chromatographic materials for high-performance liquid chromatography (HPLC).

Central to any enantioselective HPLC (e-HPLC) method based on chiral stationary phases (CSPs) is the choice of the appropriate chiral selector. Low molecular mass selectors linked to a solid support form a class of stationary phases, the so-called brush-type phases, in which the individual chiral molecules are more or less evenly distributed onto

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the surface of the — ideally — inert matrix and are easily accessible to analyte molecules. It is assumed that silica based, brush type phases are characterized by a monomolecular organic layer on the silica surface, resulting from the attachment of a single chiral molecule to the silanol groups. Oligomeric or polymeric phases may in principle originate from low molecular mass selectors that carry a trifunctional (usually a trialkoxy or trichloro) silane terminated arm through which they are anchored to the silica surface. An intermediate situation may arise when the final stationary phase is constructed via a multistep procedure, whereby the silica surface is first activated with a trifunctional silane and then allowed to react with the chiral selector or a precursor of the selector itself [1]. In this case, the chiral selector will be anchored to a polymeric layer of achiral molecules formed on the silica surface. Non-monomeric, silica-based chiral phases are likely to be formed when the surface silanization is carried out with trifunctional silanes in the presence of water. The situation here is similar to that encountered in the preparation of achiral stationary phases [2,3]. The actual structure of the final stationary phase and the distribution density of the chiral selector on the surface are thus somewhat difficult to define. Nonetheless, irrespective of the binding outcome, brush-type phases are characterized by independent selectors, as opposed to polymeric phases in which the selector superstructure may play a major role in the recognition events. Within polymeric CSPs, the role of the superstructure in the recognition process has been investigated for triacetylcellulose [4] and for poly(triphenylmethyl methacrylate), a polymer that is chiral by virtue of its helical superstructure [5]. CSPs based on low molecular mass selectors share some favourable characteristics that are partly responsible for their widespread use in modern enantioselective analysis. These include: good kinetic performance, broad applicability, chemical and thermal inertness, compatibility with any mobile phase. In favourable cases, their low structural complexity and the availability of good models for their recognition properties facilitate the refinement of existing structures and the design of improved selectors.

Several books and reviews describe in detail the vast repertoire of modern brush-type HPLC chiral stationary phases [6–10]. Here we present a selection of recent developments in the field, with emphasis on two distinct subjects: (1) new chiral stationary phases, including those featuring immobilized synthetic receptors, (2) application of brush-type phases to the study of enantiomer interconversion phenomena.

2. New chiral stationary phases

Several new selectors for HPLC applications have been described in recent years. Some of them are closely related to the original phases developed by Pirkle and co-workers [11], based on aminoacid derivatives carrying π-acidic or π-basic sites. A π-basic CSP was prepared by immobilization of *N*-butanoyl-(*R*)-*p*-hydroxyphenylglycine propylamide via its phenolic oxygen on silica gel [12]. The resulting CSP 1 (Fig. 1) shows high enantiorecognition ability for *N*-(3,5-dinitrobenzoyl)-aminoacid amides, with enantioselectivity values (α) up to 26.59 for the enantiomers of derivatized leucine, using *n*-hexane/isopropyl alcohol as eluent. CSP 1 more strongly retains the *S* enantiomers, with a preference for derivatives with secondary amides over tertiary amides or esters at the carboxyl terminus and a preference for branched, unfunctionalized side chains on the stereogenic carbon. Related CSPs with trifluroacetylated amino terminus or tertiary amide at the carboxyl terminus showed decreased enantioselectivity. A chromatographically inferred mechanism was presented in which the selector π-basic phenyl ring stacks parallel to the 3,5-dinitrobenzoyl ring of the analyte, while two intermolecular H-bonding interactions are established between the amides functionalities. An improved version of the Pirkle *N*-(3,5-dinitrobenzoyl)leucine stationary phase was prepared by replacing the amide hydrogen at the carboxyl end with a phenyl ring (CSP 2, Fig. 1) [13]. As this amide hydrogen was considered to be a potential superfluous interaction site with enantiomeric analytes, its replacement with the bulky aromatic ring was expected to result in enhanced enantioselectivity for racemic test compounds. This was indeed observed for a range of chiral analytes derived from aminoacids and carrying either π-acidic or π-basic aromatic rings. Recently, CSP 2 was used successfully in the enantiomer

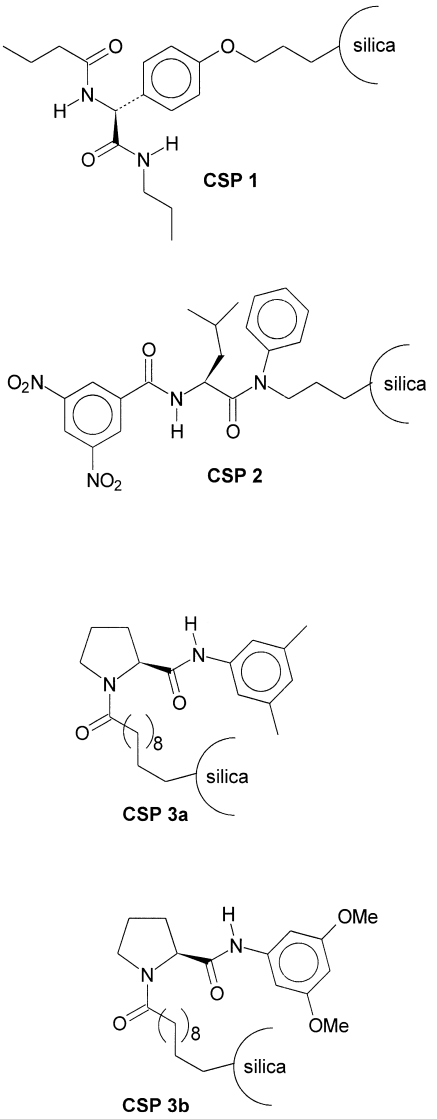


Fig. 1. Chiral stationary phases with selectors derived from aminoacids.

separation of fully protected 2-hydroxy carboxylic acids having π-basic anilide moieties. Enantioselectivity values reaching 6.76 with the *S*-enantiomer of mandelic acid being eluted first were recorded under standard normal-phase conditions [14]. The structure of a chiral selector based on a proline derivative [15–17] was fine-tuned and optimized for the preparation of a chiral stationary phase for preparative applications [18]. The immobilized-target protocol was followed in this work. Basically, a number of diverse chiral compounds are analyzed on a CSP containing as chiral selector the immobilized target molecule for the enantiomers of which a new selector is desired [19]. The compound showing the highest enantioselectivity on this system is chosen and a chiral stationary phase is then prepared from one of its enantiomers: assuming the reciprocity of enantioselective recognition is not disturbed by the binding chemistry, the new CSP should display the same degree of enantioselectivity of the immobilized-target CSP. Thus, starting from CSP 3a and searching for a related selector with the same enantioselectivity and improved affinity for the precursor of two target analytes, CSP 3b was identified, prepared and shown to have higher loading ability than its precursor (Fig. 1). In this particular case, the three to fourfold increase in loading was due to the greater retention afforded by CSP 3b, which enabled the use of more polar mobile phases with enhanced solvation power for the analytes of interest.

Several new CSPs have been described in which a chiral selector is assembled around a 1,3,5-triazine skeleton (Fig. 2). Starting from 2,4,6-trichloro-1,3,5-triazine (*s*-triclorotriazine) and taking advantage of the different reactivities of the three chlorine atoms as leaving groups, one can introduce different chiral fragments on the triazine ring and thus obtain a polyfunctional selector with a potentially widened application range.

CSPs 4a–h were prepared by sequential displacement of two chlorine atoms of the *s*-trichlorotriazine with a free aminoacid and with pyrrolidine or naphthyl amines and finally by coupling the chiral substituted triazines to aminopropyl silica [20,21]. These chiral sorbents showed modest selectivities for the enantiomers of *N*-3,5-dinitrobenzoylated aminoacid methyl esters and aminoalcohols. A sizeable effect of the relative configurations of the two chiral moieties in CSPs 4d–e (R_1=benzyl, R_2=methyl) was noted, with the heterochiral selector performing better than the homochiral one. A different synthetic procedure was followed to prepare CSP 5 [22]: here, a polyfunctional selector was constructed around *s*-trichlorotriazine from a C-protected valyl tripeptide and (*S*)-1-(1-naphthyl)ethylamine. The remaining chlorine atom on the *s*-triazine ring was displaced by the amino group of aminopropyl silica in the last

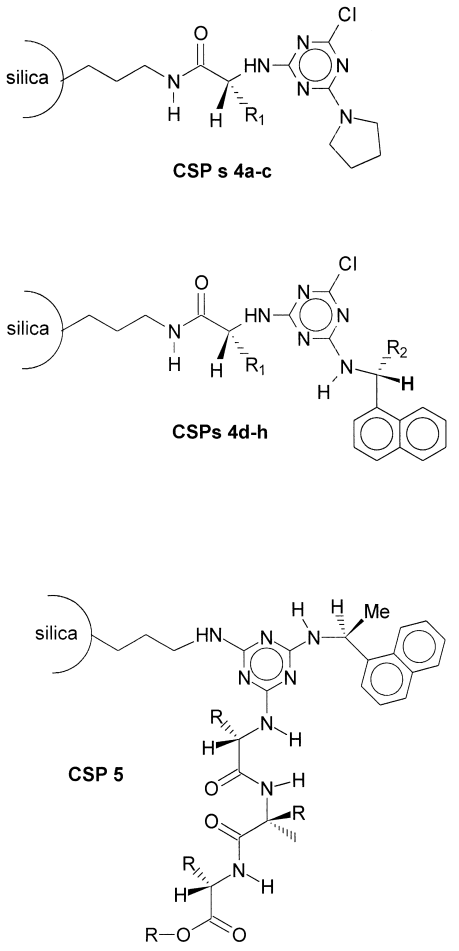


Fig. 2. Chiral stationary phases with selectors assembled around 1,3,5-triazine.

step, thereby assuring the covalent bonding of the chiral selector to the silica matrix. CSP 5 resolves the enantiomers of *N*-3,5-dinitrobenzoylated amino-acid alkyl esters and of 2,2′-dihydroxy-1,1′-binaphthyl derivatives.

(*R*)-1-(1-Naphthyl)ethylamine based selectors were prepared in similar ways starting from 2,4,5,6-tetrachloro-1,3-dicyanobenzene: sequential substitution of chlorine atoms on the aromatic ring by nitrogen nucleophiles (the chiral amine or some of its derivatives, sarcosine as a spacer, 3-aminopropyltriethoxysilane) afforded various selectors that, after immobilization on silica gel yielded a range of CSPs with significant enantioselectivities for π-acidic compounds [23].

Starting from (*R,R*)-diacetyltartaric acid anhydride, a diamide derivative carrying a *p*-chlorophenyl and a 10-undecenyl groups at the two carboxyl ends was prepared, silylated with dimethylchlorosilane and immobilized onto silica gel to afford, after end-capping and aminolysis of the acetyl groups, CSP 6 (Fig. 3) [24]. This chiral sorbent, like its isopropylamide analog [25], discriminates between the enantiomers of 1,2-diols and of 2,2′-dihydroxy-1,1′-binaphthyl. Modest enantioselectivities were observed for 1,2-aminoalcohols.

Cholic acid and deoxycholic acid derivatives were used as selectors for the preparation of new chiral stationary phases (Fig. 3). Cholic acid and 3-phenylcarbamoyl cholic acid allyl esters were grafted to hydride activated silica gel to afford CSPs 7a and b, respectively [26]. The latter showed enantioselectivity values up to 1.83 in the resolution of derivatized aminoacids and amines, alcohols, hydantoins

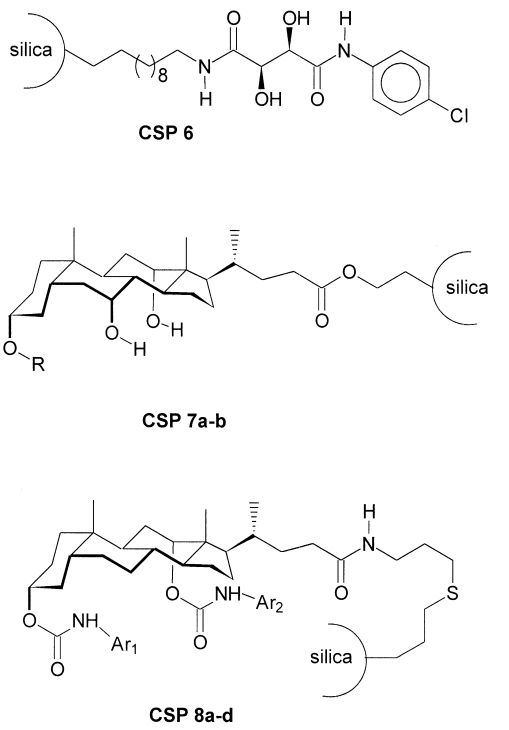


Fig. 3. Chiral stationary phases with selectors derived from tartaric, cholic and deoxycholic acids.

and 2,2′-dihydroxy-1,1′-binaphthyl. Deoxycholic acid derivatives were immobilized onto silica gel by conversion of the acid to the allylamide followed by (a) the introduction of two identical or two different arylcarbamate groups at the 3 and 12 positions, (b) radical addition of 3-mercaptopropyltriethoxysilane to the allyl double bond and (c) final reaction of the chiral silanes with silica gel to give CSPs 8a–d [27]. These new phases are quite effective in the resolution of amines, acids, aminoacid derivatives and 3-hydroxy-benzodiazepin-2-ones.

A chiral stationary phase containing an ergot alkaloid (CSP 9, Fig. 4) was prepared and shown to be effective in the resolution of acidic compounds in buffered aqueous media. Good levels of enantioselectivity were observed for 2-aryloxypropionic acids, chrysanthemic acid and analogs, and profens [28–30].

Cinchona alkaloid quinine and quinidine have been extensively used as starting materials for the preparation of chiral anion-exchange stationary phases [31–33]. A recently described stationary phase having *tert*-butylcarbamoylated quinine linked to mercaptopropylsilica (CSP 10a, Fig. 4) was able to separate the enantiomers of *N*-3,5-dinitrobenzoylated leucine and phenylalanine with α-values of 15.87 and 10.78, respectively, in buffered aqueous media [34]. The large selectivity observed for leucine dropped to 1.07 for *N*-3,5-dinitrobenzoyl-*N*-methylleucine. Replacement of the *N*-3,5-dinitrobenzoyl group with the 2,4-dinitrophenyl ring resulted in a drop of α to 1.3 for both leucine and phenylalanine and in a change of the elution order. The chromatographic behaviour of the same analytes on the native quinine based stationary phase and on a *N*-methyl-*tert*-butylcarbamoylated quinine revealed a strong, beneficial effect on enantioselectivity of both the carbonyl oxygen and the amide *N*-H at the C9 position. A chiral recognition mechanism based on chromatographic data and corroborated by spectroscopic investigations (FT-IR and X-Ray) was proposed in which one enantiomer of the analyte molecule undergoes simultaneous ionic, π–π and hydrogen bonding interactions with the immobilized selector. Dimeric versions [35] of CSP 10a, in which two quinine units are connected by a difunctional spacer, have also been prepared (CSPs 11a–k, Fig. 4).

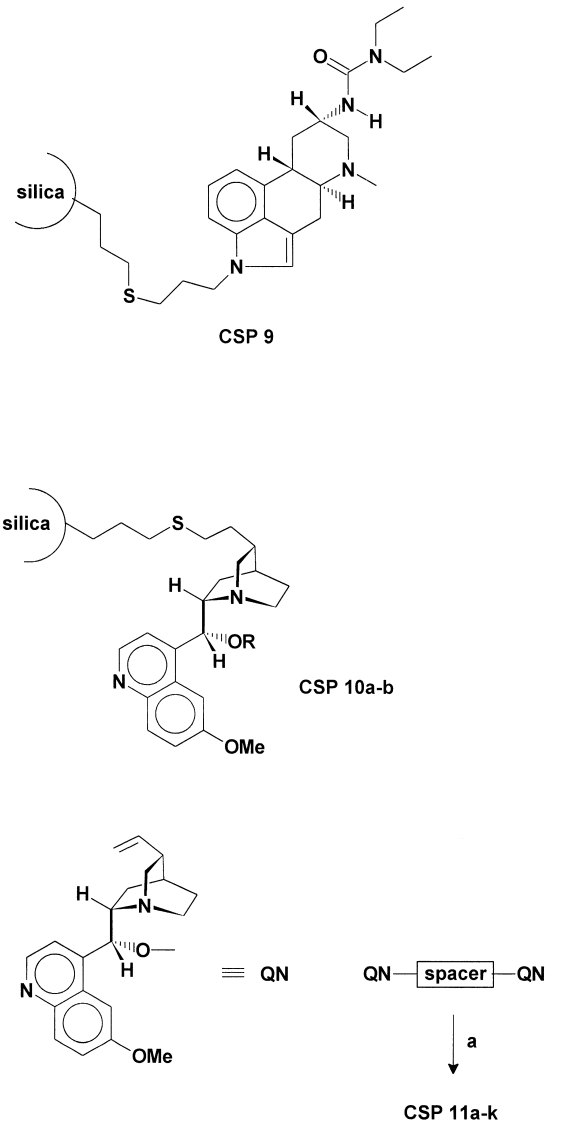


Fig. 4. Chiral stationary phases with alkaloid selectors.(a) 3-mercaptopropyl silica gel, ΔT, AIBN.

Molecules that are not based on the chiral pool are also attractive for the preparation of CSPs. Indeed, totally synthetic chiral molecules have the added advantages of an equally easy access to both enantiomers and a larger potential for structural modification and optimization.

New selectors assembled around bis-3,5-dinitrobenzoylated C_2 symmetric diamines were de-

scribed and evaluated in the enantioresolution of a broad set of compounds under normal-phase conditions. Following a multistep procedure used to prepare synthetic CSPs derived from the enantiomers of *trans*-1,2-diaminocyclohexane (CSP 12a, Fig. 5) [36–39], two new CSPs were prepared starting from the enantiomers of 1,2-diphenylethane-1,2-diamine and of 11,12-diamino-9,10-dihydro-9,10-ethanoanthracene (CSP 12b, Fig. 5) [40]. The latter CSP was successfully used in the resolution of five-membered cyclic oxazolidinones and lactones. Selectors having the 1,2-diphenyl-1,2-ethanediamine skeleton have been optimized in the structure, stereochemistry and binding mode [41–43]. Syn-type selectors connected to the silica surface through a short carbon chain (CSP 13, Fig. 5) were shown to be the most effective in the enantioresolution of aromatic secondary alcohols and some carboxylic acids. Removal of the amide function connecting the chiral selector to the silica matrix gave anti- and syn-type CSPs 14a–b (Fig. 5) [44]. When compared to their precursor CSPs, the new phases showed reduced selectivity for alcohols and carboxylic acid enantiomers while improved selectivities were observed towards the enantiomers of amides, ureas, carbamates and esters. Selectors derived from C_2 symmetric (*S*)-2,2′-dihydroxy-1,1′-binaphthyl derivatives and carrying a carboxyl terminated alkyl chain at the 6 position of one binaphthyl unit were prepared and grafted to aminopropyl silica gel by standard peptide coupling procedures (CSPs 15a–d, Fig. 6). The enantiomers

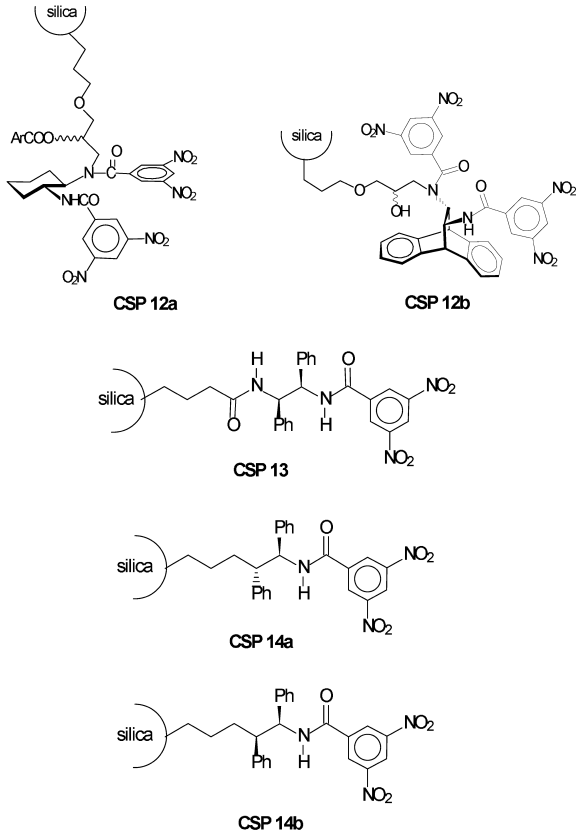


Fig. 5. Chiral stationary phases with totally synthetic selectors.

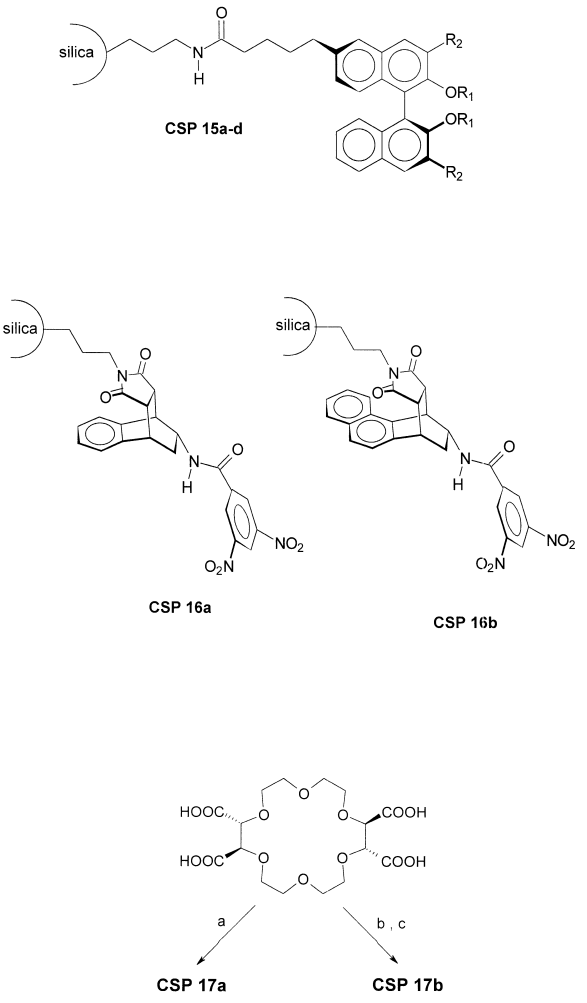


Fig. 6. Chiral stationary phases with totally synthetic selectors. Bottom: (a) EEDQ, aminopropyl silica gel; (b) acetyl chloride; (c) aminopropyl silica gel, Et_3N.

of primary, secondary and tertiary amines were resolved with α-values in the 1.02–1.18 range, using hexane based eluents containing trifluoroacetic acid [45].

An improved version of a previously reported selector (CSP 16a) with selectivity for the enantiomers of profens [46] was prepared by introducing in a rigid bicyclooctane skeleton two nearly orthogonal aromatic rings with π-basic (naphthyl) and π-acidic (3,5-dinitrobenzoyl) properties (CSPs 16b). The new selector [47] (Fig. 6) was designed to contain a cleft delimited by the two aromatic systems. This cleft is able to accommodate one enantiomer of the analyte while undergoing simultaneous face-to-face and face-to-edge aromatic–aromatic interactions and H-bonding interaction. The extended, flat aromatic surface present in CSP 16b compared to CSP 16a had a beneficial effect on enantioselectivity towards profen analytes. Using a mobile phase consisting of *n*-hexane/isopropyl alcohol containing ammonium acetate, CSP 16b resolved the enantiomers of underivatized 2-arylpropionic acids with α-values between 1.34 and 3.81. A two fold increase in enantioselectivity was observed for the enantiomers of Naproxen when going from CSP 16a to 16b.

A chiral C_2 symmetric crown ether tetracarboxylic acid was grafted to aminopropyl silica gel, either using a direct coupling with 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as condensing agent or using a two-step procedure with the dianhydride as intermediate, to give CSPs 17a [48] and 17b [49,50], respectively (Fig. 6, bottom). These CSPs were able to resolve the enantiomers of compounds bearing a primary amino group in an acidic–aqueous mobile phase system.

As an alternative way to discover new chiral molecular structures with improved recognition abilities, combinatorial procedures have been recently explored for the design of HPLC selectors. Using the immobilized-target approach, new selectors for the enantiomers of *N*-3,5-dinitrobenzoyl leucine were found among a library of 140 different 4-aryl-1,4-dihydropyrimidines, with the members of the library individually available through a three-component cyclocondensation reaction. A single enantiomer of one of the best resolved dihydropyrimidines was obtained by enantioselective chromatography on a CSP, converted to a reactive bromoderivative and coupled to a polymeric organic solid support carrying pendant *N*-methyl-aminoethyl groups (CSP 18, Fig. 7). The new phase showed good enantioselectivity not only for the original immobilized target, but also for other carboxylic acid derivatives and

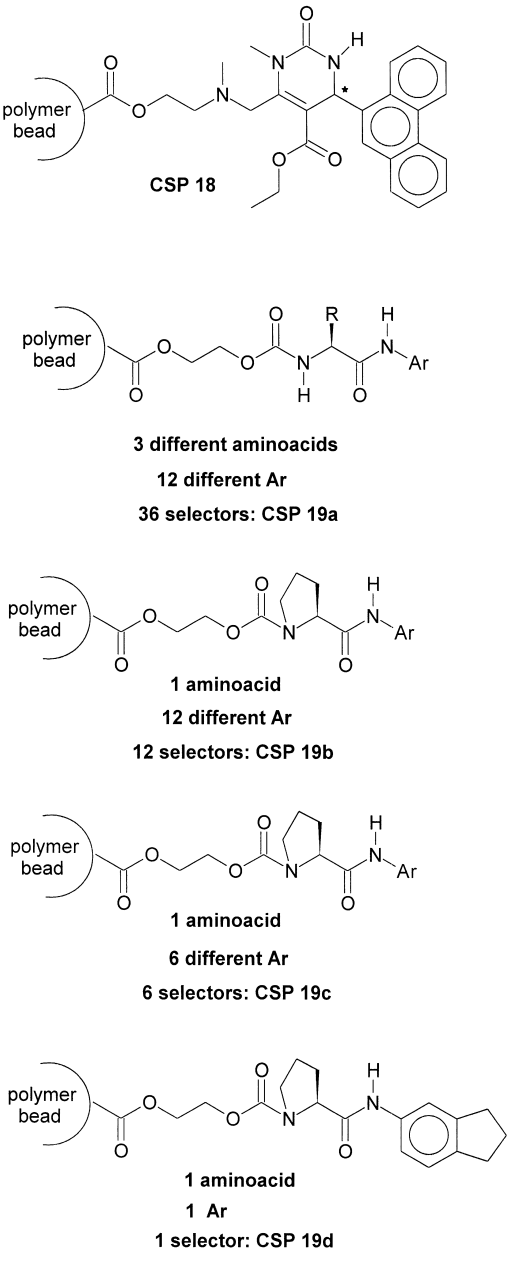


Fig. 7. Chiral stationary phases with selectors obtained from combinatorial processes.

dihydropyrimidines [51]. Another approach was followed by the same authors to prepare a chiral selector for *N*-3,5-dinitrobenzoylated aminoacid derivatives [52]. A library of 36 different L-aminoacid anilides, prepared in solution from three aminoacids and twelve aromatic primary amines, was attached to activated polymer beads through an ester linkage (CSP 19a, Fig. 7). This chiral polymer, containing a small library of potentially useful selectors, was packed into a column and used as an HPLC CSP: if some enantioselectivity is observed for the target analyte, the selector library is deconvoluted by preparing a subset of chiral packings containing only a smaller number of library members and screening their enantioselection ability. A set of proline-based selectors with high enantioselectivity was identified by this procedure (CSPs 19b–c) and among them one was found that exhibited a high level of recognition ability (CSP 19d, $\alpha=23.1$ for *N*-3,5-dinitrobenzoyl leucine diallylamide). This method has the advantage of rapidly finding a good selector out of a large number of potential candidates. The major limitation resides in the possibility of diluting the enantioselectivity of a single lead selector that coexists in low concentration with other non selective species (or with species having opposite selectivity) at the early stages of the screening procedure.

A screening procedure for parallel selector libraries based on their solid-phase synthesis and binding evaluation by circular dichroism measurements was presented. The method was applied to the production of a selector for a 1-naphthyl leucine ester derivative [53,54].

Another combinatorial approach to the discovery of new CSP selectors is based on the microscale solid-phase (silica) parallel synthesis of 3,5-dinitrobenzoyl *N*-terminated dipeptides and their evaluation by a simple solid-phase extraction procedure [55,56]. Standard solid-phase peptide chemistry was used to assemble several dipeptides on aminopropyl silica gel starting from the BOC or FMOC protected aminoacids glutamine, asparagine, serine, hystidine, arginine, aspartic and glutamic acid at the AA1 position (Fig. 8) and either the *R* or *S* BOC or FMOC protected aminoacids leucine, isoleucine, *tert*-leucine, valine, phenylanine, tryptophan and tyrosine at AA2 position. Using *N*-(2-naphthyl)alanine diethylamide as racemic test analyte, four homochiral dipeptides having phenylalanine, leucine or isoleucine at the AA2 and glutamine or arginine at the AA1 positions were discovered. The dipeptide containing the (*S*)-Glu-(*S*)-Leu fragment was chosen, the corresponding CSP 20 was prepared in gram amounts and packed into a standard analytical column. The latter was evaluated under analytical ($\alpha=20$ for the test analyte) and overloaded conditions and was shown to be capable of resolving 100 mg of the test compound in a single run.

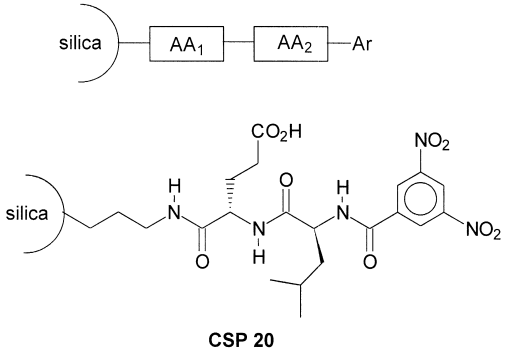


Fig. 8. A chiral stationary phase with a dipeptide selector obtained from a combinatorial process.

Novel synthetic CSPs that incorporate highly preorganized, receptor-like chiral selectors have been described recently and presented as powerful tools in the study of enantiorecognition processes characterized by high degrees of selectivity ($\alpha>20$). The investigation of molecular recognition phenomena occurring in living systems is greatly facilitated by synthetic model structures that mimic the mode of action of their natural counterparts. One convenient way to study in detail the specific interactions between artificial receptors and their binding partners is offered by HPLC systems in which surface-linked species are screened for the ability to differently retain the components of a pool of potential ligands. This approach has been recently applied in both achiral [57,58] and chiral systems and proved to give results that closely match those observed in free solution. Highly enantioselective synthetic receptors share some structural properties that seem closely related to their discrimination ability: conformational homogeneity, cage-like structure, functional groups with high degree of directionality (e.g. H-bond donor–acceptors) [59].

A C_3 symmetric, cup-shaped receptor derived from *O*-allylated tyrosine and 1,3,5-trimercaptoben-

zene was grafted to 3-mercaptopropyl silica gel to afford CSP 21 [60]. This new receptor-like stationary phase (Fig. 9) revealed very high levels of enantioselectivity for small peptidic compounds, with differences in the free energy of binding for the enantiomers up to 2.5 Kcal/mol at room temperature in organic solvents. CSP 21 was found to bind the enantiomers of *N*-BOC protected aminoacids methylamides with enantioselectivities between 3 and 43, with a marked dependence of selectivity on the aminoacid side chain shape and functionality. Comparison of free solution [61,62] (binding evaluated by NMR titrations in $CDCl_3$) and HPLC enantioselectivity data (binding evaluated in CH_2Cl_2 with 1–5% MeOH) showed that the immobilized receptor retains the same sense and extent of enantioselectivity after immobilization (Fig. 10). The stereochemical preference of the immobilized receptor was found to switch from the *S* configurated *N*-BOC-aminoacids methylamides to the *R* configurated *N*-3,5-dinitrobenzoyl aminoacid hexylamides, a finding that was interpreted in terms of two different recognition mechanisms, one based on the inclusion of the small *N*-methylamide terminus inside the basket cavity and the other based on π-stacking of the π-acidic dinitrobenzoyl rings outside of the basket surface. Large diastereoselectivities were also observed for the protected tripeptide *N*-cyclopropanoyl-Ala-Pro-Ala *n*-dodecylamide (see Fig. 9, bottom): the stereoisomer with D,L,L configuration was loosely bound by the immobilized receptor while that with inverted configuration at the *N*-terminal alanyl residue (L,L,L) was effectively retained ($\alpha \sim 21$ with 1% MeOH in CH_2Cl_2). The high affinity of *N*-cyclopropanoyl peptidic substrates with L configuration at the *N*-terminus was found to be quite general, and perhaps is due to the ability of the guests to insert the cyclopropyl ring inside the basket cavity and establish H-bond interactions at the basket opening while maintaining a low energy conformation. When CSP 21 was operated with aqueous mobile phases, the large enantioselectivities observed in organic solvents for *N*-BOC protected aminoacids methylamides were found to decrease, mainly as a result of increased retention of the less retained

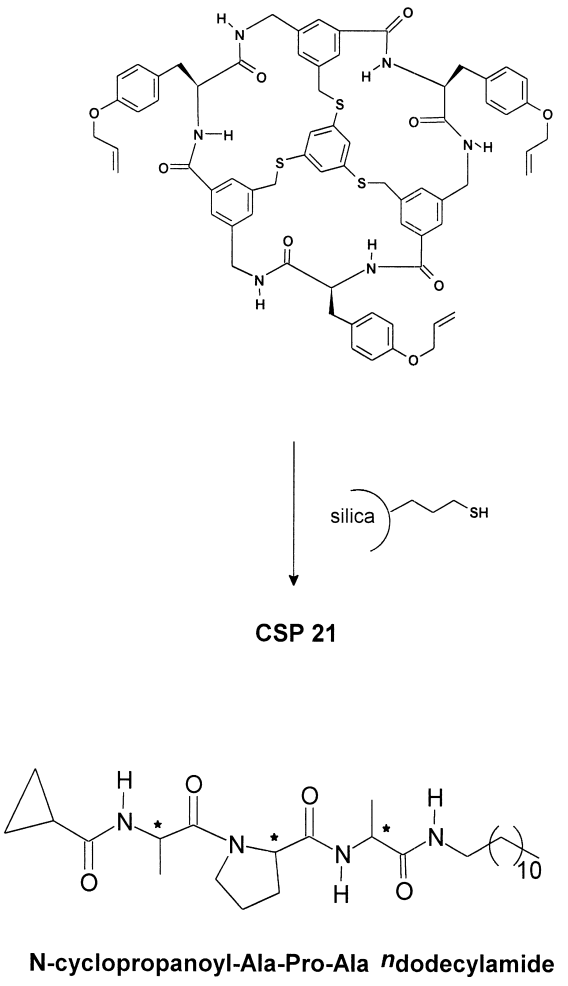


Fig. 9. A chiral stationary phase incorporating a totally synthetic C_3 symmetric receptor.

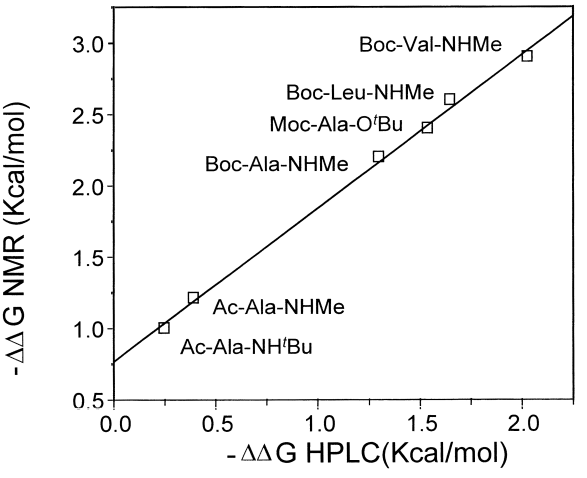
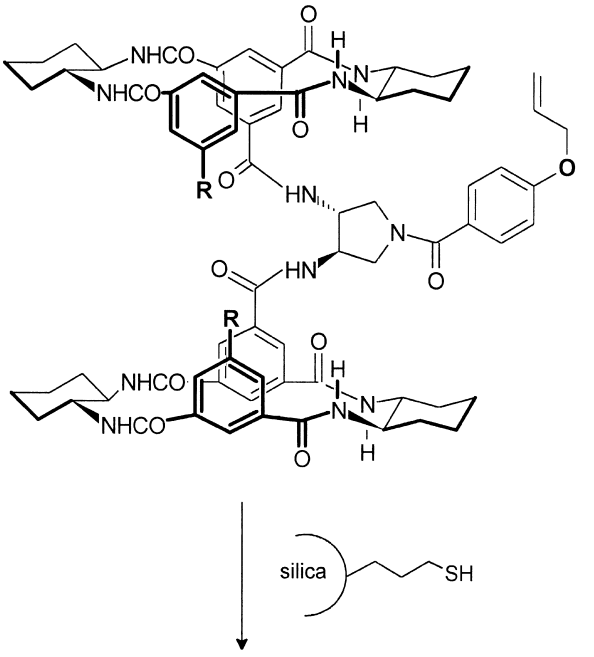
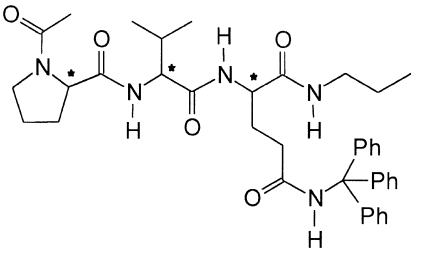


Fig. 10. Comparison of enantioselctivity data obtained by NMR (free solution) and by HPLC on CSP 20 for fully protected aminoacids.

enantiomers. Overall retention in aqueous media was controlled mainly by side chain hydrophobicity, with phenylalanine and leucine derivatives being among the most retained and suggesting an association mode dominated by non-enantioselective contacts between the apolar walls of the host and the apolar side chains of the guests. Hydrophobic interactions arising from burial of the small *N*-methyl group inside the host cavity (enantioselective) contributed less to overall retention. The enantiomers of *N*-cyclopropanoyl alanine *tert*-butylamide showed the highest enantioselectivity under reversed-phase conditions (α=4.95 with 10% acetonitrile in water, compared with α=20.99 with 0.5% MeOH in CH_2Cl_2), suggesting that hydrophobic interactions here were mainly due to the inclusion of the cyclopropyl ring inside the receptor cavity.

Two C_2 symmetric two-armed receptors (Fig. 11) derived from identical tetra-amide subunits (constructed from (*R*,*R*)-1,2-diaminocyclohexane and phthalic or trimesic acid) connected to a *N*-(4-allyloxy benzoylated)-(*R*,*R*)-2,3-diaminopyrrolidine were immobilized on the surface of 3-mercaptopropyl silica gel to afford CSPs 22a–b [63]. These new CSPs were highly selective for a broad set of π-acidic aromatic guests, binaphthols (Fig. 12) and for some particular sequences of protected tripeptides, in the latter case with a strong dependence of the affinity on stereochemistry. CSPs 22a–b were also evaluated as potential tools in the binding ability when screening small peptide libraries and the results were in excellent agreement with a solid-phase binding assay in which the chiral receptors were in solution while the potential binding peptides were fixed to an insoluble polystyrene matrix [64]. Thus, the components of a small peptide library (the eight stereoisomers of the tripeptide Acetyl-Pro-Val-Gln propylamide, see Fig. 11, bottom) were individually prepared and their affinities for the immobilized receptors evaluated by HPLC using CH_2Cl_2-based eluents. Both phases behaved similarly, in that they showed high affinity for a single stereoisomer only (CSP 22a: D,L,D. CSP 22b: L,L,D), accompanied by large values of enantioselectivities (α=16 and α=17 for CSPs 22a and b, respectively). Chromatographic runs carried out in the 25–75°C temperature range revealed an unusual behaviour of the two CSPs towards the tripeptide library members: retention of loosely bound tripeptides was entropy driven, i.e.





Actyl-Pro-Val-Gln(γ-trityl)-NHC3H7

Fig. 11. Chiral stationary phases incorporating totally synthetic C_2 symmetric receptors.

retention increased with temperature, while retention of the strongly retained tripeptide was enthalpy driven. In addition, retention of the enantiomers of cyclic 1,2-diols derivatized as 3,5-dinitrophenylcarbamates [65] on CSP 22a showed unusual dependence on temperature and led, using 1%

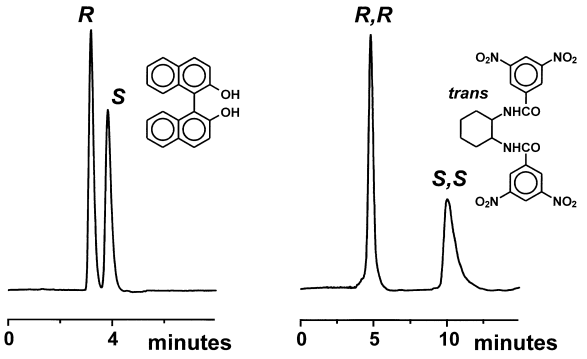


Fig. 12. Chromatographic resolution on CSP 22a and b. Left: 250×1.8 mm column packed with CSP 22a, eluent 0.1% MeOH in CH_2Cl_2, flow-rate 0.5 ml/min. Right: 250×1.8 mm column packed with CSP 22b, eluent 0.1% MeOH in CH_2Cl_2, flow-rate 0.5 ml/min.

MeOH in $CHCl_3$, to a net inversion of the elution order above 65°C. These results were interpreted in terms of solute-CSP association equilibria dominated by solvation–desolvation processes.

A cage-like C_3 symmetric receptor was prepared and immobilized onto 3-mercaptopropyl silica gel through pendant allyloxy groups (CSP 23a, Fig. 13) [66]. The receptor has two 1,3,5-triaryl benzene units at the bottom and at the top of a boxlike molecular structure, and the two aromatic units are connected by three identical peptidic spacers. A reference receptor lacking one of the two triaryl rings was also prepared and immobilized in a similar way (CSP 23b, Fig. 13). While a soluble receptor precursor of CSP 22a showed enantioselectivity in the binding of *N*-protected aminoacids, with differences in free energy of binding between the enantiomers of up to 1 kcal/mol in organic solvents, the same selectivity was not observed with the immobilized selector in the HPLC experiments using either CSP 23a or b. The different behaviour of the free-solution host-guest system and the same system in which the host is surface-linked was ascribed to the different solvents used: while complexation studies were carried out in $CDCl_2CDCl_2$, HPLC runs were conducted with CH_2Cl_2 containing 1–5% MeOH and the alcoholic modifier was considered to be a competitive solvent for the guests, whose associations with the host were driven by H-bond formation. However, both CSPs were found capable of resolving the enantiomers of 2,2′-dihydroxy-1,1′-binaphthyls in non-competitive eluents.

A cleft-type C_2 symmetrical receptor consisting of a 9,9′-spirobi[9*H*-fluorene] skeleton bearing two polar arms at the 2 and 2′ positions was immobilized on silica gel to give CSP 24, (Fig. 14) [67]. CSP 24 showed enantioselectivity for *N*-carboxybenzyl glutamic acid (*N*-Cbz-Glu) and for two 9,9′-spirobi[9*H*-fluorene]2,2′ bicarboxylic acids, with α-values between 1.18 and 1.24 using methanol-CH_2Cl_2 mixtures as eluents. The same stereochemical preferences were observed in free solution between a CSP precursor and the enantiomeric acids. However the magnitude of enantioselection was considerably reduced for the silica bound receptor (in $CDCl_3$ free solution the enantioselection for *N*-CBZ-Glu corresponds to $\alpha=3.24$), and the effect was attributed to the different solvent systems used.

3. Dynamic enantioselective chromatography

Chiral compounds with stereolabile units can be conveniently investigated by dynamic HPLC on a CSP, either in the form of variable temperature or variable flow chromatography (DHPLC) [68,69]. During a DHPLC experiment, two processes occur as the stereolabile species travel across the column: the reversible $R \rightleftharpoons S$ enantiomerization process and the separation process (Fig. 15, top). In cases where the two events take place on the same time scale, temperature and flow dependent chromatographic profiles, with an interference regime (plateau) between the *R* and *S* resolved peaks are observed. Such peak deformations have been reported during HPLC on brush-type CSPs 12a [36–39] and 25 [19] (Fig. 15, bottom) for a range of stereolabile axially chiral compounds and for compounds with both a stereostable stereogenic heteroatom (S, P) and a labile stereogenic axis (Fig. 16) [70–75]. The low exchange regime (the combination of eluent flow-rate and column temperature that yields a chromatogram with no evidence of on-column interconversion) found for these compounds ranged from −80°C to room temperature.

Computer simulation of the experimentally observed elution profile can be used to extract overall rate constants for the enantiomerization process during DHPLC on chiral phases. These rate constants are averaged values for the process occurring in the mobile phase (k^m) and in the stationary phase

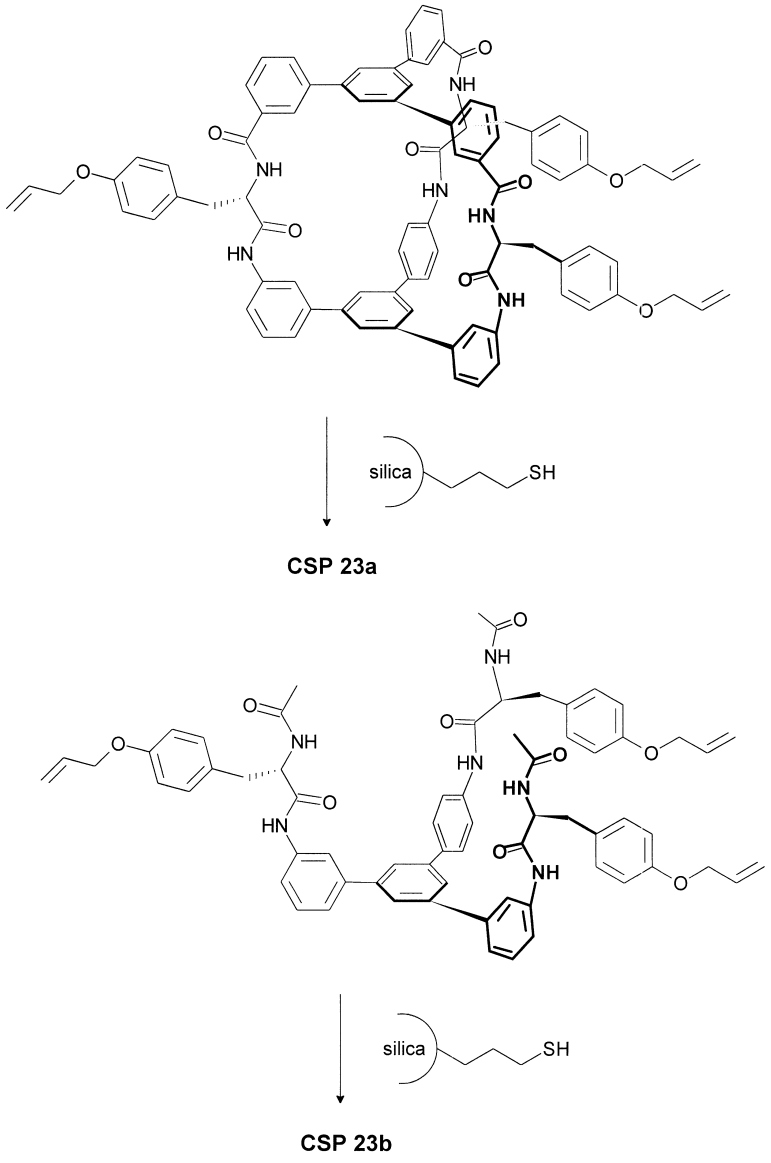
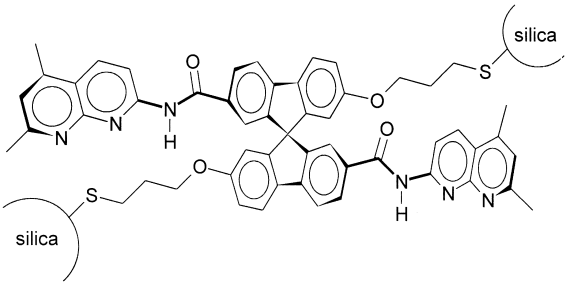


Fig. 13. Chiral stationary phases incorporating totally synthetic C_3 symmetric receptors.

(k^s). With no available preliminary kinetic data on the interconversion, the simulation procedure yields the overall or apparent rate constants for the interconversions of the two enantiomers. Simulated apparent rate constants are usually very close to those measured by independent measurements in the absence of other potentially disturbing species. On the other hand, if k^m is available from independent measurements, the rate constant in the adsorbed state k^s can be obtained by simulation. Valuable kinetic data were collected by DHPLC on CSPs 12a and 25 and simulation of the experimental chromatograms with a computer program based on the discontinuous plate model.

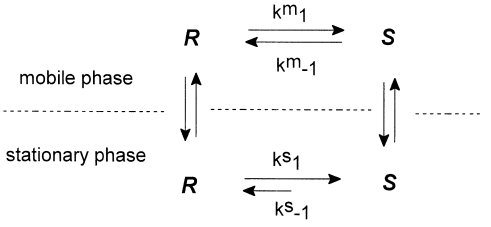
Amides of 2-methyl- or 2-ethoxy-1-naphthoic acids carrying identical or symmetrical substituents at the amide nitrogen (Fig. 17, top) showed hindered rotation around the C_{Ar}–CO bond at room tempera-

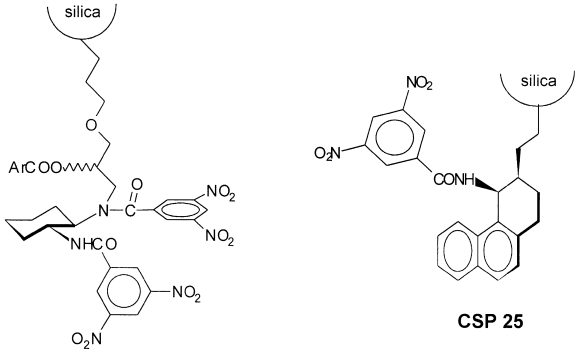


CSP 24

Fig. 14. A chiral stationary phase incorporating a totally synthetic C_2 symmetric receptor.

ture. The carboxamide and naphthyl groups take an almost orthogonal relative disposition in the lowest energy conformation and the molecules display conformational enantiomerism. Chromatography on CSP 25 [76] revealed the existence of interconver-





CSP 12a **CSP 25**

Fig. 15. Top: equilibria occurring during on-column enantiomer interconversion and migration, with the *S* enantiomer eluted last. Bottom: structures of synthetic CSPs 12a and 25 used in DHPLC experiments.

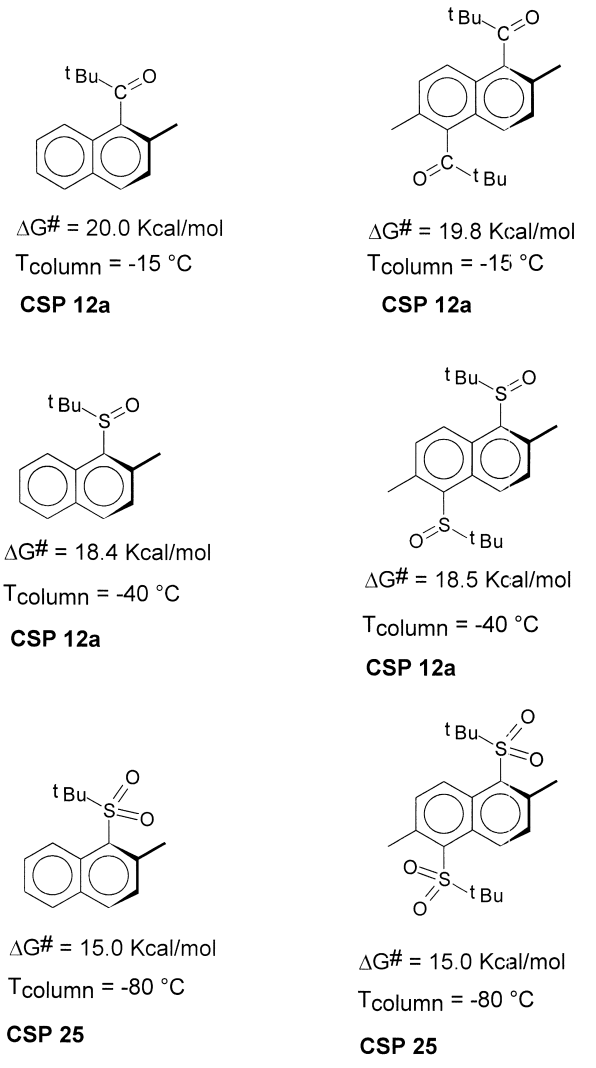


Fig. 16. Stereolabile compounds whose stereoisomers were resolved by cryogenic HPLC on CSPs 12a and 25. T_{column} is the column temperature in the slow exchange regime.

sion phenomena in the temperature range 45–75°C, with averaged elution times between 5 and 50 min (Fig. 17, bottom). Thermal racemization of the individual enantiomers gave the rate constants for the interconversion in free solution, while simulation of the dynamic elution profiles gave the rate constants in the adsorbed state. Enantiomerization barriers between 21.9 and 25.8 kcal/mol were observed, with typical deactivating effects of the CSP on the $R \rightleftharpoons S$ interconversion of the order of 0.5 and 1.0 kcal/mol

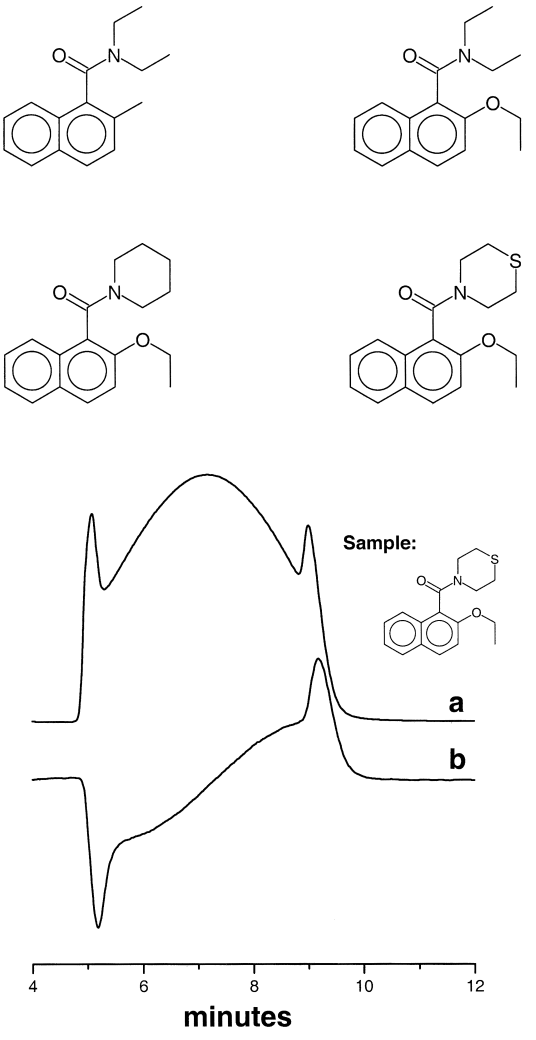


Fig. 17. Top: structures of axially chiral amides of 1-naphthoic acid resolved on CSP 25 [62]. Bottom: DHPLC on CSP 25 using dual UV (a) and polarimetric (b) detections.

for the first and second eluted enantiomers, respectively.

Enantiomerization barriers of some arylnaphthalene lignanes [77] were obtained by computer simulation and chromatography on a polymer-anchored version of CSP 25. Temperatures between −25 and 35°C were used in conjunction with enhanced fluidity mobile phases (carbon dioxide/polar modifier mixtures). The interconversion barriers thus obtained ranged from 17.9 to 21.8 kcal/mol.

Four different *N*-aryl-1,3,2-benzodithiazole 1-oxides were investigated by DHPLC on CSP 25 [78]. Computer simulation of dynamic chromatograms recorded at temperatures between −11 and 1°C gave stereomutation barriers around 19.1 kcal/mol which were not affected by the different substituents on the *N*-phenyl ring. Related studies on the *N*-benzyl derivative carried out by a combination of CD monitored off-column racemizations and DHPLC experiments on CSP 25 revealed a negligible effect of the CSP on the interconversion barrier (23.1 kcal/mol at 35°C in a mixed hexane–CH_2Cl_2–MeOH solvent) [79].

Recently, the mathematical treatment of the equilibria outlined in Fig. 15 was extended to enable the simulation of non-enantiomeric species during their separation on a stationary phase [80]. The secondary *tert*-butyl-1-(2-methylnaphthyl)phosphineoxide, with a stable stereogenic P center and a labile C_{Ar}-P axis, was chosen to test the accuracy of the discontinuous plate model in the simulation of

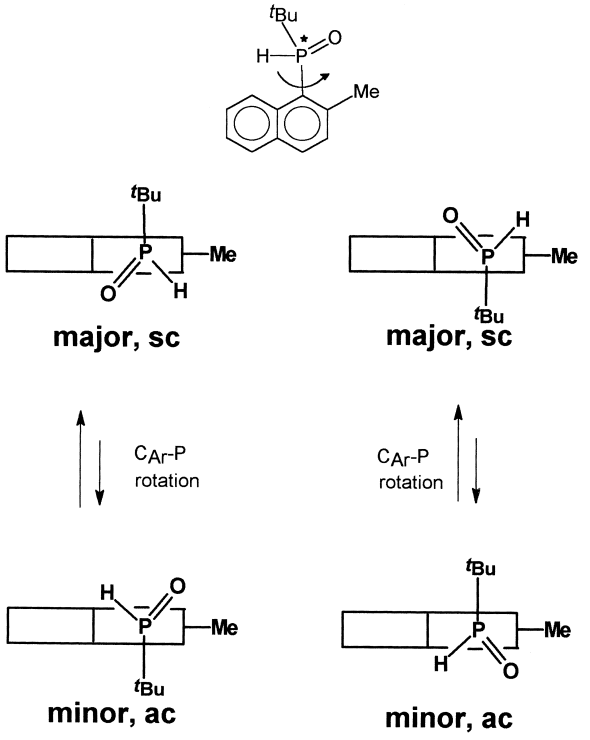


Fig. 18. Structures of the four stereoisomers of *tert*-butyl-1-(2-naphthyl)phosphineoxide resolved on CSP 12a.

the dynamic chromatographic profiles due to on-column interconversion of the synclinal (sc) and anticlinal (ac) diastereomers (Fig. 18). The individual residual enantiomers of the phosphineoxide were analyzed by HPLC at cryogenic temperatures (−68°C) on CSP 12a: non symmetrical plateaus connecting the unequally intense peaks were observed and their simulation yielded a barrier for the sc to ac interconversion of 14.7 Kcal/mol, in close agreement with the results obtained by dynamic ^{31}P NMR. It is interesting to note that, due to its extreme thermal inertness, the same chiral packing (CSP 12a) has been used successfully in a range of temperatures extending from −80°C [75] to 100°C [37].

4. Conclusions

Evolution in the design and synthesis of new chiral stationary phases with low-molecular mass selectors for HPLC applications has led to new materials with overall enhanced properties in terms of enantioselectivity and broad applicability at both the analytical and preparative scales.

New phases incorporating molecular selectors coming from the chiral pool (aminoacids, peptides, cholic acids, alkaloids) often show unprecedented levels of enantioselectivity in normal-phase and reversed-phase mode.

De novo design of molecular structure with enantioselection abilities has also reached a mature stage and rests mainly on a deep understanding of the recognition events at molecular level gained by spectroscopic investigations of model systems. As a parallel line of research, combinatorial processes applied to the discovery of efficient chiral selectors have been shown to be a promising new methodology.

Chiral phases with receptor-like enantioselectivity have been prepared and shown to be a powerful tool in the study of the intermolecular interactions controlling binding affinity of several guests under different experimental conditions.

Chiral stationary phases with small synthetic selectors are especially well suited for variable temperature applications, as their structure and performance are insensitive to even large thermal excursions. This property has provided the opportunity to investigate a range of chiral stereolabile compounds by dynamic HPLC at extreme temperatures.

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