Journal of Chromatography, 521 (1990) 134–140
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 667

Note

Resolution of free aromatic amino acid enantiomers by host–guest complexation using reversed-phase liquid chromatography

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(First received March 2nd, 1990; revised manuscript received June 26th, 1990)

The determination of the enantiomeric composition of amino acids can be accomplished in one of three basic ways: prederivatization of the enantiomers with a chiral reagent [1], addition of a chiral reagent to the mobile phase [2] or use of a chiral stationary phase (CSP) [3]. Although the first method is still the most efficient, it seems to be limited to analytical purposes. The second, especially the ion-exchange systems with chiral mobile phases on an achiral stationary phase, allows the direct separation of underivatized amino acids but requires additional steps to isolate the desired enantiomer from the components of the mobile phase [4].

A wide variety of CSPs have been studied in the attempt to resolve racemic amino acids but none has yet been able to resolve all of the common amino acids. Early attempts with CSPs involved ligand-exchange chromatography on covalently bonded copper(II) ion–L-proline complexes [5]. Kyba *et al.* [6] developed the synthesis of optically active crown ethers bearing binaphthyl units. By anchoring covalently some of these compounds, called hosts, on silica gel or polystyrene resin, they were able to resolve many amino acids as guests mainly as their methyl ester salts in mixtures of organic solvents as eluents [7]. More recently, Shinbo *et al* [8] extended the *in situ* or dynamic coating procedure first introduced by Kirkland and Dilks in 1973 [9] to the preparation of chiral crown ether packings for the chromatographic separation of many racemic underivatized amino acids with dilute perchloric acid as the eluent at 2°C.

However, the synthesis of such chiral crown ethers as highly pure enantiomers always requires extra resolution of racemic mixtures by recrystallization of diastereomeric species [10] or by liquid chromatography on a CSP [11]. For future separations of amino acids on CSPs preparative-scale systems should ideally use cheap aqueous mobile phases without any prederivatization. With the same approach we recently reported the synthesis of new chiral macrocyclic ethers derived from D-mannitol as a source of an inexpensive chiral framework and their immobilization on an

0021-9673/90/$03.50

octadecylsilanized silica [12]. We describe here the preparation and some of the chromatographic characteristics of a similarly designed CSP.

EXPERIMENTAL

Reagents

All common chemicals and solvents were purchased from Aldrich (Strasbourg, France) or Prolabo (Paris, France) and were used without further purification. DL-α-Phenylglycine and D-tryptophan were obtained from Fluka (Buchs, Switzerland), (*R*)-(−)- and (*S*)-(+)-2-phenylglycine, DL-4-chlorophenylalanine and *p*-nitro-DL-phenylalanine from Aldrich, and *p*-nitro-L-phenylalanine, DL-tryptophan and DL-phenylalanine from Sigma (St. Louis, MO, U.S.A.). Rapid chromatography [13] was performed using Merck 9385 silica gel (40–63 μm). The chiral crown ether **2** was immobilized by dynamic coating on a reversed-phase column (Merck 19637; LiChrospher 100 RP-18, end-capped, d_p = 5 μm).

General

^{1}H and ^{13}C NMR spectra were recorded using a Bruker AM 400 spectrometer at 400.13 and 100.58 MHz, respectively, with tetramethylsilane or chloroform as internal references. Optical rotations were measured at 293 K using a Perkin-Elmer 141 polarimeter and a 1-dm cell. Analytical chromatography was performed using a Waters Assoc. Model 6000 pump, a Rheodyne Model 7125 injector, a Waters Model 440 UV detector (254 nm) and a Spectra-Physics Model 4290 integrator.

1,2:5,6-Di-O-isopropylidene-3,4-bis-O-[(2-chloroethoxy)ethyl]-D-mannitol (1)

A 5.25-g (20.0-mmol) amount of 1,2:5,6-diisopropylidene-D-mannitol (from D-mannitol, methoxy-2-propene and *p*-toluenesulphonic acid [14]) was reacted in a two-phase system with 100 ml of bis(2-chloroethyl) ether as solvent and reagent, 100 ml of 50% sodium hydroxide solution and 13.60 g of tetrabutylammonium hydrogensulphate (2 equiv) below 20°C for 14 h [15]. The mixture was diluted with water (100 ml) and dichloromethane (100 ml), decanted and the aqueous phase was washed with dichloromethane (2 × 50 ml). The organic extracts were combined, washed with water (2 × 50 ml), dried and concentrated. After evaporation of excess chloroethyl ether the resulting material was purified by rapid chromatography [25 × 5 cm I.D. column, ethyl acetate–*n*-hexane (1:2, v/v) as eluent to afford 7.13 g (75%) of **1** as a colourless oil: $[\alpha]_D = +14.9^0$ (c = 16; CCl_4). Microanalysis: calculated for $C_{20}H_{36}O_8Cl_2$, C 50.53, H 7.63, Cl, 14.91; found, C 50.30, H 7.54, Cl 15.41%.

1,2:5,6-Di-O-isopropylidene-3,4-O-[1,2-(4-tert.-butylbenzenediyl)bis(oxyethoxyethyl)]-D-mannitol (2)

A solution of 4-*tert.*-butylcatechol (1.03 g, 6 mmol) in *n*-butanol (40 ml) was stirred for 30 min at room temperature under argon. To this solution was added dry potassium carbonate (0.992 g, 6 mmol) and then, after having heated the resulting mixture to a gentle reflux, 2.282 g (4.8 mmol) of **1** were added. The resulting solution was boiled for 5 h, allowed to cool to room temperature and the butanol was evaporated under reduced pressure. The residue was dissolved in dichloromethane (50 ml), washed with distilled water (2 × 20 ml) and the organic phase was dried and

prepurified by elution through a neutral alumina column (about 30 g) with 100 ml of ethyl acetate. After evaporation, the resulting gum was purified by rapid chromatography [15 × 5 cm I.D column, ethyl acetate–*n*-hexane (3:2, v/v) as eluent to afford 1.73 g (63%) of **2** as a homogeneous gum: $[\alpha]_D = +3.4°$, $[\alpha]_{435} = +11.6°$ ($c=2$; CCl_4). ^1H NMR (C^2HCl_3):δ1.28 (s, 12 H, *t*-butyl + 1-methylisopropylidene), 1.30 (s, 3 H, 1-methylisopropylidene), 1.38 (2 s, 6 H, 2-methylisopropylidene), 3.48 (m, 2 H), 3.65–3.73 (m, 4 H), 3.74–3.99 (m, 4 H), 4.02 (dd, 2 H, H-1a/H-6a), 4.04 4.19 (m, 4 H, H-1e/H-6e + 2 H), 4.32 (m, 2 H, H-2/H-5), 6.80 (d, 1 H, H-5 of catechol), 6.90 (dd, 1 H, H-4 of catechol, 1 H), 6.93 (d, 1 H, H-2 of catechol). ^{13}C NMR (C^2HCl_3) : δ 148.30 (C-4 of catechol), 146.78 (C-2 or C-1 of catechol), 144.33 (C-1 or C-2 of catechol), 117.83 (C-3 or C-5 of catechol), 113.45 (C-5 or C-3 of catechol), 112.42 (C-6 of catechol), 108.57 (ketal), 80.61 (C-3/C-4 of mannitol), 75.11 (C-2 or C-5 of mannitol), 75.10 (C-5 or C-2 of mannitol), 72.60 (C-1 or C-6 of mannitol), 72.44 (C-6 or C-1 of mannitol), 70.76, 69.87, 69.19, 68.89 and 66.53 (crown methylenes), 34.16 (*t*-butyl), 31.45 (*t*-butyl *methyls*), 26.57 and 25.31 (isopropylidenes). Microanalysis: calculated for $C_{30}H_{48}O_{10}$, C 63.36, H 8.51; found, C 63.49, H 8.76%.

In situ coating of the crown ether 2

Commercial octadecylsilanized silica (*ca.* 2.3 g) was packed into a 250 × 4.6 mm I.D. stainless-steel column as a toluene–isopropanol–96% ethanol (1:1:1, v/v/v) slurry under a pressure of 450 bar [16]. The column was then washed with chloroform (50 ml) and methanol (50 ml) and equilibrated with 100 ml of methanol–water (55:45, v/v). To a solution of 250 mg of **2** in 55 ml of dry methanol were added 45 ml of distilled water, leading to a slight opalescence without any precipitation. Immobilization of the crown ether on the packing was carried out by pumping the above solution at 0.5 ml/min at 25°C. To achieve a regular coating, the proportion of methanol was decreased stepwise from 55% to 15% by passing 5 × 50 ml of appropriate methanolic aqueous solution at 0.5 ml/min. Finally, the column was carefully equilibrated with distilled water (300 ml at 0.1 ml/min) and the amount of crown ether immobilized on the silica (*ca.* 200 mg) was calculated from the difference between the initial 250 mg and the recovered amount of **2** after combination and evaporation of the eluted methanolic solutions.

RESULTS AND DISCUSSION

Synthesis of the crown ether 2 and its immobilization on C_{18} silica

The synthesis of crown ether **2** according to Fig. 1 allowed us to investigate the effects of the bulky *tert.*-butyl group on the catechol on chiral separation. Earlier results with related crown ethers where the 4-position on the catechol was substituted by a methyl or a nitro group led to the conclusion that a π-donor or π-acceptor group on the stationary phase could increase the separation factor of certain free aromatic amino acid enantiomers. A more lipophilic crown ether was also expected to enhance hydrophobic interactions with the alkyl groups of the silanized silica. A *tert.*-butylcatechol dipotassium salt in 25% excess was used to cyclize **1** with maximum template effect in boiling *n*-butanol; no starting material could be seen after 5 h of reaction by thin-layer chromatography. No attempt was made to improve the moderate 63% yield (*e.g.*, by using neutral alumina instead of silica for the final purification).

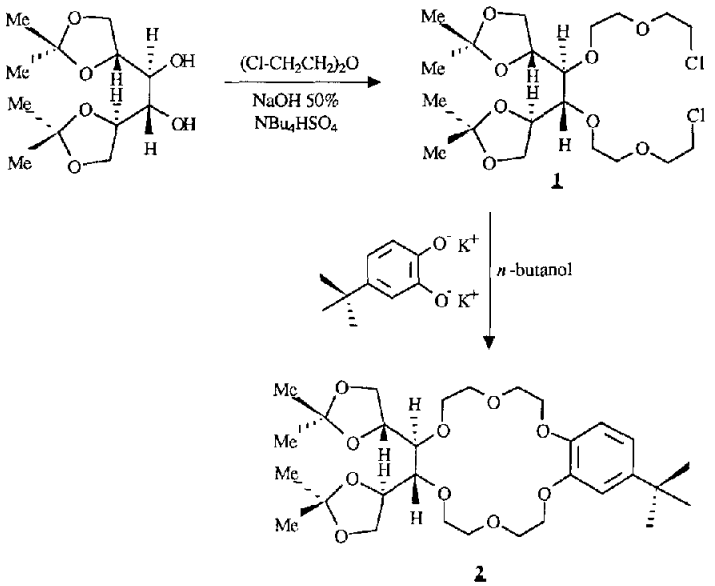

Fig. 1. Two-step synthesis of crown ether **2** from diisopropylidene-D-mannitol. Bu = Butyl; Me = methyl.

Replacement of methanol with acetonitrile as a miscible organic solvent for *in situ* coating led to very slow loading as **2** was either insufficients soluble or not retained on the C_{18} packing, depending on the proportion of acetonitrile. Attention was paid to careful equilibration of the packing before and after the dynamic coating. Reduced equilibration times or by-passing some dilution steps afforded less effective packings with the same amount of crown ether immobilized on the silica. Therefore, the lack of homogeneity in the distribution of the adsorbed crown ether throughout the column can lead to different overall thermodynamic behaviour of the resulting CSP.

Liquid liquid extraction experiments

Chiral differentiation in complexation of the enantiomers of phenylglycine by crown ether **2** was classically measured by ^{1}H NMR after partitioning phenylglycine methyl ester perchlorate between water and chloroform [6]. For this purpose, 28.44 mg of **2** (0.05 m*M*) were dissolved in 1.0 ml of C^2HCl_3 and shaken for 1 min at 0°C with 1.0 ml of lithium perchlorate (4 *M*) in ^2H_2O containing 40.33 mg (0.2 m*M*) of racemic phenylglycine methyl ester hydrochloride. The mixture was allowed to settle for 30 min at 0°C and, the organic layer was then carefully separated, dried over lithium perchlorate, filtered and its ^{1}H NMR spectrum immediately taken at 27°C. The most obvious change was the splitting of the singlet of the benzylic proton on the asymmetric centre of the guest into two broad overlapping singlets around 4.95 ppm, which did not allow separated integration. The ratio of expanded aromatic signal surfaces (10 Hz/cm) up- and downfield from 7.00 ppm led to a guest/host (G/H) stoichiometry of 1.15. The splitting of the diastereomeric methyl groups of the guest into two well separated singlets at 3.73 ppm for the *R* and at 3.64 ppm for the *S* enantiomer with an approximate ratio of 63:37 in favour of the *R* isomer was unexpected.

In order to establish the ability of chiral recognition of **2** towards free racemic

phenylglycine, a more polar organic phase (23.1% C^2H_3CN in C^2HCl_3) was used at 0°C and led to variable results in relation to the proportion of perchloric acid initially added to the aqueous phase. An excess of acid (initial pH < 1) caused partial hydrolysis of the isopropylidene groups but also important complexation (G/H around 1). Duplication of the run with an aqueous phase of initial pH 3 did not cause any hydrolysis of ketal but only a modest extraction (G/H < 0.2). Triplication with an aqueous phase of initial pH 2 did not improve significantly the extraction rate of free phenylglycine but caused a slight measurable hydrolysis of the protective groups (around 5%). No splitting of the singlet around 4.95 ppm could be observed, even on heating the isolated and dried chloroform solution to 40°C.

In brief, host **2** was able to extract phenylglycine as a free acid or as its related methyl ester when associated with the perchlorate anion. Major changes in chemical shifts and multiplicity in the ^1H NMR spectra and unusually low solubilities in C^2HCl_3 were proof of the rapid formation of highly structured 1:1 complexes with an enantiomeric excess of at least 26% in the case of the methyl ester salt.

Chromatographic experiments

Earlier results showed that no resolution of racemic phenylglycine methyl ester could be observed with such a related crown ether immobilized on a C_{18} silica when dilute perchloric acid was used as the eluent, although a high capacity factor was measured for this compound (capacity factor, $k' = 17$). No attempt was made to study other amino acid methyl esters or different mobile phases as the final aim was the resolution of free amino acids which were tested as received, without any prederivatization.

Baseline resolution of racemic phenylglycine was obtained with pure water as the eluent with an α value of 1.92 at 0°C and 1.69 at 20°C. The *R* enantiomer was eluted after the *S* enantiomer. As a stoichiometry of 1:1 was measured for this complexation according to the results of the one-plate extraction, one can use the general equation $\Delta(\Delta G^0) = -RT \ln \alpha$ to determine the free-energy differences at the two temperatures, which were −354 and −305 cal/mol, respectively. The temperature dependance of $\Delta(\Delta G^0)$ indicates that the more stable complex (corresponding to the most retained *R* enantiomer) depends to a large extent on the enthalpic term, whereas the less stable complex (corresponding to the least retained *S* enantiomer) is mostly determined by the entropic term [17]. A maximum α value of 2.2 was measured when acetonitrile–water (20:80, v/v) was used as the eluent at 20°C. The retention time of the most retained *R* enantiomer was only half that measured with pure water but the peak symmetry was poorer, suggesting slower complexation–decomplexation kinetics.

The best results for basic tryptophan were obtained when water was replaced with 10^{-4} *M* perchloric acid in a 80:20 mixture with acetonitrile at 0°C; in this instance the capacity factors were twice as large with an almost total separation in about 49 min (see Fig. 2). In contrast, the selectivity was significantly reduced from 1.32 to 1.24. Also, the L enantiomer was eluted first.

Racemic phenylalanine gave only a single peak whether pure water, water–acetonitrile or dilute perchloric acid was used as the eluent.

Racemic *p*-nitro-DL-phenylalanine was totally resolved when 10^{-4} *M* perchloric acid–acetonitrile (80:20) was used as the eluent with an α value of 1.35 (see Fig. 3). Only partial separation was observed under neutral conditions. As for phenylglycine, the D enantiomer was eluted after the L enantiomer.

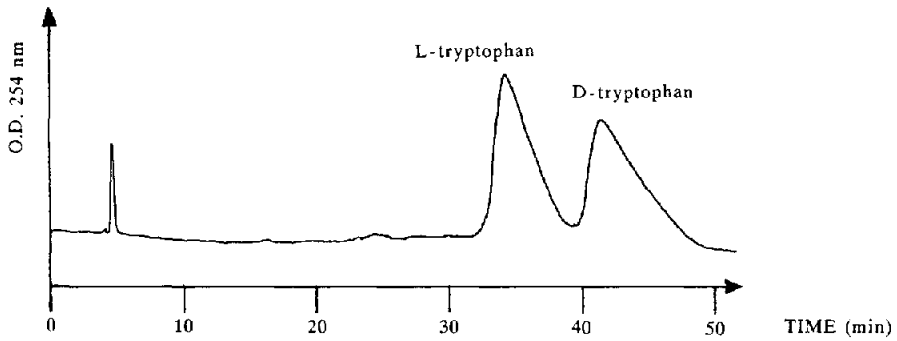

Fig. 2. Chromatographic resolution of 8 μg of racemic tryptophan on a C_{18} packing coated with *ca.* 200 mg of **2**. Mobile phase: 10^{-4} *M* perchloric acid–acetonitrile (80:20) at 0°C; flow-rate, 0.8 ml/min.

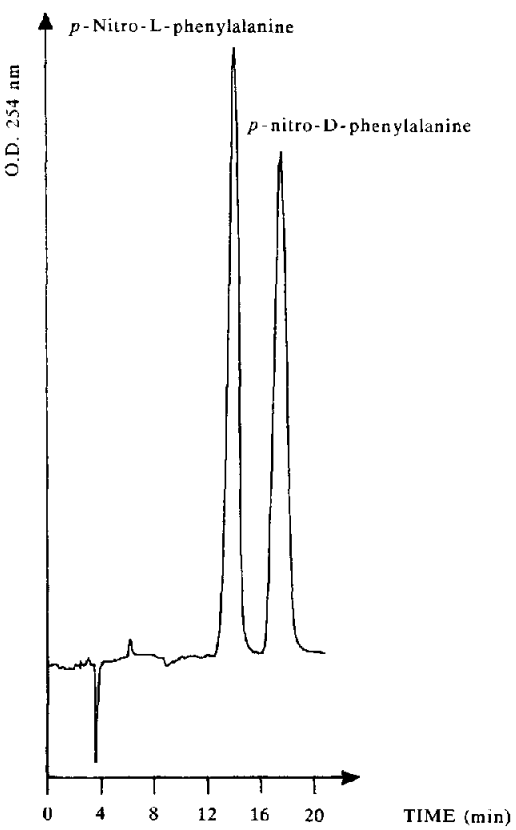

Fig. 3. Chromatographic resolution of 5 μg of racemic *p*-nitrophenylalanine on a C_{18} packing coated with *ca.* 200 mg of **2**. Mobile phase: 10^{-4} *M* perchloric acid–acetonitrile (80:20) at 0°C; flow-rate, 1.0 ml/min.

DL-4-Chlorophenylalanine was not eluted within 1 h when acetonitrile-water (25:75) was used as the mobile phase. Neither higher proportions of acetonitrile nor other organic solvents in the eluent were tested as it was feared that the crown ether could be leached out from the packing by less polar mobile phases.

CONCLUSION

It can be assumed that the magnitude of chiral recognition depends mainly on the steric bulk around the chiral centre, as the best selectivity was observed with racemic phenylglycine. If we consider the NH_3^+ group anchored in the centre and on the top of the heterotopic cavity of the host the three others groups on the stereogenic centre differ greatly in bulk, in the order $C_6H_5 > CO_2H > H$. On the other hand, π-stacking interactions are able to stabilize one of the two diastereoisomeric adsorbates; *p*-nitro-D-phenylalanine was perfectly separated from its antipode but not D-phenylalanine. Formation of an additional hydrogen bond between the hydrogen atom of the carboxylic acid function and one of the oxygen atoms of the ketal may provide a third useful interaction according to the classical three-point model. Lastly, an acidic medium or the addition of acetonitrile to the mobile phase favours strong pole–dipole interactions between the organic cation and the oxygens of the cavity but does not always increase selectivities. In brief, at least three semi-independent interactions are involved in the chiral recogition process.

We are now checking systematically the chromatograhic behaviour of host **2** and related compounds towards aliphatic amino acids in various mobile phases to improve our understanding of the chiral recognition process.

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