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# SEPARATION OF SOME ENANTIOMERIC DI- AND TRIPEPTIDES ON CHIRAL STATIONARY PHASES

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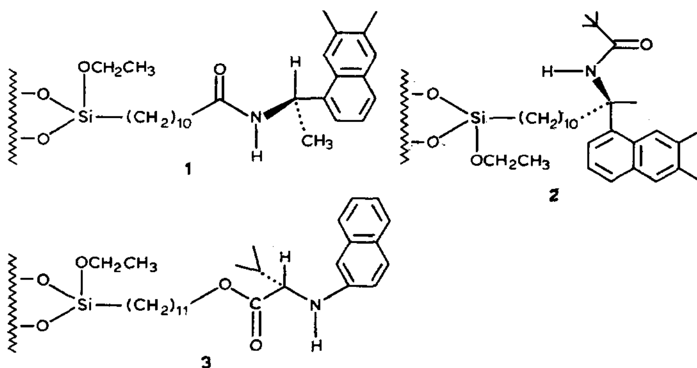
## SUMMARY

The chromatographic behavior of the N-3,5-dinitrobenzoyl derivatives of twelve dipeptide esters and two tripeptide esters was investigated on three different chiral stationary phases (CSPs). It is observed that the stereoisomers present in each sample may be cleanly separated on each chiral phase. A degree of regularity is noted in the elution order of the enantiomers and often of the diastereomers. Elution order of the enantiomers is related to a chiral recognition model for each CSP.

## INTRODUCTION

The gas and liquid chromatographic (GC and LC) resolution of the enantiomers of α-amino acids, derivatized as their esters and amides, can now be easily accomplished with a variety of chiral stationary phases (CSPs)[1–6]. While there are reports of GC[7] and LC separation of enantiomeric dipeptides[8,9] and tripeptides[9], such separations are not yet routine. In view of the increasing importance of small peptides as therapeutic and sweetening agents, techniques for the separation and stereochemical analysis of peptides will assume added significance. While the need to separate enantiomeric peptides will not often arise, the need to separate diaste-




Scheme 1. Structures of CSPs 1, 2 and 3.

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reomeric peptides is common. We herein describe LC separations of enantiomeric and diastereomeric di- and tripeptides on chiral high-performance liquid chromatographic (HPLC) columns.

A number of CSPs has been developed on which one may efficiently separate the enantiomers of a variety of compounds[1,5,10,11]. Most systematic studies to date have focused on analytes which contain but a single stereogenic center. For example, stationary phases 1, 2 and 3 (Scheme 1) efficiently separate the enantiomers of α-amino amides as their N-3,5-dinitrobenzoyl (N-3,5-DNB) derivatives[1,4,5,11]. In this paper, we extend this work to encompass the chromatographic resolution of the enantiomers of compounds containing more than one chiral center, specifically, esters of N-3,5-DNB di- and tripeptides.

## EXPERIMENTAL

Chromatography was performed with a Beckman Model 100A pump, Altex 210 injector, Beckman 165 variable-wavelength detector and a Kipp and Zonen BD41 recorder. The UV detector monitored simultaneously at 254 and 280 nm. A Buchi apparatus was used for melting point determination. $^{1}H$ NMR spectra were obtained on a Varian EM-390 spectrometer using deuterated chloroform as a solvent and tetramethylsilane as an internal standard.

### *α-Amino acid methyl esters*

To a 50-ml round-bottom flask was added 1 g of the amino acid and 25 ml of methanol. The solution was saturated with hydrogen chloride gas. After 20 min, the solvent was removed under vacuum and the resulting material was triturated with hexane and/or light petroleum (b.p. 60–68°C). The white solid was then collected on a fritted glass filter and allowed to air dry. Both racemates and enantiomerically enriched materials were prepared in this way as were the isopropyl esters of phenylalanine.

### *N-3,5-DNB amino acids*

Typically, 3,5-DNB chloride and the amino acid were added in a 1.5:1.0 ratio to tetrahydrofuran containing a several-fold excess of propylene oxide. After the solid dissolved, the solution was concentrated under vacuum and the resulting solid was recrystallized from acetonitrile or acetone–water. The compounds listed below are now available from Aldrich: (*R*)-N-3,5-DNB-phgly, m.p. 216–217°C (phgly = phenylglycine); (*S*)-N-3,5-DNB-leu, m.p. 186–188°C (leu = leucine); (*R,S*)-N-3,5-DNB-phgly, m.p. 240°C; (*R,S*)-N-3,5-DNB-leu, m.p. 200–202°C.

### *Methyl esters of N-3,5-DNB dipeptides*

Typically, to a round-bottom flask equipped with a magnetic stirrer were added methylene chloride and equimolar amounts of 3,5-DNB amino acid and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). The mixture was kept at 0°C until solution was obtained (20–45 min). The amino ester then was added as its hydrochloride salt. After several hours, the solution was filtered and washed first with 1 *M* hydrochloric acid and then with saturated sodium bicarbonate. The organic layer was dried with magnesium sulfate, filtered, and evaporated to dryness. Where puri-

fication was desired, the material was simply triturated with light petroleum (b.p. 60–68°C). When racemic reagents are used, the product consists of a mixture of racemic diastereomers.

For 3,5-DNB-(*R*,*S*)-leu-(*R*,*S*)-leu methyl ester: m.p. 182–190°C. $^1$H NMR (90 MHz) ($C^2HCl_3$) $\delta$ 0.70–0.90 (bs, 12H), $\delta$ 1.30–1.80 (m, 6H), $\delta$ 3.30–3.70 (m, 3H), $\delta$ 4.20–4.80 (m, 2H), $\delta$ 8.10–8.30 (bs, 1H), $\delta$ 8.30–8.80 (bs, 2H).

*Phenylalanine N-carboxyanhydride*

An amount of 2 g (13.4 mmol) of racemic phenylalanine was suspended in 50 ml of dry tetrahydrofuran and heated to 55°C. In a well ventilated fume cupboard, phosgene (danger, toxic) was bubbled into the hot suspension for 1 h, then air was passed through the solution for 1 h to remove excess phosgene. The solvent was removed under vacuum and the resulting solid was washed thoroughly with diethyl ether and hexane. Off-white crystals of the carboxyanhydride were obtained in 68% yield. m.p. 122–125°C (literature value: 127°C)[12].

*p-Methylbenzyl ester of phenylalanine*

An amount of 1 g (5.2 mmol) of phenylalanine N-carboxyanhydride was suspended in 50 ml anhydrous diethyl ether. The slurry was cooled to 0°C and saturated with hydrogen chloride gas. Then, 1 g (8.0 mmol) of *p*-methylbenzyl alcohol was added. After 24 h, the ether was removed under vacuum and the remaining white solid ester (49% yield) was washed with diethyl ether. m.p. 178–185°C. $^1$H NMR (90 MHz) ($C^2HCl_3$) $\delta$ 2.10 (s, 3H), $\delta$ 2.80 (bs, 2H), $\delta$ 3.80–4.10 (m, 1H), $\delta$ 6.70–7.00 (m, 9H), $\delta$ 8.00–8.50 (m, 3H). Anal. calc. for $C_{17}H_{20}NO_2Cl$: C, 66.88; H, 6.56; N, 4.59; Cl, 11.48%. Found: C, 66.93; H, 6.62; N, 4.53; Cl, 11.36%.

This amino ester was used to make 3,5-DNB dipeptides following the previously described procedure.

*N-3,5-Dinitrobenzoyl tripeptide methyl esters*

These tripeptides were prepared in basically the same manner as the dipeptides, using commercial samples (Sigma) of glycyl-methionine or glycyl-phenylalanine which had been esterified using methanol–hydrogen chloride.

## RESULTS AND DISCUSSION

Owing to the use of racemic components in the synthesis of the dipeptides, four stereoisomers are present in each sample. The diastereomers, termed like [*i.e.* (*R*,*R*), (*S*,*S*)] or unlike [*i.e.* (*R*,*S*), (*S*,*R*)] are each racemic but not present in equal amounts owing to kinetic fractionation during the coupling reaction. The unlike diastereomer always predominated, diastereomeric excesses ranging between 22 and 48%. In the case of phgly-phe, both isopropyl and *p*-methylbenzyl esters of phenylalanine were employed in the coupling reactions. This increases the extent of kinetic fractionation. Diastereomeric excesses of *ca.* 60% were noted. Stereochemical assignments of chromatographic peaks were made by chromatographic comparison with samples highly enriched in independently prepared, configurationally known, stereoisomers.

The dipeptides were chromatographed on CSPs 1, 2 and 3 (Scheme 1) using

TABLE I

SEPARATION OF THE STEREOISOMERS OF 3,5-DNB DIPEPTIDES ON CSPs(*R*)-1, (*R*)-2 AND (*R*)-3 USING 2-PROPANOL–HEXANE (20:80)

Negative values for $\alpha$ indicate that the elution order for that pair of enantiomers or diastereomers departs from the usual elution order of: (*S*,*S*) before (*R*,*R*); (*S*,*R*) before (*R*,*S*); like before unlike.

| *DNB-a.a.-a.a.-$CO_2CH_3$* | *(S,S) Capacity ratio* | | | *$\alpha$ like* | | | *(S,R) Capacity ratio* | | | *$\alpha$ unlike* | | | *$\alpha$ Diast.* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* | *1* | *2* | *3* |
| ala-ala | – | 7.5 | 1.3 | – | 3.04 | 17.22 | – | 11.8 | 3.7 | – | 3.45 | 5.96 | – | 1.74 | 1.10 |
| ala-leu | 2.8 | 3.8 | – | 2.45 | 3.00 | – | 4.5 | 5.0 | – | 1.67 | 3.33 | – | 1.04 | 1.41 | – |
| ala-met | 11.4 | 6.3 | 2.8 | 2.26 | 3.16 | 10.31 | 19.3 | 9.7 | 4.1 | 1.67 | 3.37 | 6.52 | 1.19 | 1.63 | −1.01 |
| ala-phe | 9.8 | 6.5 | 2.9 | 1.80 | 2.03 | 8.91 | 15.7 | 7.9 | 5.4 | 1.51 | 2.34 | 5.00 | 1.23 | 1.42 | 1.15 |
| leu-ala | 3.7 | 4.0 | 2.4 | 1.42 | 4.83 | 10.61 | 6.2 | 5.4 | 2.4 | 1.54 | 4.39 | 11.21 | 1.49 | 1.27 | 1.06 |
| leu-leu | 4.6 | 3.7 | 1.4 | 2.41 | 6.55 | 15.71 | 8.0 | 4.6 | 1.6 | 1.40 | 3.57 | 13.01 | 1.05 | −1.30 | 1.01 |
| leu-met | 3.9 | 4.2 | 2.7 | 2.26 | 4.72 | 18.51 | 6.7 | 6.0 | 3.8 | 1.53 | 4.47 | 11.91 | 1.15 | 1.38 | −1.07 |
| leu-phe | 7.1 | 6.5 | 2.0 | 1.70 | 3.18 | 17.31 | 15.2 | 10.1 | 3.6 | 1.32 | 3.97 | 11.21 | 1.57 | 1.89 | 1.19 |
| phgly-ala | 10.3 | 8.3 | 3.7 | 1.25 | 2.70 | 5.10 | 21.5 | 12.4 | 5.2 | −1.43 | 2.85 | 3.83 | 1.35 | 1.56 | 1.13 |
| phgly-leu | 12.6 | 4.3 | 2.7 | 1.36 | 2.69 | 6.61 | 30.7 | 6.2 | 4.1 | −1.53 | 2.51 | 4.07 | 1.44 | 1.43 | 1.00 |
| phgly-met | 11.7 | 7.3 | 4.9 | 1.12 | 2.65 | 6.22 | 23.7 | 10.5 | 7.9 | −1.34 | 2.46 | 3.62 | 1.43 | 1.35 | 1.02 |
| phgly-phe | 22.7 | 7.3 | 5.1 | 1.00 | 2.11 | 6.03 | 47.8 | 10.2 | 8.0 | −1.45 | 2.20 | 3.65 | 1.43 | 1.42 | 1.03 |
| phgly-bnzala | 9.3 | 6.3 | – | 1.11 | 2.61 | – | 20.2 | 9.8 | – | −1.41 | 2.58 | – | 1.49 | 1.57 | – |

2-propanol–hexane (20:80) as the mobile phase with a flow-rate of 2 ml/min. On CSP-(*R*)-2, a general pattern in elution order was noted. With but one exception, the elution order is (*S*,*S*), (*S*,*R*), (*R*,*R*), (*R*,*S*) where the initially indicated configuration is that of the N-DNB-amino acid. The exceptional case is DNB-(*R*,*S*)-leu-(*R*,*S*)-leu-$CO_2CH_3$ where the elution order is (*S*,*S*), (*S*,*R*), (*R*,*S*), (*R*,*R*). Actually, no change has occurred in the relative elution order of either diastereomers or enantiomers; the magnitude of the separation factor for the enantiomers of the like diastereomers is unusually large and causes "cross-over" of the last two peaks. Pertinent data are presented in Table I for the chromatographic behavior of these dipeptides on CSPs 1 and 2. The separation factors listed for the diastereomers are calculated from the midpoint of each of the two sets of enantiomers. These separation factors have no rigorous meaning and are not the values which would be observed were the diastereomers chromatographed on a racemic version of the CSP. However, these values do provide a basis for discussing the relative mobility of the diastereomers. Negative values indicate exceptions to the usual elution orders [*i.e.* like before unlike; (*S*) before (*R*), these configurations referring to the N-terminal amino acid].

When the dipeptides are chromatographed on CSP(*R*)-1 using the same eluent and flow-rate, it is noted that those dipeptides having alanine or leucine as their N-terminal amino acid show the same chromatographic behavior as on CSP-(*R*)-2. However, those with phenylglycine (phgly) as their N-terminal amino acid behave differently. In these cases, the elution order is (*S*,*S*), (*R*,*R*), (*R*,*S*), (*S*,*R*) with little separation between the (*R*,*R*) and (*R*,*S*) isomers. The "abnormal" elution order of the unlike enantiomers [*i.e.* (*R*,*S*) before (*S*,*R*)] is caused by the $\alpha$-phenyl substituent. Perhaps for conformational reasons, aryl substituents $\alpha$ to the 3,5-dinitrobenzamido group lead to increased contribution toward the overall chiral recognition by what has been termed the "dipole-stacking" process[10,11]. This is a process which engenders enantioselectivity opposite to that of the normally (for amino acid derivatives) dominant "hydrogen-bonding process"[10,11]. One infers that, for the unlike diastereomers, the contribution made by the dipole-stacking process has more than counter-balanced the contribution made by the hydrogen-bonding process.

On CSP 1, the magnitude of the separation factors for the enantiomers of the like dipeptides having phenylglycine as the N-terminal amino acid are reduced relative to those of the other like dipeptides but elution orders are still normal. Here, the increased contribution by the dipole-stacking process only partially counteracts the contribution made by the hydrogen-bonding process, normally the dominant process for N-3,5-DNB amino acid derivatives on CSPs 1 and 2*. The (*S*,*S*), (*R*,*R*), (*S*,*R*), (*R*,*S*) order noted for the (*R*,*S*)-leu-(*R*,*S*)-phe diastereomers is not viewed as exceptional, since the elution order of enantiomers and diastereomers is normal despite the cross-over of the center peaks. In general, the enantiomeric and diastereomeric separation factors tend to be larger on CSP 2 than on CSP 1.

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* Work with other CSPs has indicated that $\pi$-electron clouds may serve as basic sites for hydrogen bonding[13]. Hence, one considers the possibility that, in the hydrogen-bonded process, one enantiomer of an N-terminal phenylglycine analyte uses the phenyl as a basic site, the other enantiomer uses the oxygen of the carboxamide group. The former interaction, by increasing the retention of the least retained enantiomer, would be expected to reduce the magnitude of the separation factor but would not be expected to compete so effectively as to invert elution order. Such competition would produce results similar in some regards to increased contribution from the dipole-stacking process.

The recently described CSP 3 is unusually effective for the separation of the enantiomers of amides and esters of amino acid DNB's[5]. On CSP-(*R*)-3, the (*S*,) and (*S*,*R*) isomers are first eluted for each of the enantiomeric pairs. The more strongly retained (*R*,*R*) and (*R*,*S*) enantiomers elute in no consistent order relative to each other, cross-over being common. It is to be noted that on CSP(*R*)-3, enantiomeric separation factors are larger than on CSPs 1 and 2 whereas the diastereomeric separation factors are smaller. This is not unexpected, since the chiral recognition mechanisms suggested to be operative on CSP 3 for N-3,5-DNB amino acid derivatives do not place the entity appended to the C-terminal carboxyl in close proximity to the CSP. Hence, the stereochemistry of such entities has but a modest effect upon the magnitude of the separation factor for the diastereomers.

We repeat that the separation factor calculated from the midpoints of the enantiomeric pairs of like-unlike diastereomers is not actually that which would be expected on a racemic but otherwise identical version of the CSP, this being the subject of as future discussion. We do point out that on the chiral column, separations of all four stereoisomers may be observed even when the separation factor for the diastereomers is unity. However, the separation factors for the enantiomers of each diastereomer must differ for this to occur. This is typically the case. On CSP 3, the midpoints of the peaks from each set of enantiomers are nearly coincident, yet the four peaks are, as a rule, cleanly separated. This clearly indicates an intrinsic advantage of CSPs relative to achiral stationary phases for the analysis of diastereomers. On an achiral column, analysis cannot be made when the diastereomeric separation factor is unity. This need not prevent analysis on a CSP. To further enhance selectivity, one may couple the CSP column and a racemic version of this column in series. This, too, is a subject for future discussion.

To rationalize the elution order of the enantiomers of the like and unlike dipeptides, one need but take cognizance of the chiral recognition mechanisms already postulated for the separation of the enantiomers of N-3,5-DNB amino acid derivatives on CSPs 1, 2 and 3. The factors which determine the relative elution orders of the diastereomers are less apparent. For that reason, we continue to study the effects of "remote" second chiral centers upon elution order. As a part of this study, we sought to increase the distance between the chiral centers by investigating the chromatographic behavior of tripeptide derivatives in which glycine is the central amino acid. Two tripeptides of the general structure DNB-a.a-gly-a.a.-$CO_2CH_3$ were prepared and examined. CSP 2 is unable to separate all four of the stereoisomers of these tripeptides. However, CSP 1 does effect such separations; the elution order of the stereoisomers of the tripeptide DNB-leu-gly-phe-$CO_2CH_3$ is (*S*,*S*), (*S*,*R*), (*R*,*R*), (*R*,*S*). On CSP 3, the elution order of the stereoisomers of both DNB-leu-gly-phe-$CO_2CH_3$ and DNB-leu-gly-met-$CO_2CH_3$ is (*S*,*S*), (*S*,*R*), (*R*,*R*), (*R*,*S*).

Thus, even with the second chiral center quite far from the principle site of interaction with the CSP, the stereochemistry of the second center still manifests itself on chromatographic behavior. How this is accomplished is a question of major importance, for insight into the details of such remote interactions seems likely to aid in the design of CSPs intended to enhance such interactions.

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