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LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS OF β-AMINO ACIDS USING A CHIRAL STATIONARY PHASE

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SUMMARY

The enantiomers of both α-substituted β-alanines and β-substituted β-alanines may be chromatographically separated using silica-bonded chiral stationary phases derived from N-acetylated α-arylalkylamines. The amino acids are chromatographed as alkyl esters of N-3,5-dinitrobenzoyl derivatives; separability factors range from 1.11 to 1.65 for nine α-substituted β-alanines and from 1.08 to 1.20 for nine β-substituted β-alanines. The enantiomers of β-aminoisobutyrate and β-leucine, chiral β-amino acids occurring in animal tissues and physiological fluids, are among those resolved. The enantiomers of *R,S*-β-aminoisobutyrate and several related α-alkyl-β-alanines were prepared by chromatographic resolution of diastereomeric dipeptides.

INTRODUCTION

Four β-amino carboxylic acids participate in mammalian metabolism. β-Alanine and *R*-β-aminoisobutyrate are metabolites of uracil and thymine, respectively, whereas *S*-β-aminoisobutyrate is formed by transamination of *S*-methylmalonate semialdehyde, a metabolite of L-valine[1]. β-Leucine (3-amino-5-methylpentanoic acid) is an intermediate in a recently described pathway linking L-leucine with β-ketoisocaproyl-coenzyme A and isobutyryl-coenzyme A, the last also a metabolite of L-valine[1,2]. The enantiomer of β-leucine occurring in animals is not yet established.

Studies of β-amino acid metabolism have been considerably impeded by difficulties encountered in obtaining and analyzing the biochemically important chiral β-amino acids. Enantiomers of some β-substituted β-alanines (*e.g.*, those which are higher homologues of protein α-amino acids) can be prepared by Arndt–Eistert synthesis from an appropriate α-amino acid [*e.g.*, L(*R*)-β-leucine from L-valine], but no generally useful synthesis of enantiomeric α-substituted β-alanines is yet available. In previous studies Kakimoto and Armstrong[3] resolved the enantiomers of β-aminoisobutyrate by fractional crystallization of the cinchonidine salt of N-acetyl-*R,S*-β-aminoisobutyrate, and Pollock[4] obtained *R*-β-aminoisobutyrate from its racemate

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following selective catabolism of the *S*-isomer by Baker's yeast. In the present studies, enantiomers of several α-alkyl-β-alanines including *R,S*-β-aminoisobutyrate were resolved by preparative chromatography of *R,S*-β-amino acyl-*S*,(L)-aspartic acid diastereomeric dipeptides on Dowex 50 resin.

Analytical separation of the enantiomers of derivatized β-amino acids has previously been accomplished by gas chromatography (GC) on N-lauroyl amino acid-*tert.*-butylamide stationary phases by Gil-Av and co-workers[5]. GC resolution of diastereomeric derivatives of β-amino acids using camphanic acid[6], trifluoroacetylproline[7] or menthol[8] and liquid chromatographic (LC) resolution of the diastereomeric dipeptide pair L-glutamyl-D,L-β-amino-*n*-butyric acid[9] have also been reported. Pirkle and co-workers[10–12] previously described the preparation of a series of chiral stationary phases (CSPs) derived from N-acylated α-arylalkylamines and demonstrated their ability to separate the N-3,5-dinitrobenzoyl derivatives of an assortment of enantiomeric amines and amino acids. The present report describes the separation of the enantiomers of similarly derivatized β-amino acids. A preliminary report of the findings has been made[13].

EXPERIMENTAL

Materials

R,S-β-Aminoisobutyrate was prepared by reduction and hydrolysis of thymine[14]. Other α-alkyl-β-alanines were prepared from the appropriate diethyl alkylmalonate and N-(chloromethyl)phthalimide as described[15]. β-Alkyl-β-alanines were prepared by addition of ammonia to the corresponding α,β-unsaturated acid[16]; the latter were prepared from malonic acid and the appropriate aldehyde. New compounds gave correct analytical and (or) spectroscopic data. The preparation of CSP 3 has been described previously[10].

Methods

N-Boc-α-alkyl-β-alanine derivatives were prepared in nearly quantiative yield using either 2-(*tert.*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON)[17] or di-*tert.*-butyldicarbonate[18]. The N-hydroxysuccinimide derivatives of the protected amino acids were prepared using dicyclohexylcarbodiimide in dimethoxyethane and were filtered without isolation directly into an aqueous, sodium hydrogen carbonate buffered solution containing 1.5 equiv. of L-aspartate. The resulting diastereomeric N-Boc-*R,S*-β-amino acyl-*S*-(L)-aspartate dipeptides were extracted from the acidified reaction mixtures into ethyl acetate, and, after drying the organic solution, were obtained in solid and often crystalline form by evaporation of the solvent. Without further purification, the N-Boc group was removed by treatment with trifluoroacetic acid, and the crude dipeptide mixtures, typically containing 80–90% dipeptide and 10–20% free amino acids, were applied directly to a column (120 × 2.5 cm I.D.) of Dowex 50 × 8 (200–400 mesh). The column was equilibrated and eluted with an aqueous buffer containing 25 ml of pyridine and 63 ml of 88% formic acid per liter; 10–30 mmol of dipeptide were applied in each run. Amino acid and dipeptide containing fractions were detected using *o*-phthalaldehyde, and the species present were determined by comparison with standards using an amino acid analyzer. Appropriate fractions were pooled, concentrated to a small volume by rotary evaporation at re-

duced pressure, and applied to a column (25 × 2.5 cm I.D.) of Dowex 50 (H^+). The resin was washed with water to elute residual formic acid, and then the dipeptide was eluted with 3 *M* ammonium hydroxide. Evaporation of the latter solution yielded diastereomerically pure dipeptide as judged by amino acid analysis (< 0.1% contaminating diastereomeric dipeptide). The dipeptide was hydrolyzed for 6 h in 250 ml of 6 *M* hydrochloric acid. After evaporation of the solvent, the residue (pH 6–8) was applied to a column (45 × 2.5 cm I.D.) of Dowex 1 × 8 (200–400 mesh, acetate form). The resin was washed with water which elutes the α-amino acid; L-aspartate and chloride are bound quantitatively. Rotary evaporation at reduced pressure yields crystalline β-amino acids of 97–99% enantiomeric purity as judged by either CSP or chiral solvent high-performance liquid chromatography (HPLC) (see below).

β-Amino acids and β-amino acid-containing dipeptides were analyzed for chemical and/or diastereomeric purity using a Durrum Model 500 amino acid analyzer operating with Li^+ citrate buffers according to the manufacturer's recommendations for 'hydrolysis samples'. For all α-alkyl-β-alanines examined, aspartate, *S*-β-amino acyl-L-aspartate, *R*-β-amino acyl-L-aspartate and free β-amino acid eluted as clearly resolved peaks in the order listed.

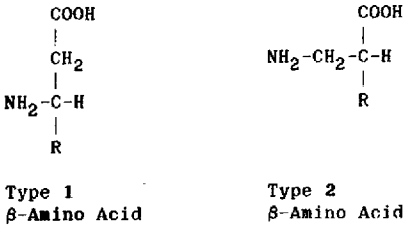
Specific optical rotations of the resolved α-substituted-β-alanines were measured at 1% concentration in water using a Rudolph Autopol III. Rotations, $(\alpha)_D$, for β-aminoisobutyrate, α-ethyl-β-alanine and α-propyl-β-alanine derived from the dipeptide eluting earliest from Dowex 50 were +13.9° (21°C), +6.0° (27°C) and +0.2° (24°C), respectively. β-Aminoisobutyrate, α-ethyl-β-alanine, and α-propyl-β-alanine derived from the second Dowex 50 peak gave specific optical rotations of −13.8° (26°C), −6.5° (24°C) and −0.2° (24°C), respectively. *R*(D)-β-Aminoisobutyrate has been reported previously to have a specific optical rotation of −15.4° at 27°C[3], −15° at 23°C (2.5% in water)[4], and −13° (temperature not specified)[19].

For HPLC analysis on chiral stationary phases the β-amino acids were derivatized as the alkyl esters of the 3,5-dinitrobenzamides before chromatography. The alkyl esters were prepared by allowing a solution of the β-amino acid in the appropriate alcohol to stand for a few hours after saturation with gaseous hydrochloric acid. The esters were then converted to the dinitrobenzamides by the Schotten–Bauman procedure (3,5-dinitrobenzoyl chloride in dichloromethane–dilute sodium hydroxide). The entire derivatization sequence frequently has been carried out on milligram samples of amino acids. Such quantities are more than adequate. Although no attempt has been made to do so, it is clearly possible to determine enantiomeric composition using much smaller samples.

Chromatography on CSPs was performed using an Altex 100A pump, Altex 210 injector, and an Altex Model 165 detector operated at 254 and 280 nm. A Kipp & Zonen BD 41 recorder was used.

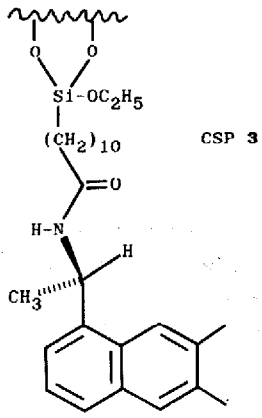
RESULTS AND DISCUSSION

Considered as homologues of the more common α-amino acids, β-amino acids are of two distinct structural types, β-substituted β-alanines (type 1) and α-substituted β-alanines (type 2). As noted, both types occur naturally, and, in the case of β-aminoisobutyrate, both the *R*(D)- and *S*(L)-enantiomers are found in mammalian tissues



Type 1
β-Amino Acid

Type 2
β-Amino Acid



CSP 3

and physiological fluids*. *A priori*, enantiodistinction is expected to be more difficult for type 2 β-amino acids where the amino group is one carbon removed from the chiral center. Both types of β-amino acids will be more difficult to resolve than the corresponding α-amino acid due to the increased separation between the amino and carboxyl groups. Preliminary studies attempting to resolve β-amino acids by reversed-phase ion-pair chromatography with chiral eluents or by ion-exchange chromatography of diastereomeric dipeptide derivatives confirmed this expectation. Thus several type 1 and type 2 β-amino acids were chromatographed on C_{18} HPLC columns using eluents containing Cu^{2+}-L-proline[20] or Cu^{2+}-N,N,dipropyl-L-alanine[21]; both eluent systems were developed on the basis of their ability to resolve the enantiomers of essentially all of the common protein α-amino acids[20,21]. Several type 2 β-amino acids in which R was a C_3 or larger alkyl group are resolved by both systems, whereas *R,S*-α-ethyl-β-alanine is resolved only by the N,N-dipropyl-L-alanine system. *R,S*-α-Aminoisobutyrate (α-methyl-β-alanine) is not resolved by either system (data not shown). None of several type 1 β-amino acids in which R was a C_1–C_4 *n*-alkyl or iso-alkyl group are usefully resolved by the Cu^{2+}-L-proline system. The N,N-dipropyl-L-alanine system was more effective with type 1 β-amino acids and resolved the enantiomers of all of the examples tested. Details on the utility of this system with β-amino acids will be reported elsewhere.

Manning and Moore[22] reported in 1968 that enantiomers of the protein α-amino acids could be resolved as diastereomeric dipeptides on conventional ion-exchange amino acid analyzers. For most amino acids, both L-leucine and L-glutamate

* Type 1 β-amino acids are unambiguously assigned to the L- or D-configurational series on the basis of their structural analogy to α-amino acid enantiomers. Thus compounds belonging to the series illustrated are type 1 L-β-amino acids. Type 2 β-amino acids belonging to the series illustrated could be assigned L-configurations as α-amino acid analogues or could be assigned D-configurations as substituted β-alanine derivatives. In their early studies Kakimoto and Armstrong[3] took the latter approach and refer to *R*-β-aminoisobutyrate as D-(−)-α-methyl-β-alanine. For consistency with the literature we follow their approach and consider type 2 β-amino acids of the configuration illustrated to be D-enantiomers. Although the use of D and L to designate configurations is particularly convenient when comparing properties of enantiomers within a homologous series, it should be noted that *R* and *S* designations are generally and preferably used with type 2 β-amino acid enantiomers.

are effective resolving agents with the resulting L-leucyl-D,L-α-amino acid diastereomeric mixture or the L-glutamyl-D,L-α-amino acid mixture each giving two distinct dipeptide peaks. As noted, Winnacker *et al.*[9] subsequently showed that D,L-α-amino-*n*-butyrate was similarly resolved as the L-glutamyl derivative. In preliminary studies, we found that the enantiomers of several higher homologues of α-amino-*n*-butyrate were also resolved. In contrast, L-glutamyl derivatives of type 2 *R,S*-β-amino acids are not resolved on a Durrum amino acid analyzer; L-leucyl, L-valyl, L-methionyl and L-alanyl derivatives were tested less extensively with no evidence of resolution. Interestingly, reversal of the dipeptide sequence results in diastereomeric peptides which are resolved by the amino acid analyzer. Thus *S*-β-aminoisobutyryl-*S*(L)-aspartate and *R*-β-aminoisobutyryl-*S*(L)-aspartate elute at 52 min and 60 min, respectively, whereas *S*(L)-aspartyl-*R,S*-β-aminoisobutyrate elutes as a single peak at 69 min (aspartate, valine, phenylalanine and ammonia elute at 40, 82, 109, and 135 min, respectively, under these conditions). It is noted that the chiral centers in L-aspartyl-*R,S*-β-aminoisobutyrate are separated by four bonds whereas the chiral centers in all of the resolvable diastereomeric dipeptide pairs (*i.e.*, dipeptides of α-amino acids as well as dipeptides having amino acids in the sequence type 2 β,α or α, type 1 β) are separated by only three bonds. Separation of the chiral centers by four bonds apparently decreases their interaction to the point where the diastereomeric dipeptides bind equally to a non-chiral sulfonated polystyrene resin.

Analytical resolution of type 2 β-amino acids as diastereomeric dipeptides is disadvantaged by the need to protect and activate the β-amino acid under consideration; the possibility of partial racemization during peptide synthesis is difficult to exclude. Preparative resolutions beginning with racemates of type 2 β-amino acids are not subject to this negative consideration, and are facilitated by the large scale on which Dowex 50 ion-exchange chromatography can be accomplished. As shown in Fig. 1, the enantiomers of *R,S*-β-aminoisobutyrate are conveniently separated using L-aspartate as resolving agent. L-aspartate and L-glutamate are particularly useful chiral agents because they counter the high basicity of the β-amino acid amino group and because following dipeptide hydrolysis they are readily separated from the resolved β-amino acid by filtration through Dowex 1. In addition to *R,S*-β-aminoisobutyrate, enantiomers of α-ethyl-β-alanine and α-propyl-β-alanine have been resolved preparatively; in all cases the dipeptide containing the levorotatory β-amino acid eluted second. (−)-β-Aminoisobutyrate is known to be the *R*(D)-isomer[3,4] and it is probable from the consistency of the elution patterns on Dowex 50 and on CSP 3 (see below) that the levorotatory higher homologues are also of the *R*(D)-configuration.

CSPs derived from N-acylated α-arylalkylamines were shown previously to resolve derivatives of α-amino acids and several additional classes of enantiomeric amines. In general, CSPs based on amides of arylalkylamines, such as CSP 3, provide resolution through a combination of π-basicity and hydrogen bond donor and acceptor sites. 3,5-Dinitrobenzoyl derivatives of β-amino acid esters contain sites of complementary functionality capable of undergoing the necessary interactions. In addition, such derivatives are easy to prepare and contain a strong chromophore to facilitate detection. Since the derivatizing agents are achiral and are used in dilute solution, errors due to kinetic fractionation of the analyte enantiomers do not occur. Octyl esters exhibit lower capacity factors (k') than the corresponding butyl or methyl

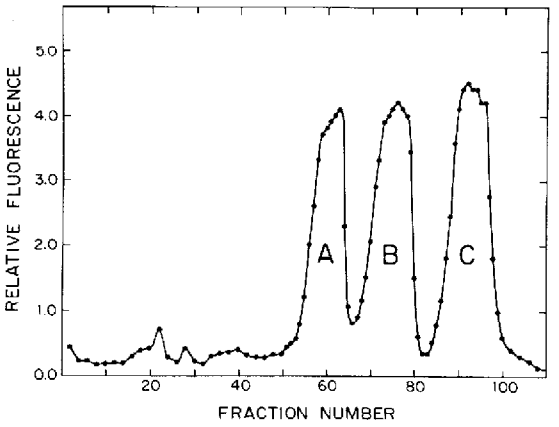


Fig. 1. Preparative resolution of *R,S*-β-aminoisobutyrate-*S*(L)-aspartate diastereomers. A reaction mixture containing the two diastereomers (about 15 mmol) was prepared and chromatographed as described in the Methods section. Fractions of 25 ml were collected at a flow-rate of 2.5 ml min^{-1}. Portions (5 μl) of the fractions were assayed fluorometrically for amino acids or peptides using *o*-phthalaldehyde. Peaks A, B and C correspond to aspartic acid, *S*-β-aminoisobutyryl-L-aspartate and *R*-β-aminoisobutyryl-*S*(L)-aspartate, respectively; free β-aminoisobutyric acid is also present in the crude reaction mixture but elutes much later. The yield of fluorescent product from aspartic acid is *ca.* 5-fold higher than that from the dipeptides; in the experiment shown the yield of dipeptides was about 80%. Dipeptides derived from higher homologues of β-aminoisobutyrate chromatograph with base-line separation between the diastereomers.

esters and afford somewhat greater separability factors for type 1 β-amino acids when CSP 3 is used. For type 2 β-amino acids, separability factors are very similar regardless of the alcohol used for esterification.

Table I contains data pertinent to the separation of the enantiomers of derivatized β-amino acids on CSP 3. Although type 1 β-amino acids can be readily resolved on any of several arylalkylamine-derived CSPs, CSP 3 consistently affords satisfactory resolutions for the enantiomers of both type 1 and type 2 β-amino acids. Reso-

TABLE I

SEPARATION OF THE ENANTIOMERIC *n*-OCTYL ESTER N-3,5-DINITROBENZOYL DERIVATIVES OF β-MINO ACIDS ON CSP 3

Type 1	*R*	α	k_1'	*Type 2*	*R*	α	k_1'
a	CH_3	1.21	28.6	a	CH_3	1.08	24.0
b	C_2H_5	1.43	19.2	b	C_2H_5	1.12	22.0
c	*n*-C_3H_7	1.28	18.9	c	*n*-C_3H_7	1.16	21.3
d	iso-C_3H_7	1.65	16.2	d	iso-C_3H_7	1.19	16.8
e	*n*-C_4H_9	1.27	18.5	e	*n*-C_4H_9	1.16	18.3
f	*n*-C_5H_{11}	1.27	18.3	f	*n*-C_5H_{11}	1.17	16.7
g	CH_3SCH_2	1.20	48.7	g	iso-C_5H_{11}	1.18	16.3
h	$(CH_2)_2COOC_8H_{11}$	1.11	15.9	h	*sec.*-C_5H_{11}	1.19	14.6
						1.20	15.6
i	cyclo-C_6H_{11}	1.32	18.7	i	$CH_2CO_2C_8H_{17}$	1.10	22.5

lution of the derivatized enantiomers of β-aminoisobutyrate is shown in Fig. 2. It is notable that the chromatographic separation of β-aminoisobutyrate enantiomers in amounts adequate for the determination of, for example, specific radioactivities should be readily achieved. The derivatized enantiomers of β-leucine (compound 1d) are very well resolved on CSP 3; LC resolution of a diastereomeric derivative of β-leucine was very recently reported by Aberhart *et al.*[23].

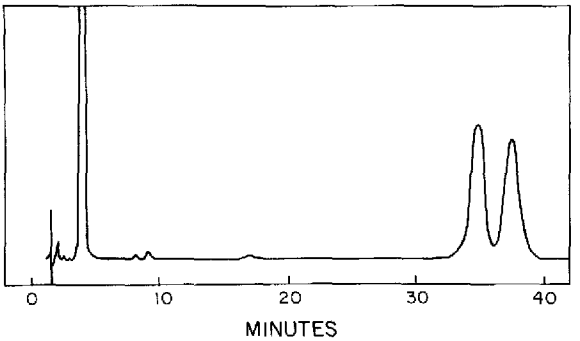


Fig. 2. Separation of N-3,5-dinitrobenzoyl-β-aminoisobutyrate octyl ester enantiomers on CSP 3. Derivatization and chromatography was carried out as described in the Methods section. The peak at 4.0 min corresponds to octyl 3,5-dinitrobenzoate; the peaks at 35 and 37.2 min correspond to the derivatives of *S*(L)- and *R*(D)-β-aminoisobutyrate, respectively.

Elution orders appear to be uniform within each subgroup of β-amino acids. For compounds 1a and 1d, the D-enantiomer is most strongly retained, a finding established by chromatographing samples of known absolute configuration. For compounds 2a–c, it was similarly established that the D(*R*)-enantiomer is most strongly retained (see footnote on p. 348). For the remaining members of each subgroup "tracking of absolute configuration" strongly suggests a uniform elution order, at least when no additional functionality is present in the R group to afford further interactions with the CSP. The mechanistic origin of the observed chiral recognition is under study.

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