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Note

Resolution of underivatized polyfluoro-2,2-dialkylglycine enantiomers by reversed-phase chromatography

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Reversed-phase high-performance liquid chromatography (HPLC) using copper(II)–L-amino acid complexes has been used to separate the optical isomers of underivatized common amino acids[1–3] and 2-methyl amino acids[4]. HPLC resolution of the nearly symmetric amino acid isovaline has not been reported, although it has been resolved enzymatically[5] and by gas chromatography[6]. We show here that introduction of fluorine substituents into isovaline or the related 2-methylalanine leads to increased retention and chiral discrimination in a Cu(II)–L-phenylalanine reversed-phase system.

We have been studying several chiral fluorinated amino acids (Table I) as potential inhibitors of a bacterial decarboxylase[7]. Since the biological properties of most amino acid enzyme inhibitors are configuration dependent, we have sought general methods for separating the stereoisomers of these compounds. On the preparative scale we have successfully used the classical method of stereoselective enzymatic hydrolysis of an N-acyl derivative for resolution of one of these, 2-trifluoromethylalanine[8,9], but for analytical-scale optical purity determinations a chromatographic method is preferable. Our results show that the Cu(II)–L-Phe HPLC system readily resolves these amino acids, enabling precise optical purity measurements to be made. We also include descriptions of two new polyfluoro amino acids, 2-amino-2-trifluoromethylbutanoic acid and 2-pentafluoroethylalanine.

MATERIALS AND METHODS

L-Phenylalanine and 2-methylalanine were obtained from Sigma and racemic isovaline was obtained from Research Plus Labs., Bayonne, NJ, U.S.A. 2-Trifluoromethylalanine was synthesized by literature methods[10]. 2-Fluoromethylalanine and 2-difluoromethylalanine were the generous gifts, respectively, of Dr. M. H. O'Leary of the University of Wisconsin and Dr. J. Lee of Lee's Bio-organic Labs., Marcus Hook, PA, U.S.A. Elemental analysis was performed by Galbraith Labs., Knoxville, TN, U.S.A.

2-Amino-2-trifluoromethylbutanoic acid (ATB) was synthesized by adding 11 g of 1,1,1-trifluoro-2-butanone[11] (0.09 mol) portionwise to a solution of 7.7 g (0.12 mol) of potassium cyanide in 15 ml of water. The mixture was heated to 70°C for 10 min, mixed with 100 ml cold concentrated ammonia, and heated to 100°C for 7 h in

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a Parr bomb. The contents were evaporated to dryness, hydrolyzed overnight in 120 ml of refluxing 6 *M* hydrochloric acid, evaporated to dryness, and extracted with four 200-ml portions of ethanol–diethyl ether (1:1). The filtered extracts were evaporated to dryness, dissolved in concentrated ammonia and eluted with water from a 30 × 2 cm Dowex 50 column (H^+). Fractions positive to ninhydrin (300–600 ml) were combined, dried and recrystallized from isopropanol to give 3.2 g (21%) of white powder, m.p. 243–243.5°C (sealed capillary); IR(KBr)1640, 1150 cm^{-1}; ^1H NMR(^2H_2O) δ 0.91 (3H, t), 2.01 (2H, m). Analysis calculated for $C_5H_8F_3NO_2$: C, 35.10; H, 4.71; N, 8.19; F, 33.31. Found: C, 35.12; H, 4.77; N, 8.18; F, 33.17.

2-Pentafluoroethylalanine (PEA) was synthesized in low yield by essentially the same procedure, in which 6 g of 3,3,4,4,4-pentafluoro-2-butanone[11] was converted into 0.32 g (3%) of white powder, m.p. 201–203°C (sealed capillary); IR(KBr)1660, 1550, 1220, 1140 cm^{-1}; ^1H NMR(^2H_2O) δ 1.1 (s); ^{13}C NMR(^2H_2O) δ 18.0 (q, J = 132 Hz), 63.3 (t, J = 19 Hz), 109–118 (m), 168.9 (s).

HPLC was carried out on a 250 × 4.6 mm Altech C_{18} (10 μm) column; the mobile phase was 2.0 m*M* L-phenylalanine, 1.0 m*M* copper(II) acetate adjusted to pH 4.4 with acetic acid, to which was added acetonitrile to a concentration of either 5% or 10% (v/v). A Beckman Model 110A pump and a Gilson Holochrome variable-wavelength detector (280 nm) were used. Samples (20 μl, usually 1 mg/ml dissolved in eluent) were injected with a Rheodyne valve. Detector output was digitized, stored, and plotted using an IBM-PC microcomputer equipped with a Data Translation DT2805 A/D board and ASYST software; a polynomial function fitted to the baseline was subtracted from each data array prior to plotting.

RESULTS AND DISCUSSION

When chromatographed in the Cu(II)–L-Phe reversed-phase system, all fluoro compounds tested except FMA appeared as two peaks of equal area (Figs. 1 and 2 and Table I); IVA was not resolved. Amino acids with more fluorine atoms or larger alkyl groups produced later-eluting pairs of peaks. These results are similar to Wernicke's finding that the enantiomers of 3,3,3-trifluoroalanine are retained about ten times longer in a Cu(II)–L-Phe reversed-phase system than the enantiomers of alanine itself[3]. Increased acetonitrile in the eluent decreased retention (Table I).

TMA has been resolved in previous work in our laboratory by an enzymatic method[8] and the absolute configuration determined by X-ray diffraction[9]. When TMA samples highly enriched in each isomer were chromatographed separately, the isomer with the *S* configuration eluted first (Fig. 2). In the *S* isomer the larger, more hydrophobic C-2 substituent (trifluoromethyl) occupies the pro-*R* position, which is the same position occupied by alkyl or aryl side chains in the first-eluting D isomers of the common alkyl amino acids[3]. In fact, the hydrophobicities (or steric requirements) of both pro-*R* and pro-*S* amino acid substituents affect retention of the amino acid in this chromatography system. Thus for example, the first-eluting *S*-TMA elutes substantially later than IVA.

Chiral discrimination between polyfluoro amino acid stereoisomers in the Cu(II)–L-Phe reversed-phase system is probably due to formation of diastereomeric mixed complexes, *i.e.* L-Phe–Cu(II)–amino acid, with different formation constants, affinities for the hydrophobic phase and kinetics of complex formation. Complexes

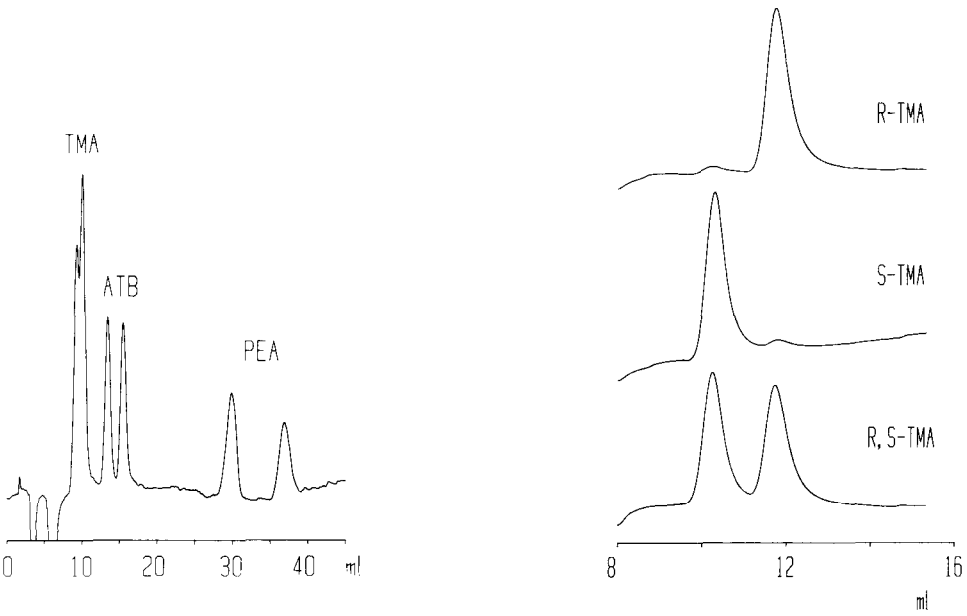

Fig. 1. Chromatogram of a mixture of racemic TMA, ATB and PEA, each 1 mg/ml in the eluent. Eluent: 2 m*M* L-Phe, 1 m*M* copper(II) acetate, pH 4.4, 10% (v/v) acetonitrile. Flow-rate: 0.75 ml/min.

Fig. 2. Chromatograms of racemic TMA (1 mg/ml), *R*-TMA (0.5 mg/ml) and *S*-TMA (0.5 mg/ml). Eluent: 2 m*M* L-Phe, 1 m*M* copper(II) acetate, pH 4.4, 5% (v/v) acetonitrile. Flow-rate: 0.5 ml/min.

containing polyfluoro amino acids would be expected to have a strong attraction for the stationary phase due to the extreme hydrophobicity of polyfluoroalkyl groups[12]. An alternative mechanism recently proposed for the case of Cu(II)–di-*n*-propyl-L-alanine reversed-phase chromatography[13], is that in some cases chiral discrimination could result from differing tendencies of amino acid stereoisomers to add to the axial position of a stable Cu(II)–bis(di-*n*-propylalanine) complex bound to the stationary phase. A mechanism of this type is less likely in the present case since these polyfluoro

TABLE I

STRUCTURES, CAPACITY FACTORS (k, k'), AND ENANTIOSELECTIVITIES ($\alpha = k/k'$) OF 2,2-DIALKYLGLYCINES ($R_1R_2CNH_2COOH$) IN Cu(II)–L-PHENYLALANINE REVERSED-PHASE CHROMATOGRAPHY

Compound	*Abbreviation*	R_1	R_2	*5% Acetonitrile*		*10% Acetonitrile*	
				k,k'	α	k,k'	α
Isovaline	IVA	CH_3	CH_2CH_3	1.0	–	nd	–
3-Fluoromethylalanine	FMA	CH_2F	CH_3	1.0	–	nd	–
3-Difluoromethylalanine	DMA	CHF_2	CH_3	1.6, 1.9	1.2	nd	–
3-Trifluoromethylalanine	TMA	CF_3	CH_3	3.4, 3.9	1.1	3.1, 3.4	1.1
2-Amino-2-trifluoromethylbutanoic acid	ATB	CF_3	CH_2CH_3	5.4, 6.6	1.2	4.6, 5.4	1.2
3-Pentafluoroethylalanine	PEA	CF_2CF_3	CH_3	11.4, 16.5	1.4	10.2, 12.6	1.2

amino acids are strongly retained in spite of having a hindered steric environment at C-2 which would discourage axial attachment. Also, ATB and PEA have nearly identical pK_2 values and steric environments at C-2 and yet PEA is retained much more strongly than ATB. This result suggests that the PEA isomers are retained more strongly due to the greater combined hydrophobicities of CH_3 and CF_2CF_3 compared to CH_2CH_3 and CF_3.

Detection sensitivity for the polyfluoro amino acids in the Cu(II)–L-Phe system was similar to that of the common amino acids[3]. In fact, detection of TMA, PEA and ATB was possible only if they were complexed to the UV-absorbing Cu, since these compounds are extremely difficult to derivatize with standard pre- or post-column chemistry. This low chemical reactivity is due to a combination of steric hindrance at C-2 and low basicity (pK_2 values of TMA, PEA and ATB as determined by titration are about 6 compared to 9.5 for most non-fluorinated amino acids).

Racemic samples produced two chromatographic peaks with areas equal within experimental error (Fig. 2), thus the optical purity of an amino acid sample could be easily estimated from the relative areas of the two peaks. Fig. 2 shows chromatograms of samples of the two stereoisomers of TMA obtained from an enzymatic resolution experiment[8] along with racemic TMA. Peak integration showed that both of the enriched samples contain 1% of the opposite stereoisomer, which were likely produced by non-stereoselective enzymatic reaction[8]. Such levels of stereochemical contaminants may be significant in enzyme inhibition studies if the minor component is biologically active.

Negative peaks with k values of 1.5 and 2.5 and amplitudes proportional to the amount of amino acid injected were observed in chromatograms of the more strongly retained amino acids such as DMA, TMA, ATB and PEA. These peaks probably are due to transfer of Cu from eluent to Cu–amino acid complexes bound to the stationary phase. They did not interfere with optical purity measurements.

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