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Note

Chiral thin-layer chromatographic separation of phenylalanine and tyrosine derivatives

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Ligand-exchange chromatography is extremely useful for the separation of enantiomers of amino acids, peptides and other optically active substances[1]. Recently the commercially available thin-layer chromatography (TLC) plates of Macherey-Nagel, Chiralplate, have become routine equipment for laboratories synthesizing amino acids and peptides[2].

Our interest has focused on the asymmetric synthesis of phenylalanine and tyrosine *derivatives* and *analogues*, with special attention to analogues which can conformationally fix or bias such residues in biologically active peptides. Conformational constraint or topographic bias residues have been shown to provide an important new approach in the design of selectively acting hormone analogues[3–5]. The synthesis of appropriately modified phenylalanine and tyrosine derivatives and their characterization is obviously the first step in this task. We have employed chiral TLC plates in the evaluation of reaction products and in their stereochemical characterization, and we would like to report here the chromatographic behavior of these sterically constrained or biased phenylalanine and tyrosine derivatives.

EXPERIMENTAL

Materials and reagents

The amino acids phenylalanine (I, Fig. 1), tyrosine (IX), and 3′,5′-dibromotyrosine (XIII) were purchased from Sigma. The other unusual aromatic amino acids used in these studies were synthesized in our laboratories at the Department of Chemistry, University of Arizona, Tucson, and the Department of Peptide Chemistry, Institute of Organic Chemistry and Biochemistry, Prague. Their structures were determined by a variety of physico-chemical methods, including high-field NMR, mass spectrometry, elemental analysis and optical rotation. The new synthetic procedures developed

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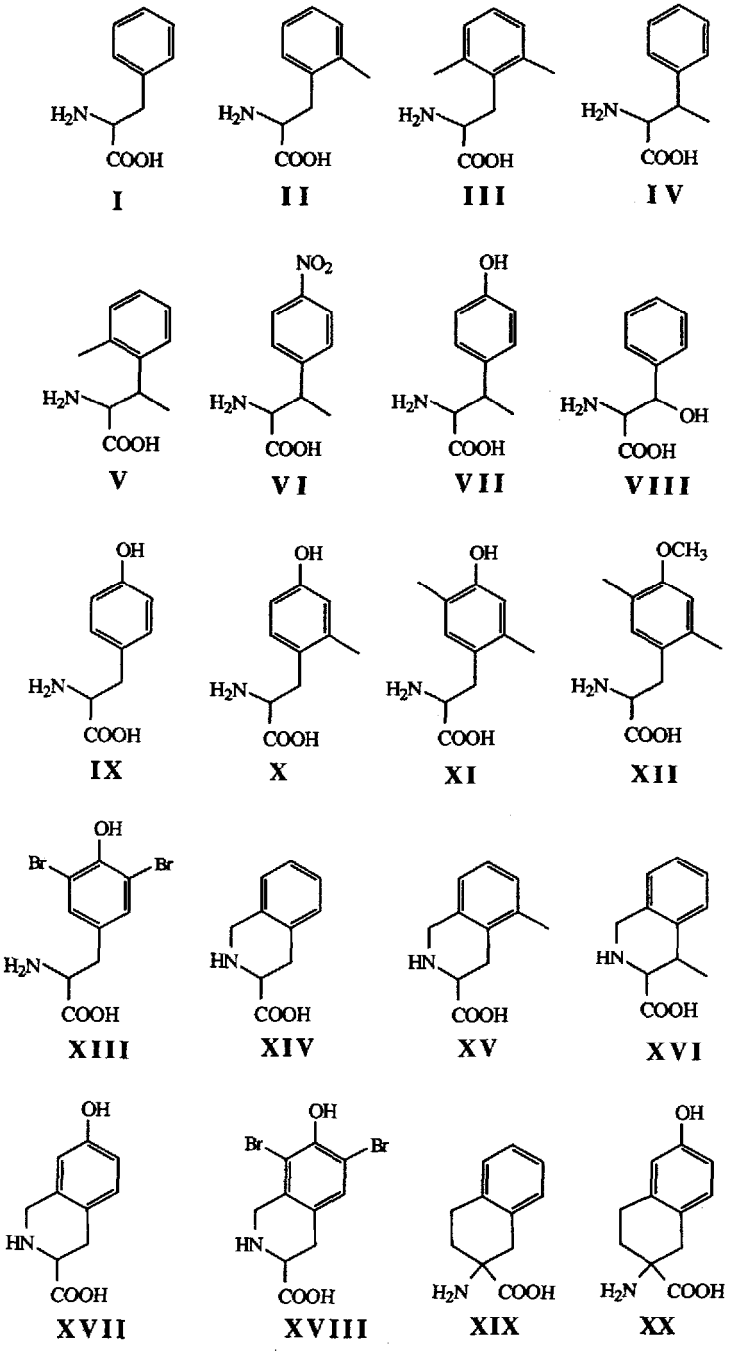

Fig. 1. Structures of the amino acid derivatives examined.

for the synthesis of many other new amino acids will be published elsewhere, as will the peptide analogues described in the Results and Discussion section. The following amino acids were prepared for this study (see superscript references for synthetic methodology, the Roman numbers used refer to Fig. 1): 2′-methylphenylalanine (II)[6]; 2′,6′-dimethylphenylalanine (III)[6]; β-methylphenylalanine (IV)[7]; 2′-methyl-β-methylphenylalanine (V)[8]; β-methyl-*p*-nitrophenylalanine (VI)[9]; β-methyltyrosine (VII)[10]; β-hydroxyphenylalanine (VIII)[11]; 2′-methyltyrosine (X)[12]; 2′,5′-dimethyltyrosine (XI)[12]; 2′,5′-dimethyl-4-methoxyphenylalanine (XII)[12]; tetrahydroisoquinoline carboxylic acid (XIV)[13]; 2′-methyltetrahydroisoquinolinecarboxylic acid (XV)[13,14]; β-methyltetrahydroisoquinolinecaboxylic acid (XVI)[13,14]; 4′-hydroxytetrahyhydroisoquinolinecarboxylic acid (XVII)[14]; 3′,5′-dibromo-4′-hydroxytetrahydroisoquinolinecarboxylic acid (XVIII)[14]; 2-aminotetralincarboxylic acid (XIX)[15]; 2-amino-6-hydroxytetralin carboxylic acid (XX)[15].

L-Amino acid oxidase (*Crotalus adamateus* crude venom) was purchased from Sigma. Chiral TLC plates were from Macherey-Nagel, trifluoroacetic acid was purchased from Chemical Dynamics Corporation, acetonitrile was obtained from American Burdick and Jackson (American Scientific Products).

The peptides Tyr-D-Pen-Gly-X-D-Pen (Pen = penicillamine or β,β-dimethylcysteine, X = β-methylphenylalanine, *p*-NO_2-β-methylphenylalanine) and oxytocin with β-methylphenylalanine in position 2 were synthesized in our laboratory by solid-phase peptide synthesis methods we have developed[16,17]. HPLC experiments were performed on a Perkin-Elmer 3D instrument equipped with an LP 90 UV detector and an LC1-100 Laboratory Computing Integrator. The HPLC columns used were Vydac 218TP1004 (25 cm × 0.46 cm) columns. Fourier transform ^{1}H NMR spectra were run on a Bruker AM 250 spectrometer.

The R_F values of the individual amino acids on chiral plates were identical to those previously reported by Günther[1]. The solvent system used was acetonitrile–methanol–water (4:1:1).

Enzymatic digestion of aromatic amino acids[18]

Amino acids I–XII (0.3 mg) were dissolved in Tris buffer (0.1 *M*, pH 7.2), the pH was adjusted by the addition of 0.2 *M* sodium hydroxide to 7.2 and 0.3 mg of L-amino acid oxidase (*Crotalus adamanteus* crude venom, Sigma) was added. The test tube was filled with oxygen, tightly closed and incubated for 24 h at 37°C. After 24 h new enzyme was added and the incubation was continued for another 24 h. The reaction mixture was spotted directly on a Chiralplate without any dilution or concentration. Development was performed in the system specified in Table I.

Determination of configuration of aromatic amino acid in peptide (general method)

An oxytocin analogue containing an aromatic amino acid in position 2 (0.2 mg) was hydrolysed in 6 *M* hydrochloric acid at 100°C for 20 h. The solution was evaporated *in vacuo*, and the residue was dissolved in 50 μl of water and injected into a C_{18} Vydac column (25 × 0.46 cm) mentioned above, which had been equilibrated with 0.1% trifluoroacetic acid in water. Gradient elution (0–3 min: 0% acetonitrile, 3–23 min: to 50% acetonitrile) afforded a strong peak at the dead volume of the column (containing Asp, Glu, Gly, Pro, Leu, Ile and Cys, as determined by TLC comparison with standards), and a second major peak eluted at 12–15 min [depending on the

TABLE I

R_F DATA FOR THE PHENYLALANINE AND TYROSINE DERIVATIVES

Chiralplate, acetonitrile–methanol–water (4:1:1), detection by ninhydrin.

Compound	*Configuration*	$R_{F(L)}$	$R_{F(D)}$	$R_{F(L)}/R_{F(D)}$
I		0.56	0.42	1.33
II		0.54	0.43	1.26
III		0.52	0.38	1.37
IV	*S,S+R,R (erythro)*	0.56	0.36	1.56
	R,S+S,R (threo)	0.55	0.47	1.17
V	*S,S+R,R (erythro)*	0.57	0.33	1.73
	R,S+S,R (threo)	0.55	0.48	1.15
VI	*S,S+R,R (erythro)*	0.62	0.43	1.44
	R,S+S,R (threo)	0.60	0.52	1.15
VII	*S,S+R,R (erythro)*	0.67	0.52	1.29
	R,S+S,R (threo)	0.67	0.55	1.22
VIII	*S,S+R,R (erythro))*	0.63	0.49	1.29
IX		0.63	0.51	1.24
X		0.62	0.54	1.15
XI		0.67	0.56	1.20
XII		0.57	0.45	1.27
XIII		0.62	–	–
XIV		0.54	0.50	1.08
XV		0.51	0.49	1.04
XVI	*S,S+R,R (erythro)*	0.51	0.45	1.13
XVII		0.60	–	–
XVIII		0.58	–	–
XIX		0.55	0.47	1.17
XX		0.64	0.59	1.08

structure of the aromatic amino acid(s)]. All peaks were collected, lyophilized, and the residue was dissolved in water (30 μl) and spotted onto a Chiralplate. Comparison with standards revealed the configuration of the aromatic amino acids.

RESULTS AND DISCUSSION

The structures of the amino acid derivatives examined are given in Fig. 1. Table I shows the R_F values of all available optical isomers. We were able to determine the absolute configuration of these derivatives containing the primary amino group (with the exception of XIX and XX) by enzymatic digestion using L-amino acid oxidase[18]. The configuration of XIX was determined with the use of carboxypeptidase[7]. The configuration of compounds containing a secondary amino group was secured by synthesis starting from an optically pure amino acid. Due to the substitution of the side chain moiety of the phenylalanine analogues, the enzymatic digestion was significantly slower in comparison to Phe itself. However, complete digestion of the L-isomer was obtained using a larger excess of enzyme and longer reaction times. Results obtained with Chiralplate (enzymatic conversions of L-enantiomer) were further verified by quantitative amino acid analysis[18].

Only the D-amino acids are then detectable by amino acid analysis. It is in-

teresting to compare the amino acids containing a tetrahydroisoquinoline ring structure with the corresponding amino acids in which the rotation of the aromatic side chain group is not so constrained. The more constrained compounds have displayed much poorer separation of enantiomers. Comparison of β-substituted amino acids shows that the *erythro* compounds have very different R_F values in comparison with compounds of *threo* configuration. Compounds with *threo* configuration of substituents can be compared to the β-unsubstituted compounds. The influence of aromatic ring substitution on separation can be observed in compounds I–III, IV–VII, IX–XII, XIV and XV.

The use of chiral plates was extremely useful in the cases of amino acid derivatives in which a mixture of D-, L-*erythro* or D-, L-*threo* stereoisomers was obtained during the synthesis. The combination of this technique with NMR spectroscopy and enzymatic digestion has enabled us to determine the stereochemistry of all chiral carbons[7,15,19].

We have also used chiral TLC separation for the determination of amino acid configuration of synthetic peptide analogues which were prepared starting from the racemic aromatic amino acid. After separation of the diastereoisomeric peptides on a reversed-phase HPLC column, we hydrolyzed the sample of each isomer and either analysed it directly on a Chiralplate (in cases when no amino acids interfered with the separation), or applied the hydrolysate to a reversed-phase HPLC column and analysed on TLC only fractions containing the aromatic amino acids. We can recommend this method for determination of the configuration of aromatic amino acids in synthetic peptides including oxytocin, enkephalin and cholecystokinin-8 analogues.

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