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Resolution of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate derivatives of α-methyl amino acid enantiomers by high-performance liquid chromatography[a]

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ABSTRACT

Diastereomers formed by precolumn derivatization of D,L-α-methyl amino acids with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate (GITC) were separated by high-performance liquid chromatography using a conventional C_{18} reversed-phase column. This method completely resolved all the α-methyl amino acids tested except D,L-4-chloro-α-methylphenylalanines which were less well separated. The D-enantiomer was eluted earlier than the L-enantiomer, which is opposite to the order of elution observed with GITC derivative of unmethylated amino acids.

INTRODUCTION

α-Methyl amino acids have been used extensively in biochemistry and drug development in recent years. They have been used as specific inhibitors of the enzymes which act on their α-amino acid counterparts [1–4]. α-Methyl amino acids have been incorporated into peptides for conformational studies because of the rigidity they provide to the peptide backbone and their tendency to promote α-helix or β-turn formation [5–10]. The use of α-methyl amino acids has been explored in peptide drugs, the so-called peptoid concept [11,12].

Even though several asymmetric synthetic methods have been elaborated for the synthesis of α-methyl amino acids [13-16], usually these do not provide an absolutely pure enantiomer but enantiomerically enriched mixtures [17–19]. Therefore it is necessary to know the enantiomeric excess (or the optical purity) of the mixture

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obtained. Furthermore, it is always desirable to know the absolute configuration of the enatiomer even in an enantiomerically enriched mixture. Most of this information is now obtained by one of the following physical measurements. ^{1}H NMR has been used either in the presence of chiral ligands [20–22] or after derivatization with (*S*)-2-chloropropionyl chloride [23]. ^{19}F NMR has been also used after derivatization with Mosher's acid (see refs. 24 and 25). One disadvantage of the NMR method is that usually it requires large amounts of samples because of its low sensitivity. Column chromatography [26,27] and thin-layer chromatography [27] have been used to determine enantiomeric excess of the enriched mixtures using a chiral stationary phase. Diastereomeric isoindole derivatives of α-methyl glutamic acid, made from *o*-phthalaldehyde (OPA) and N-acetyl-L-cysteine (AcCys), have been separated by reversed-phase high-performance liquid chromatography (HPLC) [28].

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosylisothiocyanate (GITC) was introduced by Kinoshita and co-workers [29,30] as a chiral reagent for the derivatization of amino acid enantiomers followed by reversed-phase HPLC separation of the resulting diastereomers (Fig. 1). It has been shown to be very useful in the resolution and identification of a variety of both common and unusual amino acid enantiomers [31–34]. In conjunction with our work on synthetic peptides containing α-methyl amino acids, we explored its use in the resolution of α-methyl amino acid enantiomers. The results indicate that this method works well for the analytical resolution of all the α-methyl amino acid enantiomers tested. The elution order from the C_{18} column is exactly correlated with the absolute configuration of the α-methyl amino acids and is opposite to that of GITC derivatives of the common amino acids [30]. Using molecular modeling we suggest an explanation of this phenomenon.

EXPERIMENTAL

Materials

The optically pure D- and L-α-methyl amino acids were obtained as follows. α-Methyl-4-chlorophenylalanine was synthesized [35] and resolved [36] utilizing chy-

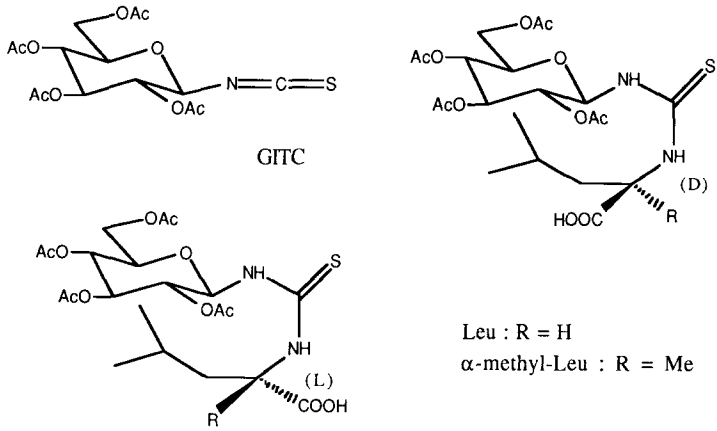

Fig. 1. Structure of GITC, diastereomeric GITC derivatives of D,L-leucine and D,L-α-methylleucine. Ac = Acetyl; Me = methyl.

motrypsin. α-Methylserine was not completely resolved but enriched by the action of chymotrypsin on O-benzyl-DL-α-methylserine [37]. α-Methyltryptophan, α-methyltyrosine, α-methyl-4-fluorophenylalanine were all obtained by the literature procedure [36]. α-Methylleucine was a gift from Dr. Jean Rivier of the Salk Institute. α-Methylornithine was synthesized and resolved by the procedure of *βey et al.* [38]. Optically pure α-methylarginine was prepared starting with optically pure α-methylornithine in our laboratory [37].

All the solvents used both for the derivatization and HPLC elution are HPLC grade. GITC was prepared according to the literature procedure [29] but is also available from Polysciences (Warrington, PA, U.S.A.). The 10 m*M* phosphate buffer was prepared from potassium phosphate (monobasic) and the pH was adjusted to 2.8 by addition of perchloric acid (70%).

Precolumn derivatization

Some modification of the original protocol was made in order to compensate for the low reactivity of the α-amino group of α-methyl amino acids [23,39]. A 5-mg amount of the α-methyl amino acid was dissolved in 5 ml of 50% (v/v) aqueous acetonitrile containing 0.4% (w/v) triethylamine. A 25-μl aliquot of this stock solution was mixed with 50 μl of a solution of 0.2% (w/v) GITC in acetonitrile. This reaction mixture was stirred at room temperature for 1 h and 4–6 μl was injected depending on the chemical purity of the synthesized α-methyl amino acids. For the resolution of α-methyl amino acids with an aromatic side chain, 10 μl of 0.25% (w/v) monoethanolamine in acetonitrile was added and the mixture was stirred for another 10 min after the 1 h reaction time.

Chromatographic conditions

Analytical HPLC analysis was performed using a Ranin Microsorb C_{18} reversed-phase column (5 μm, 250 × 4.6 mm I.D.), with a Varian-5000 liquid chromatography system, a Kipp & Zonen BD40 recorder, and a Hewlett-Packard integrator. The compounds, about 1 μg, were eluted with solvents: (A) 10 m*M* phosphate buffer (pH 2.8) and (B) methanol according to the gradient program shown and were detected by their absorbance at 250 nm, sensitivity set at 0.01 a.u.f.s.

RESULTS AND DISCUSSION

By using a longer derivatization time and higher reactant concentration than the original protocol, all the α-methyl amino acids tested react cleanly with GITC under alkaline conditions despite the steric hindrance around the α-amino group. Data obtained clearly indicate that GITC is a suitable chiral reagent for the derivatization of α-methyl amino acids. Analytical HPLC using a conventional C_{18} reversed-phase column can be used effectively to resolve the diastereomers formed from the derivatization and the elution order is well-correlated with the configuration of the α-methyl amino acids from which information on the enantiomeric excess and the configuration of the enantiomer can be extracted (Figs. 2 and 3). All the results obtained on the α-methyl amino acids tested are summarized in Table I: t_0 refers to the retention time of the unretained material, t_R, k', α, R_s refer to the retention time, capacity ratio, separation factor and resolution respectively for a pair of diastereomers.

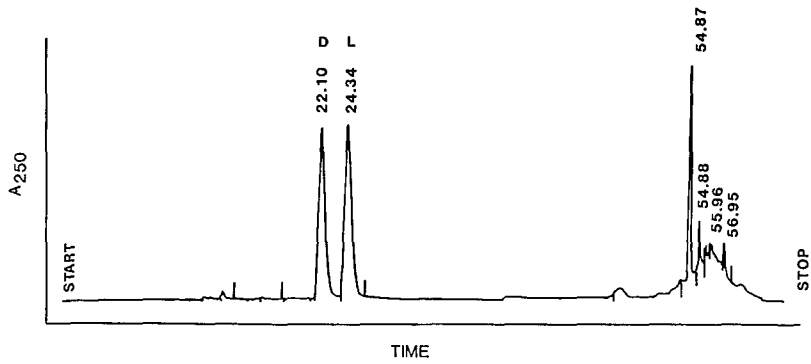

Fig. 2. Separation of diasteromeric GITC derivatives of DL-α-methyltryptophan. Gradient program: 0–10 min, 30–50%B; 10–40 min, 50%B constant; 40–50 min, 50–100%B; 50–55 min, 100–30%B. Time in min.

However, the elution order in all cases tested except for α-methylarginine is opposite that of GITC-derivatized α-amino acids. Thus GITC-L-amino acids except for histidine and arginine [40] are eluted before the D isomers, but GITC-α-methl-L-amino acids are eluted later than the D-isomers under the same conditions of chromatography. In order to understand the reversed order of elution between the common α-amino acids and α-methyl amino acids, a molecular modeling experiment was run on GITC leucine and α-methylleucine using a Silicon Graphics Workstation with QUANTA and energy minimization by CHARMm. It was shown that the GITC-amino acid derivatives have a turn at the thiourea region with the sulfur atom point-

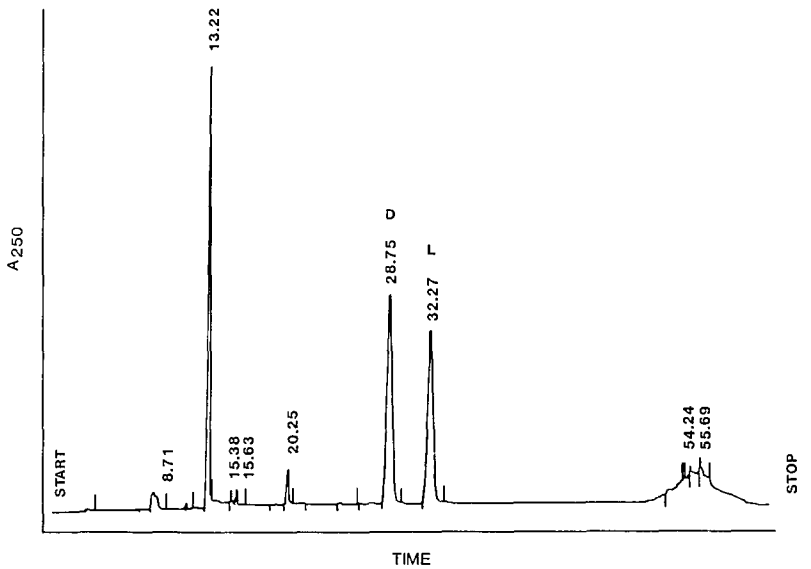

Fig. 3. Separation of diasteromeric GITC derivatives of DL-α-methylarginine. Gradient program: 0–10 min, 30–38%B; 10–45 min, 38%B constant; 45–50 min, 38–100%B; 50–55 min, 100–30%B.

TABLE I

SEPARATION OF GITC DERIVATIVES OF α-METHYL AMINO ACIDS

Gradient programs:

(A) 0–10 min, 30–53%B; 10–45 min, 53%B constant; 45–50 min, 53–100%B; 50–55 min, 100–30%B.
(B) 0–10 min, 30–57%B; 10–50 min, 57%B constant; 50–55 min, 57–100%B; 55–60 min, 100–30%B.
(C) 0–10 min, 30–40%B; 10–50 min, 40%B constant; 50–55 min, 40–100%B; 55–60 min, 100–30%B.
(D) See Fig. 2.
(E) 0 10 min, 20–30%B; 10–50 min, 30%B constant; 50–55 min, 30–100%B; 55–60 min, 100–20%B.
(F) 0–10 min, 30–46%B; 10–45 min, 46%B constant; 45–50 min, 46–100%B; 50–55 min, 100–30%B.
(G) See Fig. 3.
(H) 0–10 min, 30–48%B; 10–45 min, 48%B constant; 45–50 min, 48–100%B; 50–55 min, 100–30%B.

DL-α-Methyl amino acids	t_0 (min)	t_R(min)		k'		α	R_s	Elution gradient
		D	L	D	L			
α-Methyl--4-fluoro-Phe	2.98	33.26	35.16	10.16	10.80	1.06	1.24	A
α-Methyl-4-Cl-Phe	3.00	30.02	31.62	9.01	9.54	1.06	0.63	B
α-Methyl-Tyr	3.44	45.43	46.66	12.21	12.56	1.03	0.96	C
α-Methyl-Trp	3.03	28.75	32.27	8.49	9.65	1.14	3.49	D
α-Methyl-Ser	3.02	26.15	31.27	7.66	9.35	1.22	4.00	E
α-Methyl-Orn	3.29	33.90	44.52	9.30	12.53	1.35	3.82	F
α-Methyl-Arg	3.10	22.10	24.34	6.13	6.85	1.12	1.98	G
α-Methyl-Leu	3.00	29.35	35.94	8.78	10.98	1.25	5.81	H

ing outward. The model shows that the molecule is very rigid and crowded, which is consistent with the view of Kinoshita and co-workers [29,30] that conformational rigidity provides the basis of separation. The GITC-(L)-leucine and GITC-(D)-α-methylleucine are both crescent-shaped and present uneven surfaces whereas the later eluting diastereomers, GITC-(D)-leucine and GITC-(L)-α-methylleucine, are flat, presenting two smooth hydrophobic surfaces.

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