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STUDY OF THE CHROMATOGRAPHIC BEHAVIOUR AND RESOLUTION OF α-AMINO ACID ENANTIOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY UTILIZING PRE-COLUMN DERIVATIZATION WITH *o*-PHTHALDIALDEHYDE AND NEW CHIRAL THIOLS

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SUMMARY

The synthesis and chromatographic behaviour of a series of novel, optically pure, chiral thiols in the pre-column derivatization of various amino compounds with *o*-phthaldialdehyde (OPA) are described. The resulting OPA–amino–thiol adducts show advantages over the currently used thiols of reduced K^*, enhanced resolution and better fluorescent properties.

INTRODUCTION

Chiral amino compounds, in particular α-amino acids used in enzymological, pharmacological and neurological studies, are frequently required in their enantiomerically pure forms, as the enantiomers can possess different activities[1–6]. It is therefore of great importance to be able to assess quantitatively the enantiomeric purity of the amino compound used. Recently, there has been considerable interest in the enantioselective high-performance liquid chromatographic (HPLC) determination of amino acids and related amino enantiomers, in particular the detection and quantification of minor amounts of one enantiomer in the presence of the other. This has been successfully achieved by pre-column derivatization with *o*-phthaldialdehyde (OPA) and chiral thiols to yield diastereoisomeric isoindole derivatives which are separable by reversed-phase HPLC, and can be detected using fluorimetry[5–14]. This method gives a higher sensitivity than is attainable using direct separation on a chiral bonded stationary phase or chiral additives combined with a non-chiral stationary phase. The chiral thiols employed, to date, have been N-acetyl-L-cysteine (NAC), N-acetyl-D-penicillamine (NAP) and N-*tert*-butyloxycarbonyl-L-cysteine (BocC); the first two are commercially available in an enantiomerically pure form and the third can be synthesized simply without racemization[9]. Despite the success of the three chiral thiols, they all possess certain drawbacks: NAC fails to resolve many amino compounds[9,10,13,14], NAP derivatives possess low fluorescence intensities compared with BocC and NAC derivatives[13,14] and the BocC derivatives, especially with

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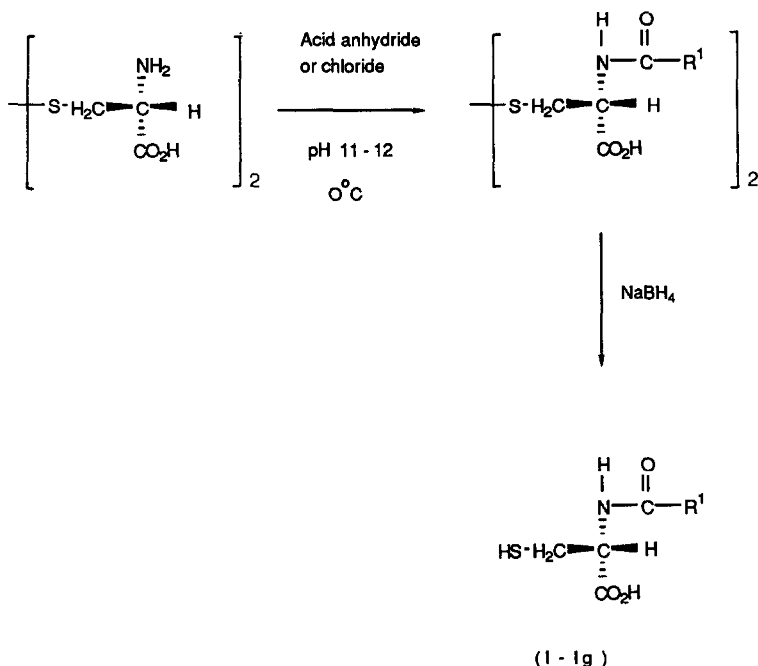
lipophilic amino compounds, can form highly lipophilic diastereoisomers which have unacceptably long retention times[14].

It seemed that a range of new chiral thiols were therefore needed that would be less lipophilic than BocC but still retain good fluorescent and resolving powers. Since the OPA–amino–thiol reaction can occur with various thiol substrates and the phys-



compound	R^1	
I	CH_3	N-Acetyl-L-cysteine **(NAC)**
Ia	CH_2CH_3	N-Propanoyl-L-cysteine **(NPC)**
Ib	$CH_2CH_2CH_3$	N-Butanoyl-L-cysteine **(NBC)**
Ic	$CH(CH_3)_2$	N-Isobutanoyl-L-cysteine **(NIBC)**
Id	$CH_2CH_2CH_2CH_3$	N-Valeroyl-L-cysteine **(NVC)**
Ie	$CH_2CH(CH_3)_2$	N-Isovaleroyl-L-cysteine **(NIVC)**
If	$C(CH_3)_3$	N-Trimethylacetyl-L-cysteine **(NTMAC)**
Ig	$OC(CH_3)_3$	N-Tert.-butoxycarbonyl-L-cysteine **(BocC)**

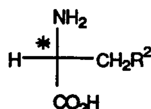
Fig. 1. Synthetic route to the chiral thiols (**Ia–g**).

ico-chemical properties of the thiol affect the chromatographic behaviour of the resulting isoindole[15], it was decided to evaluate the synthesis of a range of enantiomerically pure N-acyl-L-cysteines [based on NAC but possessing increased lipophilicity (Fig. 1, **Ia–f**)] and to assess their chromatographic properties against a variety of amino compounds with different structures (Fig. 2).

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical-reagent or HPLC grade. Ultrapure water was obtained by means of a Milli-Q-system (Millipore). OPA, sodium



Compounds	R^2	
IIa	$-CH_2CO_2H$	Glutamic acid
IIb	$-OP(=O)(OH)O(CH_2)_2NHC(=NH)NH_2$	Lombricine
IIc-e	$-(CH_2)_nP(=O)(OH)OH$	

IIc n = 1 2-Amino-4-phosphonobutanoic acid (APB)

IId n = 2 2-Amino-5-phosphonovaleric acid (APV)

II e n = 4 2-Amino-7-phosphonoheptanoic acid (APV)

Fig. 2. Structure of amino acids (the asterisk denotes the chiral centre).

borohydride, propanoic, butanoic, isobutanoic, valeric and trimethylacetic anhydrides, L-, D- and DL-cystine and L-, D- and DL-glutamic acid were purchased from Sigma, (Dorset, U.K.), isovaleric chloride and di-*tert*-butyl-dicarbonate from Aldrich (Dorset, U.K.), N-acetyl-L-cysteine from Fluka (Buchs, Switzerland) and DL-2-amino-4-phosphonobutanoic acid (APB), DL-2-amino-5-phosphonovaleric acid (APV) and DL-2-amino-7-phosphonoheptanoic acid (APH) from Cambridge Biochemical Research (Cambridge, U.K.). Synthetic DL-lombricine (LOMB) was prepared according to the method of Euerby *et al.*[16].

Chromatographic system

HPLC was performed using a Gilson gradient system (Anachem, Luton, U.K.), consisting of two Model 301 single-piston pumps (5-ml heads), a Rheodyne 7125 loop injector (20 μl), a Model 801 pressure module and a Model 121 fluorescence detector fitted with OPA filters (excitation at 344 nm and emission at 443 nm). The gradient was controlled by an Apple IIe computer using Gilson gradient manager software. Chromatograms were recorded on an LKB 2210 single-channel recorder at a sensitivity of 10 mV, a chart speed of 2 mm/min (except for BocC–OPA glutamic acid and cysteine derivatives, where 5 mm/min was used) and a fluorescence sensitivity of 0.5 range units. A Spherisorb ODS II EXCEL (5 μm) column (25 cm × 4.6 mm I.D.) was purchased from Hichrom (Reading, U.K.) and fitted with a guard column (5 cm × 2 mm I.D.) packed with CO-PELL ODS sorbent (particle size 40 μm) (Hichrom).

Preparation of standard amino acid derivatives

Stock solutions of the DL-racemates were prepared in water at a concentration of 2.5–5.0 μmol/ml for the 2-amino-ω-phosphonoalkanoic acids, 60 μmol/ml for lombricine and 7 μmol/ml for glutamic acid, and were stable for at least 1 month of continual use if stored at -14°C. Standard mixtures were prepared daily by mixing the appropriate stock solutions, followed by dilution with water to yield a final concentration of 150–300 nmol/ml for 2-amino-ω-phosphonalkanoic acids, 150 nmol/ml for lombricine and 200 nmol/ml for glutamic acid.

Mobile phases

Solvents A and B were prepared freshly every other day, filtered through a 0.22-μm membrane filter and degassed by continuous purging with helium. Solvents A and B were 50 m*M* sodium acetate (pH 7.1, adjusted with dilute acetic acid) and methanol, respectively. The flow-rate was 1 ml/min and the column pressure was re-equilibrated to the appropriate level at the beginning of each run, but this varied according to the initial percentage of solvent B, which is shown in Table I.

Pre-column derivatization procedure

The derivatizing agents were prepared freshly every other day by dissolving 10 mg each of OPA and the chiral thiol in 1 ml of HPLC-grade methanol (in order to preserve the optical purity of the chiral thiols, the alkaline borate buffer was added immediately prior to derivatization). These reagents were stored at 4°C in the dark until use. A 20 μl aliquot of the amino acid solution was mixed with the derivatizing OPA–thiol reagent (40 μl) and borate buffer (60 μl) (pH 8.4, adjusted with 2 *M* sodium hydroxide), and incubated for 5 min at ambient temperature, in the dark, before immediate injection onto the column.

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR THE ANALYSIS OF α-AMINO ACIDS

Amino acid	*Initial pressure (p.s.i.)*	*Gradient*		
		Duration (min)	*From (% solvent A:B)*	*To (% solvent A:B)*
(A) Glutamic acid	2000	0–55	91.5:9.5	91.5:9.5
		55–60	91.5:9.5	40.0:60.0
		60–65	40.0:60.0	40.0:60.0
		65–70	40.0:60.0	91.5:9.5
		70–75	91.5:9.5	91.5:9.5
(B) 2-Amino-4-phosphonobutanoic acid	1900	0–50	95:5	95:5
		50–55	95:5	40:60
		55–60	40:60	40:60
		60–65	40:60	95:5
		65–70	95:5	95:5
(C) 2-Amino-5-phosphonovaleric acid	2200	0–45	88:12	88:12
		45–50	88:12	40:60
		50–55	40:60	40:60
		55–60	40:60	88:12
		60–65	88:12	88:12
(D) 2-Amino-7-phosphonoheptanoic acid	2400	0–110	84:16	84:16
		110–115	84:16	40:60
		115–120	40:60	40:60
		120–125	40:60	84:16
		125–130	84:16	84:16
(E) Lombricine	1800	0–30	100:0	86:14
		30–65	86:14	86:14
		65–70	86:14	40:60
		70–75	40:60	40:60
		75–80	40:60	100:0
		80–85	100:0	100:0

*Preparation of N-acyl-*L-cysteines: general method

To a stirred suspension of L-cystine (1.2 g, 5 mmol) in water (5 ml) at 0°C, 5 *M* sodium hydroxide solution (*ca.* 3 ml) was added to bring the pH to between 11 and 12. The appropriate anhydride (for **Ia–d**, **f** and **g**, Fig. 1) or acid chloride (for **Ie**, Fig. 1) (15 mmol) was then added dropwise, with constant stirring, at 0°C, over a period of 20 min. After a further 10 min the reaction mixture was allowed to reach ambient temperature and constantly re-adjusted to pH 11–12 with 5 *M* sodium hydroxide until the reduction in pH had ceased (*ca.* 2–6 h, depending on the anhydride). Stirring was continued for a further 30 min.

The stirred reaction mixture was slowly acidified to pH 2 by addition of 1 *M* hydrochloric acid over 10 min, then extracted with diethyl ether (2 × 50 ml) to remove the organic acid. The aqueous layer was lyophilized to yield white solids of the appropriate bis-N-acylcystine and sodium chloride. The former was taken up in methanol (20 ml) and vacuum filtered to remove the sodium chloride. Thin-layer chromatography of the filtrate on silica gel plates with methanol–chloroform (9:1) gave one intensely positive spot with a 0.25% (w/v) ninhydrin in acetone spray for

each product. The filtrate was evaporated at ambient temperature to form a syrupy suspension (3–4 ml). Sodium borohydride (*ca.* 1 g) was added to the stirred suspension until the violent reaction had ceased and a solid cake formed. After 1 h this was dissolved in water (25 ml) and stirred until all effervescence had ceased (*ca.* 30 min). The reaction mixture was acidified over 10 min, to pH 2 by addition of 1 *M* hydrochloric acid and then the products were extracted with chloroform (3 × 15 ml). The organic layers were bulked, dried with anhydrous magnesium sulphate, filtered and evaporated at ambient temperature to remove chloroform. All of the N-acyl-L-cysteines prepared gave positive results with the sodium nitroprusside test for SH groups and thin-layer chromatography on silica gel plates with methanol–chloroform (9:1), gave one spot for each product when charred with sulphuric acid, which was not ninhydrin positive, except for **Ig** (Fig. 1). The products, which were viscous, odorous oils, were stored at −14°C.

RESULTS AND DISCUSSION

Novel N-acylated L-cysteine derivatives (Fig. 1, **Ia–f**), possessing N-acyl groups of increasing alkyl chain length and branching, have been prepared in low–moderate "non-optimized" yields (see Table II). The synthesis involves preparing the corresponding bis-N-acylated-L-cystine forms from cystine and the appropriate acid chloride or anhydride under alkaline conditions. The disulphide moiety of the bis-N-acylated-L-cystine was then reduced to the N-acylated-L-cysteine by reaction with sodium borohydride (see Fig. 1).

All of the N-acylated cysteines reacted with OPA and a variety of amino compounds (Fig. 2, IIa–e) under alkaline conditions to yield highly fluorescent diastereoisomeric isoindole derivatives (see Fig. 3) which reached their maximum fluorescence within 1–2 min and were stable for at least 10 min. All of the resulting diastereoisomers were amenable to separation by reversed-phase HPLC on a Spherisorb ODS II (5 μm) EXCEL column using mobile phases with various proportions of sodium acetate buffer (50 m*M*, pH 7.1) and methanol. Glutamic acid and lombricine were not resolved with OPA and the parent thiol of the NAC series, but on using the new thiols dramatic increases in resolution were observed (see, *e.g.*, Fig. 4). A series of four

TABLE II

YIELDS AND PERCENTAGE OF D-ENANTIOMER IMPURITY IN N-ACYL-L-CYSTEINE AND BocC PREPARATIONS AS SHOWN BY HPLC ANALYSIS OF THEIR OPA DERIVATIVES WITH L-GLUTAMIC ACID

Compound	*Yield (%)*[a]	D-*Isomer (approx. %)*
Ia (NPC)	5	1.0
Ib (NBC)	12	Not detected
Ic (NIBC)	14	Not detected
Id (NVC	10	0.4
Ie (NIVC)	12	0.3
If (NTMAC)	10	Not detected
Ig (BocC)	88	Not detected

[a] Overall yield based on L-cystine.

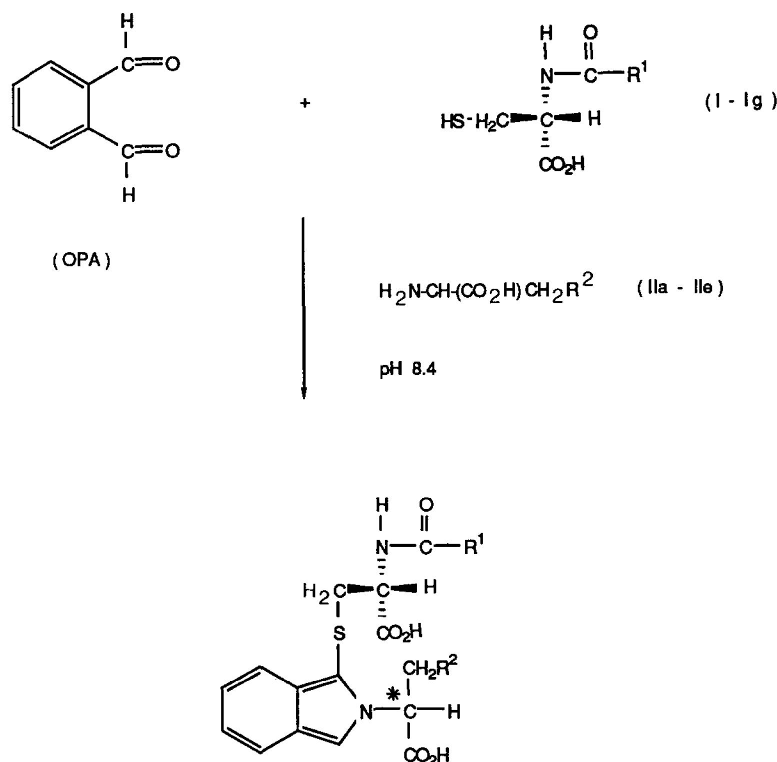


Fig. 3. Proposed structure of the OPA–thiol–amino adduct.

"runs" were performed with the N-acylated cysteines for all of the DL-amino compounds. The derivatization was completed prior to each injection with a reaction time of 5 min. Measuring peak heights and retention times, the coefficient of variation was less than 1%. The fluorescent intensities of the diastereoisomeric isoindoles formed from the new N-acyl-L-cysteines were similar to those obtained with either NAC or BocC for the amino compounds examined.

Buck and Krummen[10] have previously observed that with OPA–NAC the elution order of enantiomers is dependent on the lipophilicity of the amino compounds; for example, hydrophilic amino acids result in the L-enantiomer eluting before the D-enantiomer, whereas hydrophobic amino acids elute with the D- before the L-enantiomer. However, the elution order for all the enantiomers of various hydrophilicity with the new chiral thiols was the L-enantiomer before the corresponding D-enantiomer, and was identical with that when OPA–BocC was used[9].

It can be observed from Figs. 5 and 6 that increasing the chain length of the N-acyl group leads to increases in K^* and resolution. An increase in branching in the N-acyl moiety of the chiral thiols produced an improvement in the resolution of all of the diastereoisomers (see Table III). However, the effect of branching on K^* is more complex, as only with certain of the diastereoisomers does an increase in K^* occur.

The effect of branching on the K^* value of each diastereoisomer cannot be

rationalized in terms of the lipophilicity contribution of the N-acyl moiety[17]. Therefore, additional factors must be operative in controlling the affinity of the diastereoisomer for the lipophilic stationary phase. For example, steric interactions of the bulky N-acyl moiety may make the formation of intramolecular hydrogen bonds, resulting in a more lipophilic molecule, less energetically favourable for certain diastereoisomers, which would result in smaller K^* values than would be expected. Computational analysis of the prefered conformations of the isoindolic diastereoisomers is currently being undertaken in an attempt to gain a better understanding of the parameters that affect the resolution of these diastereoisomers on reversed-phase supports.

The enantiomers of the multifunctional amino compound lombricine have previously been separated using the OPA–BocC approach, giving a resolution value of 1.17 in an optimized HPLC analysis. Better results can be obtained by use of NTMAC in place of BocC, giving a resolution value of 3.23 in a non-optimized HPLC assay.

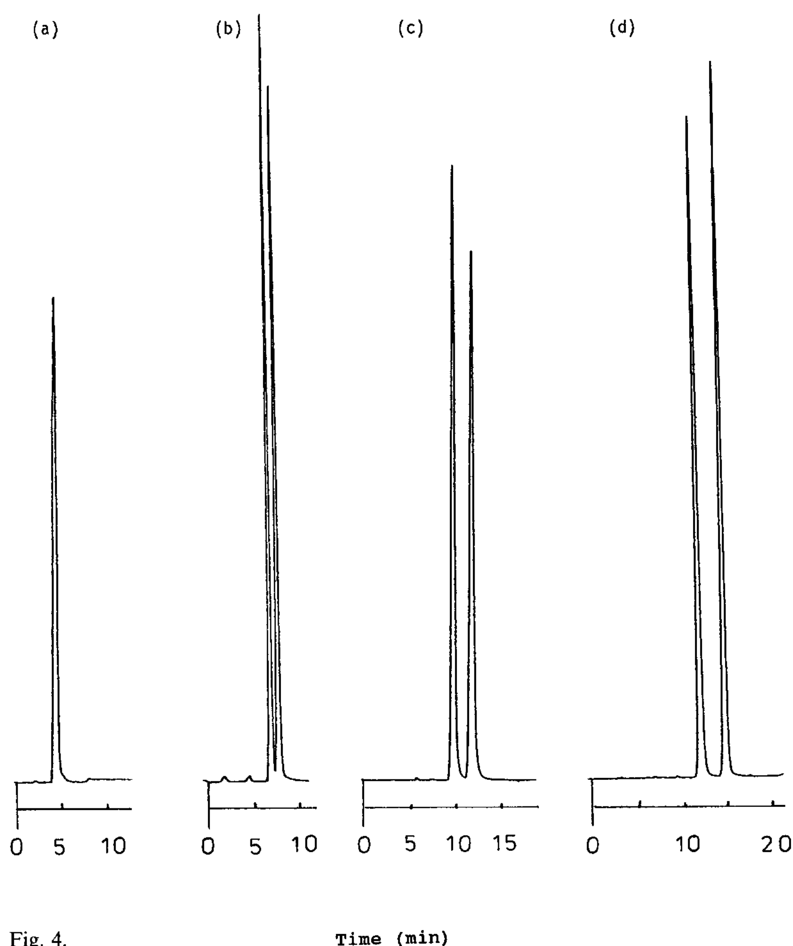


Fig. 4.

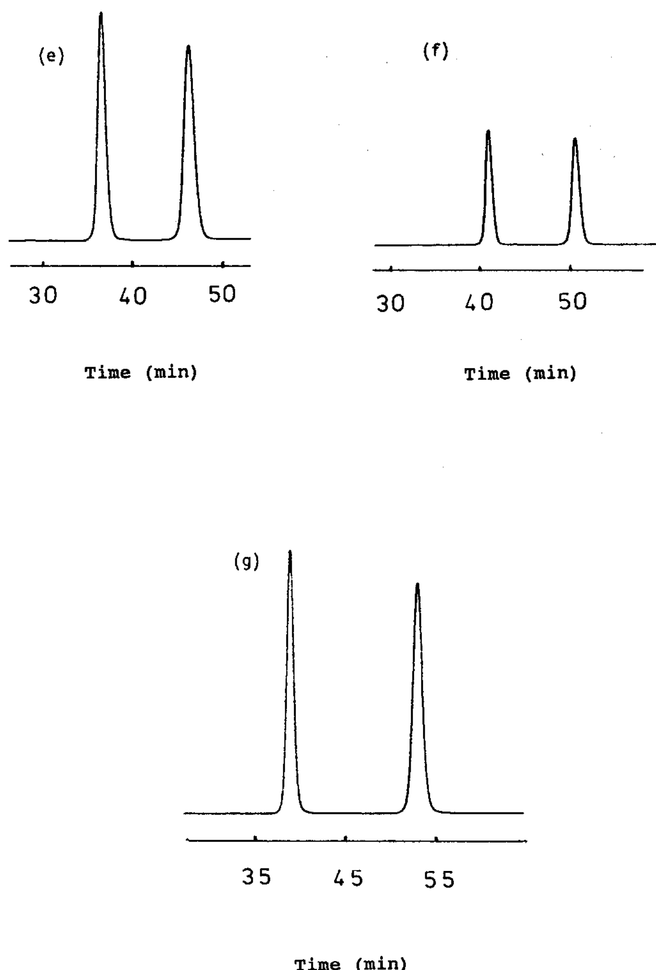


Fig. 4. HPLC of OPA–chiral thiol derivatives of DL-glutamic acid on a Spherisorb ODS II EXCEL reversed-phase column. Chromatographic conditions as in Table I (A). Peaks: (a) NAC; (b) NPC; (c) NBC; (d) NIBC; (e) NVC; (f) NIVC; (g) NTMAC.

The effect of temperature on the chromatographic behaviour of these types of diastereoisomeric isoindoles has not previously been examined; in previous work ambient temperatures were used[5–14]. However, our findings suggest that the temperature at which the chromatography is performed has a dramatic effect on the resolution of the diastereoisomers. Increasing the temperature led to a rapid decrease in the resolution (see Table IV) (almost halved by an increase from 18 to 45°C). From our initial investigations, the new N-acylcysteine thiols yield highly fluorescent isoindolic diastereoisomers when reacted with OPA and a variety of amino compounds. The diastereoisomers have K^* values intermediate between those with NAC and BocC; using gradient A (Table I), the NPC and NMTAC derivatives of L-glutamic acid had retention times of 6.5 and 38.75 min, respectively, whereas BocC derivatives failed to

elute after 120 min. The overall resolution of the diastereoisomers formed from the new chiral thiols is better than that with NAC and appears to be as good as, if not better than, that with BocC.

The synthetic route to these chiral thiols must, ideally, induce no racemization in the "optically pure" starting material. To test our proposed synthetic route, we compared a chromatogram of pure L-glutamic acid with BocC made by our method (overall yield 88%) with that from a sample of BocC made as described by Buck and Krummen[9], which has no racemization problems associated with it. Our method involved reducing the bis-N-*tert.*-butyloxycarbonylcystine (prepared from cystine and di-*tert.*-butyl dicarbonate) with methanolic sodium borohydride. Both chromatograms gave a single, sharp peak for the L-enantiomer, indicating that no racemization had occurred in the "optically pure" L-cystine. The optical purity of the latter was confirmed by reducing the L-cystine to L-cysteine with dithioerythritol followed by methylation using methyl iodide and pre-column derivatisation with OPA–BocC, as described by Buck and Krummen[10].

The N-acylated L-cysteines were initially synthesized using the appropriate acid

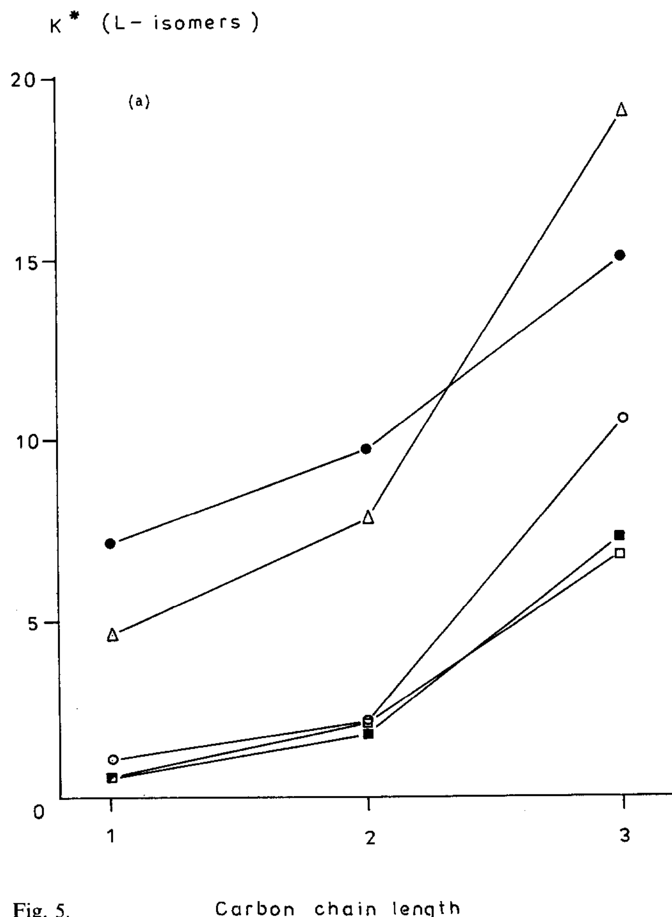


Fig. 5.

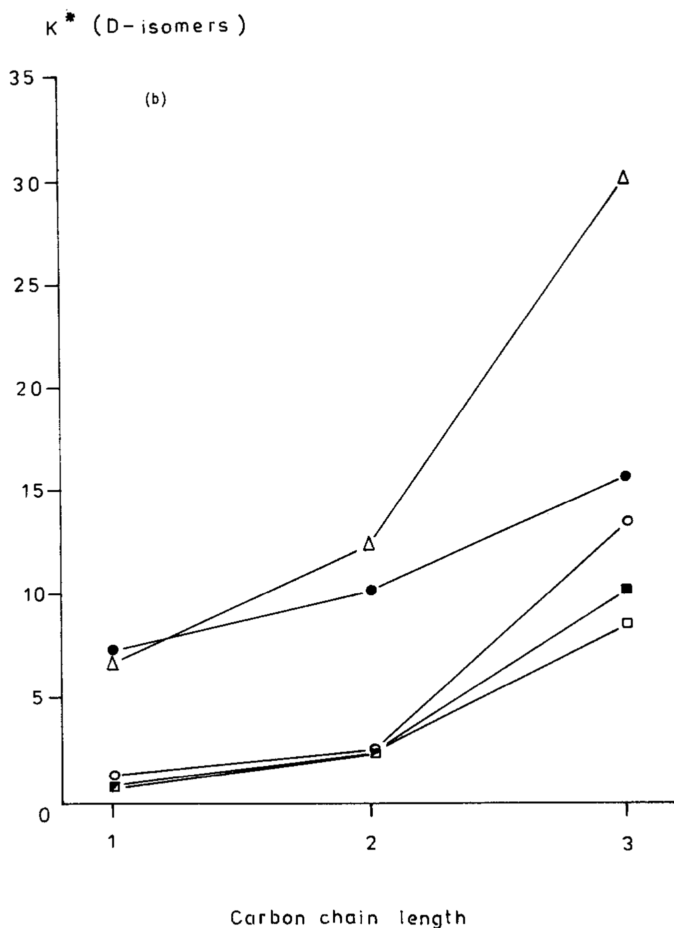


Fig. 5. Effect of increasing chain length of the chiral thiol N-acyl moiety on the K^* values for (a) the L- and (b) the D-enantiomers. (○) Glutamic acid; (●) lombricine; (□) APB; (■) APV; (△) APH.

chlorides in overall yields of approximately 20%. However, the OPA–glutamic acid purity test revealed at best 0.8% (NTMAC) and at worst 3% (NPC) racemization in the chiral thiols. The observed racemization was thought to be associated with the N-acylation stage, as no racemization had been observed with the sodium borohydride reduction in the synthesis of BocC. The less reactive acid anhydrides were therefore employed instead of the acid chlorides, in an attempt to reduce the degree of racemization. Although the "non-optimized" yields were lower (5–14%) than those obtained with the acid chlorides (18–22%), the OPA purity test revealed that the racemization problem had been dramatically reduced. The extent of racemization ranged from undetectable to *ca.* 1% NPC (see Table II).

The new N-acylated L-cysteines appeared to be stable in the methanolic OPA solutions at 4°C for at least 3 days, as judged from the peak height of the L-glutamic acid derivative and the absence of racemization. These thiols should, therefore, be

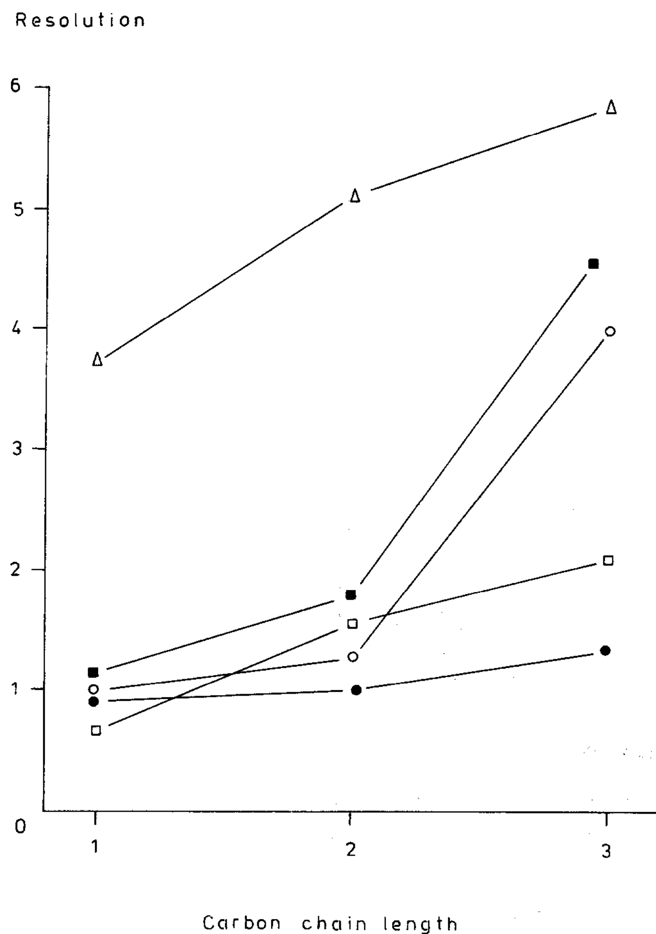


Fig. 6. Effect of increasing chain length of the chiral thiol N-acyl moiety on the resolution of (○) DL-glutamic acid, (●) DL-lombricine, (□) DL-APB, (■) DL-APV and (△) DL-APH.

TABLE III

SEPARATION OF DIASTEREOISOMERIC DERIVATIVES OF THE AMINO ACIDS (**IIa–e**) FORMED FROM OPA–CHIRAL THIOLS

Chiral thiol	*Resolution*				
	*Glut (**IIa**)*	*Lomb (**IIb**)*	*APB (**IIc**)*	*APV (**IId**)*	*APH (**IIe**)*
Ia (NBC)	1.00	0.90	0.67	1.14	3.73
Ib (NPC)	1.27	1.00	1.56	1.80	5.13
Ic (NIBC)	2.75	2.00	2.00	2.40	5.45
Id (NVC)	4.00	1.33	2.11	4.75	5.88
Ie (NIVC	5.06	1.45	3.11	5.14	6.00
If (NTMAC)	5.70	3.23	3.38	5.20	9.00

TABLE IV

SEPARATION OF DIASTEREOISOMERIC DERIVATIVES FORMED FROM GLUTAMIC ACID AND OPA–NTMAC AT VARIOUS TEMPERATURES

Temperature (°C)	*Resolution*	*Temperature (°C)*	*Resolution*
18	5.77	35	3.87
24	5.60	40	3.20
30	4.63	45	2.13

amenable to pre-column derivatization using an autosampler and injector. (The OPA–chiral thiol solution must be added to the amino compound first followed by the borate buffer prior to injection, to avoid any possibility of racemization of the chiral thiol.) In addition, the chiral thiols, which were smelly oils, appeared to be stable for at least 2 months when stored at −14°C (as determined by no additional peaks being observed in the chromatograms). Mass spectrometry revealed a small peak that corresponded to the disulphide form of the thiol, which presumably arose from oxidation of the thiol on prolonged storage. However, the disulphide is not detrimental as it does not participate in the OPA reaction. The synthetic route described produces, in most instances, enantiomerically pure N-acyl-L-cysteine derivatives which yield highly fluorescent isoindolic diastereoisomers when reacted with OPA and racemic amino compounds under alkaline conditions.

The application of these novel thiols to the assessment of the enantiomeric purity of various amino compounds of diverse chemical structure has been highlighted. The elution order of the amino enantiomers studied to date with the new N-acyl-L-cysteines and OPA appears to be L- before D-. It should be possible to synthesize the corresponding D-forms of the N-acylcysteines so that the D- and L-N-acylated cysteines will give reversed elution orders. This would be convenient when small amounts of one enantiomer have to be quantified in the presence of the other because it would be possible to use the thiol that elutes the minor enantiomer first, hence aiding quantification.

The diastereoisomeric isoindoles formed from the chiral thiols possess K^* values intermediate between the polar NAC and the lipophilic BocC-thiols that have been employed to date using reversed-phase HPLC[5–14]. The resolving power of the new thiols is excellent, with resolution values of up to 9 being achieved for certain diastereoisomers.

The study of the chromatographic behaviour of this series of novel chiral thiols with other amino compounds should provide a better understanding of the separation mechanism of these isoindolic diastereoisomers. It should then be possible to select the most appropriate thiol for use with the particular amino compound under investigation in order to achieve optimum resolution of the resulting diastereoisomers.

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