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Enantioseparation of amino compounds by derivatization with *o*-phthalaldehyde and D-3-mercapto-2-methylpropionic acid

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ABSTRACT

o-Phthalaldehyde in combination with D-3-mercapto-2-methylpropionic acid is described as a chiral reagent for the enantioseparation of amino compounds. The reagent was applied to the optical resolution of α-amino acids, α-alkyl-α-amino acids, α-amino acid amides, α-alkyl-α-amino acid amides, β-amino alcohols and β-alkyl-β-amino alcohols. Separation of the diastereomers was carried out by reversed-phase chromatography and the derivatives were detected fluorimetrically. The diastereoselectivity obtained was compared with that of other commercially available chiral thiols. The rate of the reaction of various amino compounds with *o*-phthalaldehyde–D-3-mercapto-2-methylpropionic acid was studied, together with the stability of the derivatives obtained. Application of the method to the enantioseparation of chiral monofunctional amines was demonstrated by using α-methylbenzylamine as a model compound. The method described offers good enantioselectivity combined with high sensitivity for various chiral amino compounds derived from chemo-enzymatic processes.

INTRODUCTION

o-Phthalaldehyde (OPA) in combination with a thiol compound is a widely used reagent for high-performance liquid chromatographic (HPLC) analysis of amino compounds. By using an optically active thiol compound, *e.g.*, N-acetyl-L-cysteine (NAC), in the OPA reaction, it was shown that this reaction is applicable to the enantiomeric analysis of amino compounds [1]. Since then, several chiral thiols have been employed in the OPA reaction, *e.g.*, N-acylated cysteines [2–7], thiosugars [8–10], neomenthylthiol [11] and captopril [7]. In addition to use with protein α-H-α-amino acids, the applicability of the OPA–chiral thiol approach has been demonstrated for a large number of amino compounds, *e.g.*, α-alkyl-α-amino acids [12–15], α-amino acid amides [12,14], α-amino carboxylic esters [12], β-amino alcohols [4,10,16], α-hydroxymethyl-α-amino acids [17] and non-protein α-H-α-amino acids [6,12,18–20]. Most of the separations have been performed by reversed-phase chromatography, but in some studies the diastereomeric adducts were separated by means of ligand-exchange chromatography [14,16,21,22].

An important aspect when employing the OPA–chiral thiol reaction for enantiomeric amines is how the chemical structure of the thiol influences the diastereoselectivity observed for the isoindole adducts. For OPA–N-acylcysteine derivatives, intramolecular interactions between the carboxylic group of the cysteine part and the isoindole nitrogen may give rise to the diastereoselectivity obtained [23]. However, this mechanism cannot be applied for, *e.g.*, neomenthylthiol.

Employing chiral thiols with different chemical structures in the OPA reaction may therefore lead to a better understanding of the structure requirements for the isoindole adducts to improve the dia-

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stereoselectivity. The aim of this study was to evaluate the use of D-3-mercapto-2-methylpropionic acid (MMPA) in the OPA reaction for the enantioseparation of chiral amines and to compare the diastereoselectivity obtained with that of other commercially available chiral thiols.

The OPA–MMPA reaction was applied to the enantioseparation of various amino compounds from chemo-enzymatic processes, *i.e.*, α-amino acids (AAs), α-alkyl-α-amino acids (α-alkyl-α-AAs), α-amino acid amides (AAs-NH_2), α-alkyl-α-amino acid amides (α-alkyl-α-AAs-NH_2), β-amino alcohols and β-alkyl-β-amino alcohols.

EXPERIMENTAL

Materials

AAs, N-acetyl-D-penicillamine (NAP) and 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside (TATG) were obtained from Sigma (St. Louis, MO, USA). AAs-NH_2, α-alkyl-α-AAs, α-alkyl-α-AAs-NH_2, β-amino alcohols and β-alkyl-β-amino alcohols were synthesized in our laboratory [24]. For each amino compound, both the racemic form and at least one optically pure enantiomer were available. N-*tert.*-Butyloxycarbonyl-L-cysteine (Boc-Cys), N-isobutyryl-L-cysteine (*i*-But-Cys) and D-S-acetyl-3-mercapto-2-methylpropionic acid (supplier's name: D-S-acetyl-β-mercaptoisobutyric acid) were purchased from Novabiochem (Läufelfingen, Switzerland). N-Acetyl-L-cysteine (NAC) and α-methylbenzylamine were obtained from Janssen (Beerse, Belgium). OPA and HPLC-grade methanol and acetonitrile were supplied by Merck (Darmstadt, Germany). Water was purified with a Milli-Q system (Millipore). All other chemicals were of analytical-reagent grade.

Instrumentation

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, USA) Model 1090 liquid chromatograph and a Gilson Model 231-401 autosampling injector for derivatization and injection. The injection loop had a 20-μl capacity. The columns used were Nucleosil-120-C_{18} (250 × 4.0 mm I.D., 5 μm, for isocratic elution, and 250 × 4.0 mm I.D., 3 μm, for gradient elution). The flow-rate was 1 ml/min and and the column temperature was kept at 40°C.

The derivatives were monitored with a Waters (Milford, MA, USA) Model 420 fluorescence detector. For excitation a 338-nm band-pass filter was used, and for emission a 415-nm long-pass filter was chosen. Quantification was performed with a Hewlett-Packard Model 3350 laboratory automation system.

^{1}H NMR analysis was performed with a Bruker (Karlsruhe, Germany) AM 400 instrument. Spectra were recorded at 400 MHz and samples were diluted with ^2H_2O. For LC–mass spectrometry (MS), a Finnigan MAT TSQ-70 triple quadrupole mass spectrometer equipped with a thermospray interface (Finnigan MAT, San José, CA, USA) was used. MS analysis was performed using chemical ionization with gaseous ammonia [25].

A Waters Model 481 UV detector was used for detection at 210 nm.

Eluent, reagent and derivatization procedure

The mobile phase consisted of 50 m*M* sodium acetate buffer, titrated to pH 6.0 with acetic acid. For isocratic runs, the concentration of methanol in the mobile phase was as indicated in the Tables.

The OPA reagent was prepared by dissolving 60 mg of OPA in 3 ml of methanol and 15 ml of 0.4 *M* sodium borate buffer (pH 9.4). The MMPA reagent was prepared by dissolving 6.5 mg of D-S-acetyl-3-mercapto-2-methylpropionic acid per ml of 1 *M* sodium hydroxide solution. The solution was stirred for 10 min at room temperature. The MMPA solution thus obtained was titrated to pH 7.0 with phosphoric acid solution. For the other thiols used in this study, 25 m*M* solutions were prepared in water–methanol (1:1, v/v) except TATG, which was dissolved in methanol. The amino compounds studied were dissolved in water.

Derivatization was performed automatically with a Gilson Model 231-401 system. The following volumes were mixed: 75 μl of sample solution, 300 μl of OPA reagent and 30 μl of thiol reagent. After at least 2 min (30 min for α-alkyl compounds) at room temperature, an aliquot of the reaction mixture was injected into the chromatographic system.

For LC–thermospray MS, the eluent consisted of a 10 m*M* solution of formic acid and acetonitrile. Analysis was performed using a gradient from 0 to 60% acetonitrile in 30 min.

RESULTS AND DISCUSSION

Chemical and optical purity of MMPA reagent

The chemical purity of the MMPA reagent was studied by HPLC–UV detection, LC–thermospray MS and ^{1}H NMR. HPLC–UV analysis of the MMPA solution, prepared by base-catalysed hydrolysis of the S-acetyl precursor, showed the absence of the precursor and the presence of acetate, a large peak at retention time (t_R) = 14 min and a minor peak at t_R = 22 min. Using the same elution conditions, the MMPA solution was studied by LC–thermospray MS. The mass spectrum of the compound at t_R = 14 min showed several characteristic mass peaks for MMPA : $[M + NH_4]^+$ (m/z = 138), $[M + NH_4 + CH_3CN]^+$ (m/z = 179) and $[nM + NH_4]^+$ (m/z = 258, 378, 498 for n = 2, 3 and 4, respectively). The latter type of ions probably result from intermolecular associations of MMPA molecules in the MS source. MS analysis of the compound at t_R = 22 min showed typical mass peaks of MMPA disulphide : $[MH]^+$ (m/z = 239), $[M + NH_4]^+$ (m/z = 256) and $[MH - H_2O]^+$ (m/z = 221).

^{1}H NMR analysis of the MMPA solution showed characteristic resonances for MMPA and its disulphide form: δ 2.55 (m, 2H), 2.40 (m, 1H) and 1.06 (d, 3H) for the main product, *i.e.*, MMPA, and δ 2.77 (m, 2H) and 1.09 (d, 3H) for the secondary compound, *i.e.*, MMPA disulphide (the CH was obscured by other resonances).

From the ^{1}H NMR data the concentration of MMPA and its disulphide form were calculated. The concentration of the MMPA disulphide was 10% of that of MMPA. As the disulphide form does not react with OPA, the presence of this compound in the MMPA reagent will not influence the analysis. The concentration of MMPA was 21 m*M*.

With respect to the initial concentration of D-S-acetyl-3-mercapto-2-methylpropionic acid, a conversion of 85% was found. The stability of the MMPA reagent at pH 7 was evaluated and a decrease of < 10% was found after 34 h. According to the supplier, the optical purity of D-S-acetyl-3-mercapto-2-methylpropionic acid was ≥98%. After hydrolysis, the optical purity of the resulting MMPA was measured using the "reverse" OPA reaction with L-Val (optical purity 99.9%) as chiral selector. The value found for MMPA was 98.7%.

Comparison of MMPA with other chiral thiols

The spectroscopic and chromatographic properties of MMPA were compared with those of five other commercially available thiols. For this investigation, Val was used as the test compound. The data obtained are given in Table I. The excitation spectra of the derivatives showed two maxima: one below 300 nm and the other, given in Table I, above 300 nm. The excitation wavelengths obtained for the six thiol derivatives were nearly identical. The emission spectra of the OPA derivatives of MMPA, *i*-But-Cys, Boc-Cys and NAC showed maxima in the 443–454-nm region, whereas the derivatives of NAP and TATG showed maximum fluorescence at 412 and 414 nm, respectively. With respect to the chromatographic properties of the chiral thiols, *i*-But-Cys gave the highest α-value among the N-acylcysteines tested. A comparable value was found for TATG. MMPA, however, gave the highest α-value for the whole series.

The detection limit for the first eluting valine enantiomer, based on a signal-to-noise ratio of 3, was compared for four thiols at λ_{exc} = 338 nm and λ_{em} > 415 nm. The values found for MMPA, *i*-But-Cys, Boc-Cys and NAC were 2.0, 2.8, 3.7 and 2.3 pmol, respectively.

Enantioselective analysis

The reaction of OPA and MMPA with AAs, AAs-NH_2, β-amino alcohols and the corresponding alkylated compounds yielded highly fluorescent de-

TABLE I

SPECTROSCOPIC AND CHROMATOGRAPHIC PROPERTIES OF OPA DERIVATIVES OF VALINE WITH VARIOUS CHIRAL THIOLS

For conditions, see Experimental.

Chiral thiol	Excitation maximum (nm)	Emission maximum (nm)	k'^a	α
MMPA	331	454	1.82	2.10
i-But-Cys	333	445	1.41	1.62
TATG	329	414	15.45	1.60
Boc-Cys	332	444	4.01	1.50
NAP	330	412	1.42	1.09
NAC	332	443	0.72	1.30

[a] Capacity factor of the first-eluted diastereomer; concentration of methanol in the mobile phase is 45% (v/v).

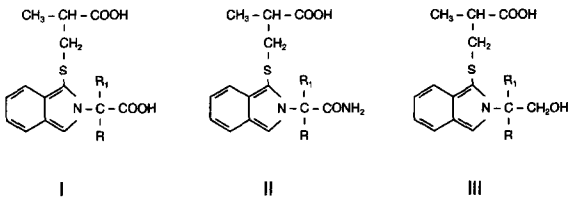

Fig. 1. Proposed structures for the diastereoisomeric adducts formed in the reaction of OPA–MMPA with (I) AAs, (II) AAs-NH_2 and (III) β-amino alcohols. R_1 = H or alkyl; R = residue of the amino compound.

rivatives, which are assumed to be analogous to the adducts from the OPA–mercaptoethanol reaction [26] (Fig. 1).

In our investigation of the OPA–MMPA reaction for amino compounds, the corresponding OPA–NAC derivatives were used for comparison. In Table II the data for the OPA–MMPA derivatives of several AAs and α-alkyl-α-AAs are presented. Using MMPA as chiral selector, enantioseparation could be obtained for all the protein AAs listed. The highest α-values were obtained for the aliphatic AAs, *i.e.*, Leu, Ile and Val. Compared with NAC, the use of MMPA resulted in higher α-values for all the AAs tested, except Trp, where equal performance was obtained. To illustrate the resolution obtained with MMPA, the enantioseparation of a standard mixture, composed of the non-chiral Gly and seventeen DL pairs of protein AAs, is shown in Fig. 2. Under the conditions employed, all the enantiomeric pairs and Gly could be separated in a single gradient run (D-Ile and L-Val are eluted together). In contrast with NAC, the elution order of the OPA–MMPA derivatives of the AAs was D before L in all instances. The OPA–MMPA deriva-

TABLE II

CAPACITY FACTORS (k') AND SELECTIVITIES (α) OF OPA–L-NAC AND OPA–D-MMPA DERIVATIVES OF AA COMPOUNDS

For chromatographic conditions, see Experimental.

AA	Reagent					
	OPA–L-NAC			OPA–D-MMPA		
	Methanol[a]	k'[b]	α	Methanol[a]	k'[b]	α
Ala	30	1.00	1.19	35	1.31	1.49
Asp	10	0.27	1.33	15	0.80	1.37
Ser	5	4.88	1.00	10	5.84	1.31
Thr	10	5.14	1.15	15	6.90	1.80
His	30	0.30	1.00	35	0.73	1.11
Tyr	30	1.62	1.27	35	1.31	1.34
Val	40	1.48	1.28	45	1.82	2.10
Met	40	1.85	1.05	45	1.51	1.64
Ile	40	2.84	1.29	45	2.87	2.13
Phe	40	2.36	1.04	45	2.25	1.47
Leu	40	4.58	1.07	45	3.72	1.80
Lys	40	12.28	1.00	45	12.10	1.56
Gln	30	0.16	1.00	35	0.23	1.53
Trp	40	1.42	1.41	45	1.30	1.40
Arg	25	0.64	1.00	30	1.66	1.37
Asn	10	2.13	1.05	15	2.62	1.32
Glu	10	1.17	1.00	15	1.67	1.60
α-Ph-Gly	40	2.14	1.10	45	2.38	1.40
α-Me-Val	40	2.45	1.15	45	6.60	1.34
α-Me-Leu	40	4.41	1.04	45	4.52	1.15
α-Me-Phe	35	9.07	1.09	40	8.63	1.20
α-Me-α-Ph-Gly	40	3.13	1.08	45	3.76	1.07

[a] Percentage of methanol in the mobile phase.

[b] Capacity factor of the first-eluted diastereomer.

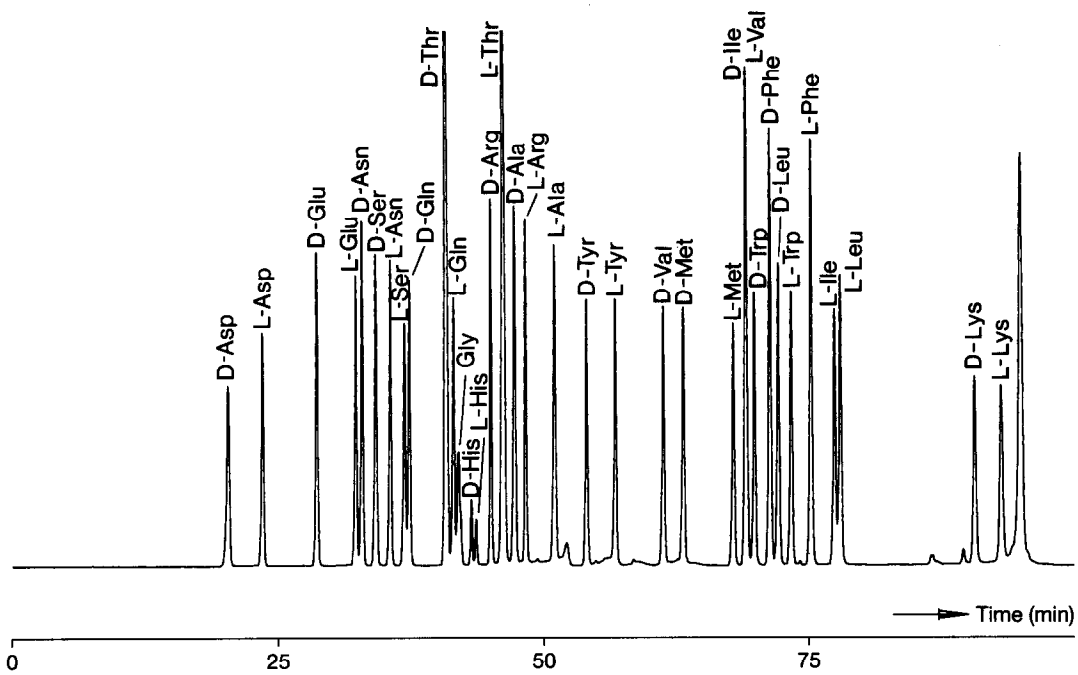

Fig. 2. Enantioseparation of an amino acid standard mixture consisting of seventeen pairs of AAs and the non-chiral Gly. Mobile phase: (A) 50 m*M* sodium acetate buffer (pH 6.0); (B) acetonitrile; gradient: 100% A, 0–10 min; 0–37.5%, 10–110 min. For other conditions, see Experimental.

tives of α-alkyl-α-AAs (Table I) showed lower α-values than the corresponding AAs. Compared with NAC, equal or better enantioselectivity was obtained with MMPA.

The k' and α-values for the OPA–MMPA and OPA–NAC derivatives of AAs-NH_2 and α-alkyl-α-AAs-NH_2 are given in Table III. For all the AAs-NH_2 investigated, enantioseparation occurred with

TABLE III

CAPACITY FACTORS (k') AND SELECTIVITIES (α) OF OPA–L-NAC AND OPA–D-MMPA DERIVATIVES OF α-AA-NH_2 COMPOUNDS

For chromatographic conditions, see Experimental.

AA-NH_2	Reagent					
	OPA–L-NAC			OPA–D-MMPA		
	Methanol[a]	k'[b]	α	Methanol[a]	k'[b]	α
Val-NH_2	40	4.88	1.17	45	11.26	1.48
Ala-NH_2	30	2.87	1.21	35	4.46	1.42
Leu-NH_2	40	10.70	1.18	45	11.46	1.51
Ser-NH_2	10	13.44	1.13	15	22.40	1.25
Phe-NH_2	40	11.35	1.00	45	9.55	1.33
α-Ph-Gly-NH_2	40	6.15	1.11	45	7.88	1.32
α-Me-Val-NH_2	40	4.81	1.23	45	5.46	1.06
α-Me-Leu-NH_2	40	8.69	1.10	45	10.00	1.03
α-MePhe-NH_2	35	20.53	1.22	40	20.43	1.06

[a] See Table II.

MMPA as selector. Analogously to the AAs, the highest α-values were obtained for the aliphatic AAs-NH_2, *i.e.*, Val-NH_2 and Leu-NH_2. The α-values obtained for the OPA–MMPA derivatives of the AAs-NH_2 are lower than those of the corresponding AAs. With respect to the elution order of the AAs-NH_2, the D-form always eluted before the L-form with MMPA, whereas for NAC both DL and LD orders occurred. As can be seen in Table III, the enantioselectivity obtained for OPA–MMPA derivatives of AAs-NH_2 is higher in all instances than for the OPA–NAC derivatives. With MMPA as selector, lower α-values were obtained for the α-alkyl-α-AAs-NH_2 in comparison with the AAs-NH_2.

The results for MMPA and NAC as selector for the enantioseparation of β-amino alcohols and the alkylated analogues are presented in Table IV. Using MMPA, resolution of the enantiomers could be obtained in all instances. For the β-amino alcohols tested, the elution order was D before L. Analogously to the AAs and AAs-NH_2, the alkylated analogues of the β-amino alcohols showed a lower enantioselectivity. For the whole series of amino alcohols, MMPA proved to be a better selector than NAC.

The rates of derivative formation of OPA–MMPA with an AA, AA-NH_2, α-alkyl-α-AA, α-alkyl-α-AA-NH_2 and β-amino alcohol were studied as a function of the reaction time. The results are presented in Fig. 3. In the two graphs, the molar fluorescence intensity for D-Val, L-Val-NH_2, L-Me-Val, D-Me-Val-NH_2 and L-Val-ol are shown at different reaction times. For D-Val, L-Val-NH_2 and L-Val-ol, maximum fluorescence was reached within 3 min, whereas L-Me-Val and D-Me-Val-NH_2 reached their maximum after 30 min. The difference in reaction rate between α-alkyl-α-amino compounds and the corresponding α-H analogues has also been observed for OPA in combination with other thiol compounds [14,27–29]. An explanation for this phenomenon may be steric hindrance of the α-alkyl substituent in the nucleophilic attack of the amine on the carbonyl function of OPA.

After their plateau value had been reached, a comparison of the molar fluorescence intensity of the compounds studied was performed. It was found that the molar fluorescence intensity increased in the order L-Val-ol > D-Val > L-Me-Val > D-Me-Val-NH_2 > L-Val-NH_2. The stability of the derivatives investigated can be seen from the right part of the graphs in Fig. 3. For up to 5 h, the derivatives of D-Me-Val-NH_2, L-Me-Val and L-Val-NH_2 show excellent stability. For D-Val, a slight decrease in the fluorescence with time is observed, *i.e.*, 1%/h. L-Val-ol formed an unstable derivative with OPA–MMPA; after 5 h only 10% of the initial fluorescence response was left. The alcohol functionality may play an important role in the instability of the derivative formed, as degradation of OPA–thiol adducts of β-amino alcohols has also been noted in other studies [4,10]. However, a possible degradation route for β-amino alcohols can-

TABLE IV

CAPACITY FACTORS (k') AND SELECTIVITIES (α) OF OPA–L-NAC AND OPA–D-MMPA DERIVATIVES OF β-AMINO ALCOHOLS

For chromatographic conditions, see Experimental.

β-Amino alcohols	Reagent					
	OPA–L-NAC			OPA–D-MMPA		
	Methanol[a]	k'[b]	α	Methanol[a]	k'[b]	α
Val-ol	40	8.53	1.04	45	11.60	1.27
Phe-ol	45	7.11	1.08	50	6.58	1.26
β-Me-Val-ol	45	10.09	1.21	50	7.98	1.38
α-Ph-Gly-ol	40	10.40	1.02	45	11.64	1.27
α-Me-Val-ol	45	4.75	1.03	50	4.86	1.21
α-et-Phe-ol	45	14.80	1.00	50	14.32	1.05

[a,b] See Table II.

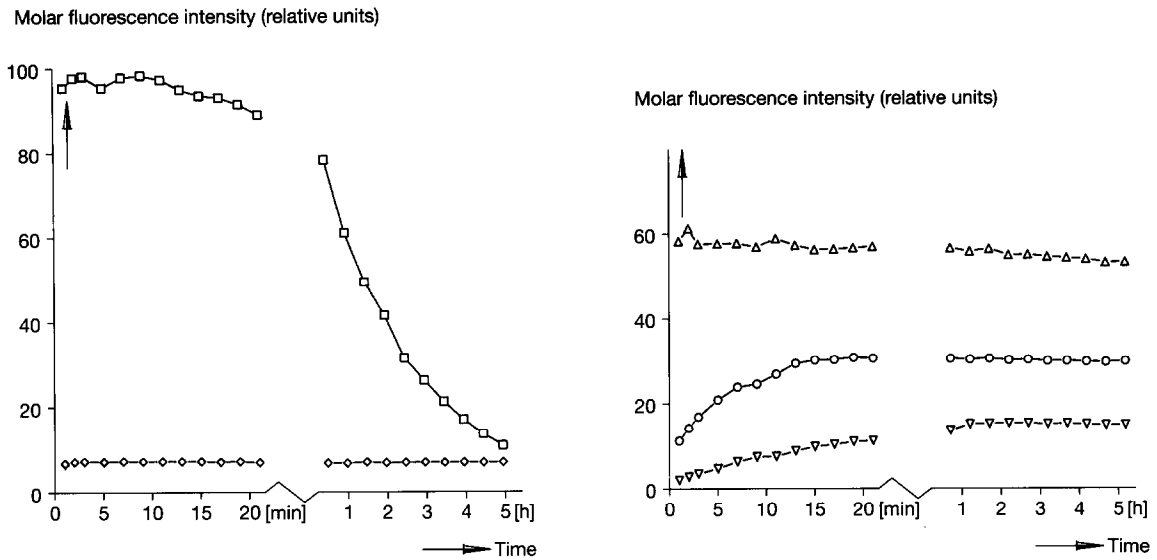

Fig. 3. Molar fluorescence response of the OPA–MMPA derivatization as a function of reaction time for (▽) D-Me-Val-NH_2 (Me = methyl), (○) L-Me-Val, (△) D-Val, (◇) L-Val-NH_2 and (□) L-Val-ol. The molar excess of OPA and MMPA was 100-fold and 10-fold, respectively, with respect to the compounds studied.

not be deduced from degradation mechanisms described earlier for isoindoles [30,31].

In our search for applications of the OPA–MMPA reaction, we noted that the OPA–chiral thiol approach has not been reported for monofunctional amines, *i.e.*, compounds possessing no other heteroatom apart from the primary amino group. As we were interested in the question of whether OPA–MMPA could be applied to this type of chiral amine, we selected α-methylbenzylamine as a model compound. Using the OPA–MMPA reagent, enantioseparation of racemic α-methylbenzylamine could be obtained (Fig. 4). Compared with other α-substituted benzylamines used in this study, *i.e.*, α-Ph-Gly-ol, α-Ph-Gly-NH_2 and α-Ph-Gly, the following ranking for the enantioselectivity can be made: α-Ph-Gly (COOH substituent; α = 1.40) > α-Ph-Gly-NH_2 ($CONH_2$ substituent; α = 1.32) > α-Ph-Gly-ol (CH_2OH substituent; α = 1.27) > α-methylbenzylamine (methyl substituent; α = 1.07).

The higher enantioselectivities obtained for α-Ph-Gly, α-Ph-Gly-NH_2 and α-Ph-Gly-ol compared with α-methylbenzylamine may be attributed to the type of α-substituent. Both the amino acid and amino acid amide and amino alcohol possess α-substituents that are capable of forming intramolecular hydrogen bonds. As this type of bond may play an important role in the diastereoselectivity obtained for the isoindole derivatives, the absence of this bonding site in α-methylbenzylamine may explain the low α-value observed.

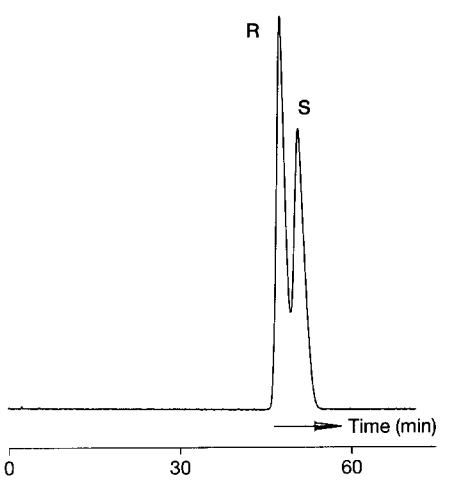

Fig. 4. Chromatogram of the OPA–MMPA derivatives of (*R,S*)-α-methylbenzylamine. Mobile phase: 50 m*M* sodium acetate buffer (pH 6.0)–methanol (55:45, v/v). For other conditions, see Experimental.

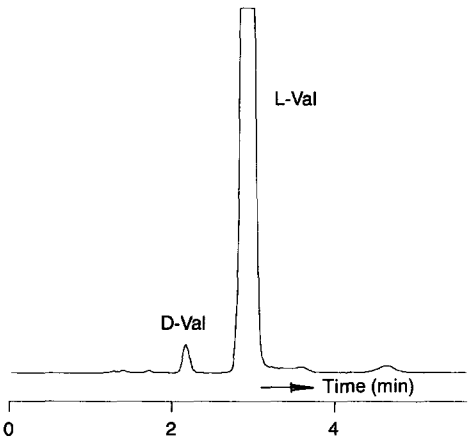

Fig. 5. Chromatogram of a sample from L-Val synthesis. Mobile phase, 50 m*M* sodium acetate buffer (pH 6.0)–methanol (55:45, v/v); flow-rate, 2 ml/min; temperature, 50°C. For other conditions, see Experimental.

Quantitative analysis

For most of the compounds studied, the diastereomeric OPA–MMPA derivatives exhibited different specific fluorescence intensities. Differences of up to 25% were obtained for some AAs-NH_2. Therefore, quantitative measurements were made by comparing the peak areas of compounds of the same enantiomeric form.

As an example of the linearity and precision of the method, these data are given for Val. The linearity of the amount *versus* response relationship was established over the range 4–67 pmol for each of the enantiomers. Linear regression analysis from the calibration graphs indicated that the correlation coefficient was 0.9999 for both enantiomers. The within-run precision of the assay gave a relative standard deviation < 2% (n = 5; 67-pmol level) and < 4% (n = 5; 4-pmol level) for both enantiomers.

As an application, a representative chromatogram of a sample from a chemo-enzymatic reaction performed on laboratory scale is given in Fig. 5.

CONCLUSIONS

It has been demonstrated that with the use of MMPA in combination with OPA, good enantioselectivity can be obtained for a broad range of amino compounds. As optically pure MMPA can be readily obtained from the commercially available S-acetylated analogue by simple hydrolysis, it may prove to be a valuable selector in other studies on the enantioselective analysis of chiral amino compounds.

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