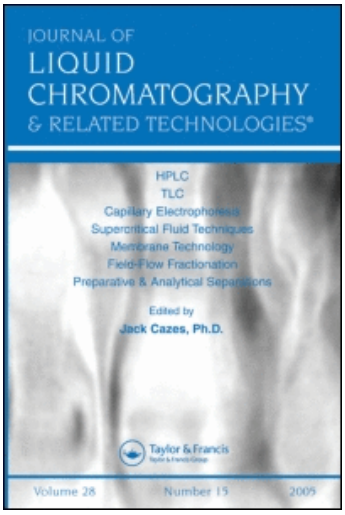

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SENSITIVE DERIVATIZATION REAGENTS FOR OPTICAL RESOLUTION OF CARBOXYLIC ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

Two highly sensitive, chiral derivatization reagents, 1-(1-anthryl)- and 1-(2-anthryl)ethylamines, have been developed. Condensation of carboxylic acids with the chiral reagent was effected in the presence of water-soluble carbodiimide and 1-hydroxybenzotriazole. The diastereomeric amides formed from N-acetylamino acid and naproxen enantiomers were efficiently resolved by normal phase chromatography (Resolve 5μ Spherical Silica column) with hexane/ethyl acetate as a mobile phase. With a fluorescence detector (excitation 260 nm, emission 400 nm), the detection limit was 100 fmol (S/N=10).

INTRODUCTION

A reliable method for the simultaneous determination of enantiomeric drug in biological fluids is a prerequisite for

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pharmacokinetic studies of the racemate. The application of high-performance liquid chromatography (HPLC) to the optical resolution has developed in two ways: the direct resolution of enantiomers on a chiral stationary phase or mobile phase (1), and derivatization with a chiral reagent followed by separation of the diastereomers using conventional column and mobile phase (2). Although the derivatization method has disadvantages in simplicity, this procedure is more favorable for the determination of racemic drugs in biological fluids with respect to sensitivity and versatility. In the previous papers we reported the preparation of chiral derivatization reagents for the resolution of enantiomeric amines (3,4), carboxylic acids (5) and alcohols (6), and their use for the quantification of racemic drugs in biological fluids by HPLC (7,8). This paper deals with the preparation of optically active 1-(1-anthryl)- and 1-(2-anthryl)ethylamines which possess an anthracene moiety highly responsive to a fluorescence detector, and the applicability of these reagents to the resolution of N-acetylamino acid enantiomers by HPLC.

EXPERIMENTAL

Materials

N-Acetylamino acids were purchased from Tokyo Kasei Co. (Tokyo, Japan). All of the chemicals were of analytical-reagent grade. Solvents were purified by distillation and deaerated prior to use. Optically active α-methoxy-α-methyl-1-naphthaleneacetic acid, anthracene-1- and -2-carboxylic acids were prepared in these laboratories by the methods previously reported (3). *dl*-Naproxen was obtained by the known method. The racemate was resolved by fractionally crystallizing the *d*- or *l*-methylbenzylamine salt from ethyl acetate. The optical purity of each enantiomer obtained was over 99.0% as judged by HPLC.

High-performance liquid chromatography

The apparatus used was a Waters Model M-45 solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Hitachi Model 650-10LC fluorescence spectrophotometer (excitation wavelength 260 nm; emission wavelength 400 nm). The test samples were applied to the chromatograph by a Waters U6K sample loop injector with an effective volume of 2 ml. A Resolve 5μ Spherical Silica column (150 mm x 4.6 mm i.d.) was used under ambient conditions.

Syntheses of derivatization reagents

1-Acetylanthracene oxime----- To a solution of anthracene-1-carboxylic acid (500 mg) in dichloromethane (10 ml) was added oxalyl chloride (2 ml), and the resulting solution was refluxed for 7 hr. After evaporation of the solvent, the residue obtained was dissolved in ether (5 ml) and added dropwise into a diazomethane-ether solution. The solution was stirred at room temperature for 1 hr and evaporated down. The residue obtained was redissolved in chloroform (5 ml), added dropwise to 55% hydroiodic acid (0.4 ml), and stirred at room temperature for 2 hr. The resulting solution was washed with 5% $Na_2S_2O_3$ and water, and evaporated down. The crude product was purified by column chromatography on silica gel (15 g). Elution with hexane-ethyl acetate (10:1) and recrystallization of the eluate from acetone-hexane gave 1-acetylanthracene (420 mg) as yellow powder. mp 69.5-71°C.

A solution of 1-acetylanthracene (300 mg), hydroxylamine hydrochloride (300 mg) and sodium acetate (600 mg) in ethanol (8 ml) was refluxed for 2 hr. After removal of the precipitate by filtration, the filtrate was evaporated down. The oily residue was purified by column chromatography on silica gel (10 g). Elution with hexane-ethyl acetate (10:1) and recrystallization of

the eluate from acetone-hexane gave 1-acetylanthracene oxime (270 mg) as colorless needles. mp 170-173°C. Anal. Calcd for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95. Found: C, 81.48; H, 5.55; N, 5.88. ^{1}H-NMR ($CDCl_3$)δ: 2.23 (3H, s, CH_3), 7.38-8.52 (9H, m, Ar-H), 8.66 (1H, broad s, OH).

dl-1-(1-Anthryl)ethylamine (Ia)----- To a stirred solution of 1-acetylanthracene oxime (400 mg) in ethanol (20 ml)-10% NaOH (20 ml) was added portionwise Raney nickel (1 g) under ice-cooling, and the suspension was stirred for 7 hr. After removal of the catalyst by filtration, the filtrate was concentrated and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate, and evaporated down. The residue obtained was purified by column chromatography on silica gel (12 g). Elution with ethyl acetate-0.1M methylamine/ethanol (10:1) gave Ia (300 mg) as an oily material. Hydrogen chloride gas was passed into a solution of Ia in ether to give a crystalline product. Recrystallization from ethyl acetate-ethanol gave the hydrochloride of Ia as colorless needles. mp 235-238°C (decomp.). Anal. Calcd for $C_{16}H_{15}N\cdot HCl$: C, 74.55; H, 6.21; N, 5.43. Found: C, 74.12; H, 6.43; N, 5.45. ^{1}H-NMR (CD_3OD)δ: 1.84 (3H, d, 7 Hz, CH_3), 5.56 (1H, q, 7 Hz, CH), 7.40-8.75 (9H, m, Ar-H).

Optical resolution of Ia----- To a solution of Ia (800 mg) in ethanol (2 ml) was added a solution of *l*-α-methoxy-α-methyl-1-naphthaleneacetic acid (800 mg) in ethanol (4 ml). The resulting precipitate was collected by filtration and fractionally crystallized from ethanol repeatedly several times. The salt was decomposed with 10% NaOH and the yielded free base was extracted with ether. The organic layer was washed with water, dried over anhydrous sodium sulfate, and evaporated down. Treatment with hydrogen chloride gas gave the hydrochloride of Ib (300 mg) as colorless needles. mp 236-238°C (decomp.). $[\alpha]_D^{25}$ -33.2° (c=0.20, methanol). The mother liquor was treated with *d*-α-methoxy-α-

methyl-1-naphthaleneacetic acid in the manner described above to give the hydrochloride of Ic (200 mg) as colorless needles. mp 235-238°C (decomp.). $[\alpha]_D^{25}$ +34.0° (c=0.30, methanol).

2-Acetylanthracene oxime----- Treatment of anthracene-2-carboxylic acid (600 mg) as described for 1-acetylanthracene oxime followed by recrystallization of the product from acetone gave 2-acetylanthracene oxime (320 mg) as colorless needles. mp 237-240°C (decomp.). Anal. Calcd for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95. Found: C, 81.76; H, 5.42; N, 5.80. ^{1}H-NMR (d_6-DMSO)δ: 2.35 (3H, s, CH_3), 7.40-8.60 (9H, m, Ar-H).

dl-1-(2-Anthryl)ethylamine (IIa)----- Treatment of 2-acetylanthracene oxime (300 mg) as described for Ia gave IIa (220 mg) as an oily material. Hydrogen chloride gas was passed into a solution of IIa in ether to give a crystalline product. Recrystallization from ethyl acetate-ethanol gave the hydrochloride of IIa as colorless needles. mp 278-280°C (decomp.). Anal. Calcd for $C_{16}H_{15}N\cdot HCl$: C, 74.55; H, 6.21; N, 5.43. Found: C, 74.40; H, 6.20; N, 5.40. ^{1}H-NMR (CD_3OD)δ: 1.76 (3H, d, 8 Hz, CH_3), 4.67 (1H, q, 8 Hz, CH), 7.40-8.52 (9H, m, Ar-H).

Optical resolution of IIa----- Treatment of IIa (1 g) as described for Ib gave the hydrochloride of IIb (320 mg) as colorless needles. mp 278-280°C (decomp.). $[\alpha]_D^{25}$ -27.4° (c=0.9, methanol). The mother liquor was treated with *d*-α-methoxy-α-methyl-1-naphthaleneacetic acid in the manner described for Ic to give the hydrochloride of IIc (100 mg) as colorless needles. mp 277-279°C (decomp.). $[\alpha]_D^{25}$ +25.8° (c=0.4, methanol).

Derivatization

N-Acetylamino acid----- To a solution of N-acetyl-D- or -L-amino acid (ca. 10 μg) in pyridine (0.1 ml) were added Ib or IIb (ca. 1 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (water-soluble carbodiimide, WSC) (ca. 1 mg), and the resulting solution was allowed to stand at room temperature for 1

hr. The reaction mixture was diluted with ether, washed successively with 5% HCl, 5% $NaHCO_3$ and water, and dried over anhydrous sodium sulfate. An aliquot of this solution was injected into the chromatograph.

Naproxen----- To a solution of *dl*-, *d*-, or *l*-naproxen (250 ng) in dichloromethane (200 µl) were added 1-hydroxybenzotriazole (HOBT) (5 µg), WSC (100 µg) and Ib or IIb (40 µg), and the resulting solution was allowed to stand at 40°C. The reaction mixture was diluted with ethyl acetate and washed successively with 5% HCl, 5% $NaHCO_3$ and water. After evaporation of the solvent under an N_2 gas stream, the residue was dissolved in ethyl acetate (500 µl), and a 10 µl aliquot of the solution was injected into the chromatograph.

RESULTS AND DISCUSSION

The design of a promising derivatization reagent for the liquid chromatographic resolution of carboxylic acid enantiomers via diastereomers requires the incorporation of suitable structural features: chirality leading to efficient resolution, a reacting group toward the carboxylic acid function, and a strong chromophore or fluorophore responding to the detector with the satisfactory sensitivity. In the previous papers, we reported the preparation of *d*- and *l*-1-(4-dimethylamino-1-naphthyl)ethylamines as derivatization reagents (5) and their application to the resolution of carboxylic acid enantiomers by HPLC (8). In the determination of a trace amount of drugs in biological fluids, however, difficulties are often encountered with elimination of unreacted reagent possessing a basic group on the chromatogram. Therefore, we have attempted to develop new chiral derivatization reagents having the anthryl moiety as a fluorophore and examine their utility for the simultaneous determination of carboxylic acid enantiomers in biological fluids.

The synthetic routes to the desired compounds are shown in Fig. 1. Reaction of anthracene-1- or -2-carboxylic acid chloride with diazomethane provided the diazo derivative, which on treatment with hydroiodic acid was easily transformed into the methyl ketone. Condensation with hydroxylamine afforded the oxime, which in turn was reduced with Raney nickel to give 1-(1- or 2-anthryl)ethylamine (Ia, IIa). The opitcal resolution was accomplished by repeated fractional crystallization of the *d*- or *l*-α-methoxy-α-methyl-1-naphthaleneacetic acid salt.

The applicability of these reagents to the separation of carboxylic acid enantiomers by HPLC was then investigated. It is sufficiently substantiated that the hydrogen bonding between amide group and stationary phase is important for the efficient resolution of the diastereomers. Therefore, a normal phase column was used together with hexane/ethyl acetate as a mobile phase. Condensation of N-acetylamino acids with the chiral reagent was effected in the presence of WSC in pyridine. The derivatized enantiomers exhibited a single peak of the theoretical shape. No racemization of the product or derivatization reagent occurred, even after prolonged reaction. The retention and resolution values of four pairs of diastereomers produced with Ib and IIb are listed in Table 1. The k' and α values refer to the capacity ratio and separation factor for a pair of diastereomers, respectively. The resolution value, R, was calculated from the equation $R=2(t_{R_2}-t_{R_1})/(W_1+W_2)$, where t_{R_1} and t_{R_2} are retention times, and W_1 and W_2 are the bases of triangles derived from the peaks. It is evident from the data that the complete separation was obtained for all the pairs of N-acetylamino acids. The α value increased with increasing carbon number of the alkyl residue at the α-position. This phenomenon may be interpreted in terms of conformational rigidity of the diastereomer which is caused by the alkyl residue of α-amino acids. No exceptions were observed in the elution order of each pair of

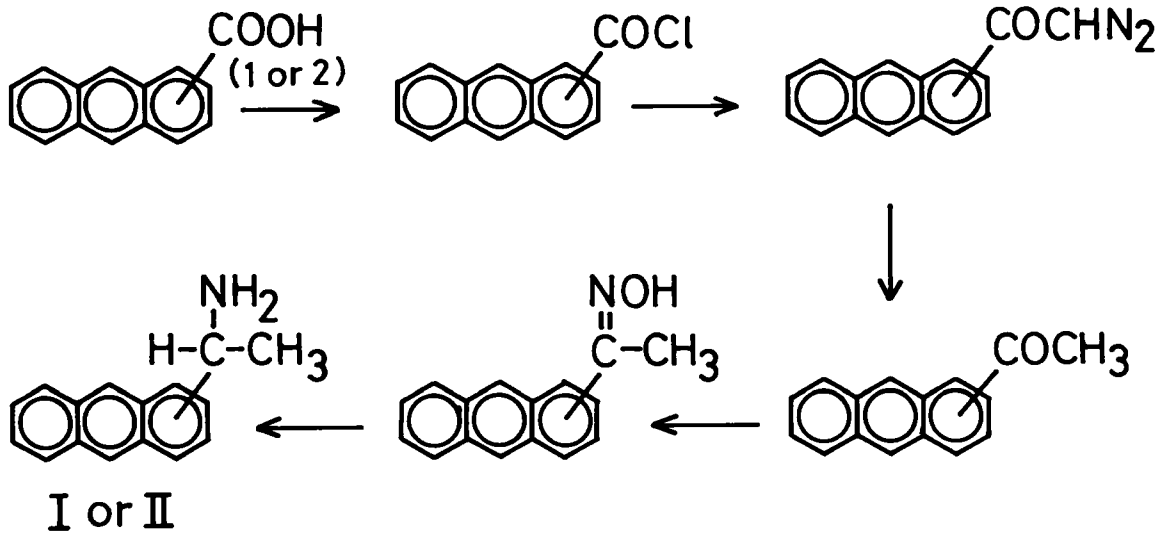

FIGURE 1 Syntheses of 1-(1-Anthryl)ethylamine (I) and 1-(2-Anthryl)ethylamine (II).
a: *dl*, b: *l*, c: *d*.

TABLE 1
HPLC Separation of Diastereomeric Amides Derived from N-Acetylamino Acids with 1-Anthrylethylamines

N-Acetylamino acid		Ib			IIb		
		k'	α	R	k'	α	R
Alanine	L	7.1	1.4	2.2	10.60	1.5	2.1
	D	10.57			15.36		
Valine	L	2.29	2.8	5.8	3.17	2.6	6.0
	D	6.32			8.38		
Leucine	L	1.57	3.9	6.9	2.13	3.4	7.0
	D	6.06			7.26		
Phenylalanine	L	1.67	3.3	5.4	2.31	3.1	6.4
	D	5.56			7.19		

Conditions: column, Resolve 5μ Spherical Silica; mobile phase, hexane/ethyl acetate (1:4), flow rate, 1.0 ml/min (t_0=1.44 min)

enantiomers; N-acetyl-L-amino acids were eluted before the corresponding D-enantiomers. This finding is in good accord with the previous result (5). In addition, no remarkable difference in the R value was observed between I and II.

The reactivity of derivatization reagents and the sensitivity of yielded diastereomers were also investigated. Several methods are available for the formation of a peptide bond. Most of them, however, are not always suitable for the quantitative derivatization of carboxylic acids. In the previous paper, we demonstrated that the combined use of WSC and HOBT is satisfactory for this purpose (8). Condensation of *dl*-naproxen with I or II was readily attained in the presence of WSC and HOBT at 40°C. The resulting solution could be directly applied to HPLC without exerting any disturbance on the chromatogram. The yield of the

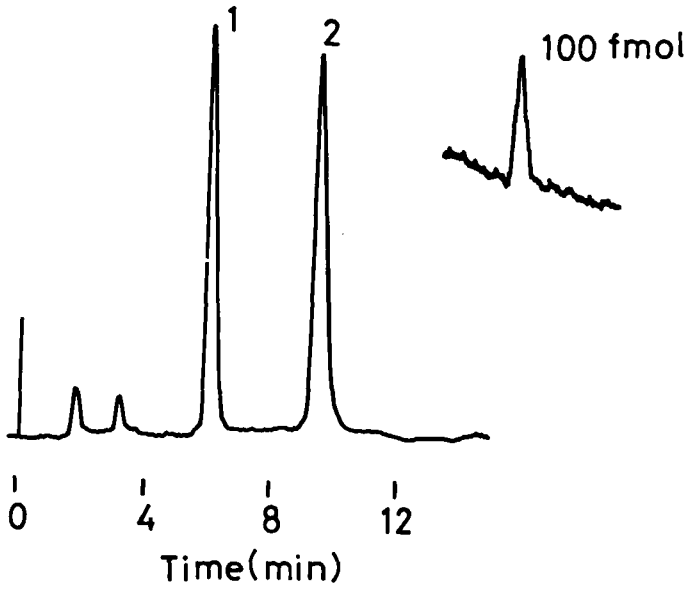

FIGURE 2 Chromatogram of Diastereomeric Amides Derived from *dl*-Naproxen with *l*-1-(1-Anthryl)ethylamine.
1: *d*-Naproxen, 2: *l*-naproxen.
Conditions: column, Resolve 5 μ Spherical Silica; mobile phase, hexane/ethyl acetate (4:1), flow rate, 1.0 ml/min (t_0=1.44 min).

diastereomer was estimated by comparison with the peak area of the standard sample.

The diastereomers formed from naproxen enantiomers with I or II were also distinctly separated on a normal phase column with hexane/ethyl acetate (4:1) as a mobile phase (R=4.5 and 6.5, respectively), exhibiting excellent sensitivity (excitation wavelength 260 nm, emission wavelength 400 nm) with a detection limit of 100 fmol (S/N=10) (Fig. 2). As illustrated in Fig. 3, the yield of the diastereomeric amides increased with increasing reaction time and reached a plateau at 60 min for II and 90 min for I. Difference in the reactivity of the two isomers toward

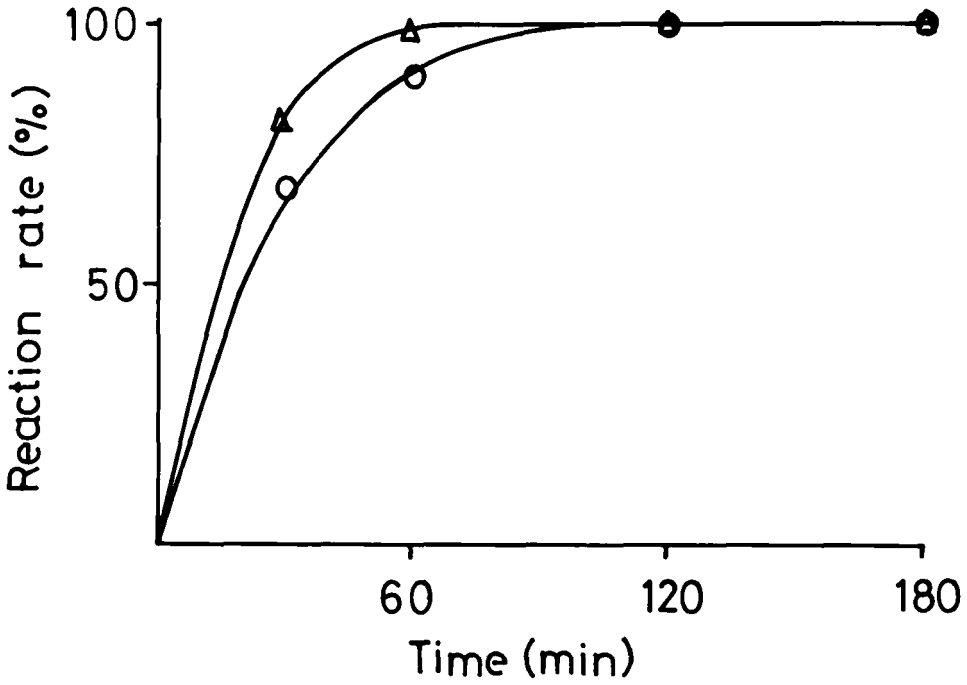

FIGURE 3 Time Course for Derivatization of *dl*-Naproxen with *l*-1-(1-Anthryl)ethylamine (-o-o-) and *l*-1-(2-Anthryl)-ethylamine (-Δ-Δ-).

carboxylic acids can be explained from a stereochemical point of view. As for the structure of I, the substituent on the anthracene nucleus may be sterically hindered by a hydrogen atom at the peri position and hence, would be somewhat unfavorable for the formation of an amide bond.

In conclusion, these chiral derivatization reagents proved to be excellent for the resolution of carboxylic acid enantiomers and their sensitive fluorometric monitoring in HPLC. Although the 1-anthracene derivative is somewhat less reactive than the positional isomer, quantitative derivatization of carboxylic acids can be attained with these two under the mild condition. The highly sensitive HPLC method may serve for the simultaneous

determination of enantiomeric drugs, such as α-arylpropionic acids, in biological fluids for pharmacokinetic studies.

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