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A gas-liquid chromatographic method for steric analysis of 2-hydroxy, 3-hydroxy, and 2,3-dihydroxy acids

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Abstract

A method was developed for assignment of the absolute configuration of oxylipin-derived 2-hydroxy acids, 3-hydroxy acids and 2,3-dihydroxy acids. The monohydroxy acids were converted into diastereomeric *N*-(propionoxyacyl)-L-phenylalanine-methyl ester (PAP) derivatives by coupling to the methyl ester of L-phenylalanine followed by propionylation, whereas the 2,3-dihydroxy acids were derivatized by treatment with L-phenylalanine methyl ester followed by acetone and perchloric acid, to afford diastereomeric *N*-(2,3-isopropylidenedioxyacyl)-L-phenylalanine-methyl ester (IAP) derivatives. The PAP and IAP derivatives were readily resolved by capillary gas-liquid chromatography. In addition, the method described allowed steric analysis of 3-hydroxy-3-methylheptanoic acid, a branched chain hydroxy acid derived from the prostaglandin analogue, misoprostol.

Keywords: Hydroxy acid; Phenylalanine; Steric analysis; Gas-liquid chromatography

1. Introduction

Steric analysis of chiral lipids by chromatographic techniques can be performed either using the so-called "direct method", which involves chromatography on a chiral stationary phase, or the "indirect method", which is based on derivatization with a chiral reagent followed by chromatographic separation of the resulting diastereomers (reviewed in [1]).

An example of steric analysis according to the indirect method was described in 1961 by Casanova and Corey [2], who were able to achieve separation by gas-liquid chromatography (GLC)

Abbreviations: DCC, *N,N'*-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; GC-MS, gas-liquid chromatography-mass spectrometry; GLC, gas-liquid chromatography; HETE, hydroxyeicosatetraenoic acid; HOT, hydroxyoctadecatrienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; HPOD, hydroperoxyoctadecadienoic acid; IAP, *N*-(isopropylidenedioxyacyl)-L-phenylalanine-methyl ester; Me_3Si, trimethylsilyl; PAP, *N*-(propionoxyacyl)-L-phenylalanine-methyl ester; SP, straight phase; TAP, *N*-(trimethylsilyloxyacyl)-L-phenylalanine-methyl ester; TLC, thin layer chromatography.

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of (±)-camphor as the diastereomeric ketals formed with (−)-2,3-butanediol. Westley and Halpern [3] used (−)-menthyl chloroformate as a derivatizing reagent to effect GLC separation of a number of 2-hydroxy acids and 2-amino acids. Subsequently, several chiral reagents, such as 2*S*-phenylpropionyl chloride [4], 1*R*-phenylethyl isocyanate [5], 2*R*-butanol [6] and *S*-Trolox™ methyl ether [7], have found use for steric analysis of a variety of chiral compounds by the indirect method. Separation of enantiomers by the direct method was described in 1939 by Henderson and Rule [8], who were able to resolve (±)-*p*-phenylene-bis-iminocamphor on a column packed with lactose. Another example was reported by Kotake et al. [9], who separated a number of racemic amino acids by paper chromatography, cellulose serving as the optically active adsorbent. During the past 10–15 years, separation of chiral compounds by the direct method has found wide use. The techniques utilized include, GLC with chiral stationary phases such as permethylated β-cyclodextrin [10], HPLC using a variety of chiral stationary phases [11], and TLC using plates impregnated with a suitable chiral selector [12].

In the oxylipin field, steric analysis by the direct method, notably chiral HPLC, is being extensively used. However, the indirect method, which as a rule is carried out with chiral fragments formed upon chemical degradation of the compound of interest, is generally more flexible and less demanding as to the number of reference compounds needed. For example, steric analysis by the direct method of hydroxy acids such as 8-HETE, 9-HETE, 11-HETE, 12-HETE, 9-HOTω6, and 13-HOTω3, requires that the *R* and *S* forms of each hydroxy acid be available as references. With the indirect method, the number of references needed is reduced to two, i.e. *R*- and *S*-malic acids.

The present work describes the use of the methyl ester of L-phenylalanine as a flexible derivatizing reagent in steric analysis of a variety of oxylipin-derived hydroxy acids.

2. Materials

2*R*,*S*-Hydroxybutanoic acid, 2*R*,*S*-hydroxyhexanoic acid, *R*,*S*- and *S*-malic acids, DL(±)-, D(−)-, and L(+)-tartaric acids, the methyl ester of L-phenylalanine hydrochloride and pig liver esterase were purchased from Sigma Chemical Co. (St. Louis, MO). 2*R*,*S*- and 2*S*-hydroxyheptanoic acids [13], 3*R*,*S*-hydroxytridecanoic acid [14], 3*R*,*S*- and 3*R*-hydroxytetradecanoic acid [14,15] and (±)-*erythro*- and (±)-*threo*-2,3-dihydroxyheptanoic acids [16] were prepared as described earlier. 2*S*,3*S*-Dihydroxyheptanoic acid was obtained by oxidative ozonolysis performed on 13*R*,14*S*-dihydroxy-9*Z*,11*E*-octadecadienoic acid, which was in turn prepared enzymatically from 13*S*-HPOD [16]. In the same way, a 1:1 mixture of 2*S*,3*S*- and 2*R*,3*S*-dihydroxyheptanoic acids was obtained from a mixture of 13*R*,14*S*- and 13*S*,14*S*-dihydroxy-9*Z*,11*E*-octadecadienoic acids [16]. *N*,*N*′-Dicyclohexylcarbodiimide and 4-dimethylaminopyridine were obtained from Fluka Chemie (Buchs, Switzerland), and 3-hydroxy-3-methylglutaric acid was purchased from Aldrich-Chemie (Steinheim, Germany). Misoprostol ((±)-15-deoxy-(16*R*,*S*)-16-hydroxy-16-methyl-prostaglandin E_1 methyl ester; mixture of the 8*R*, 11*R*, 12*R*, 16*R*/8*R*, 11*R*, 12*R*, 16*S*/8*S*, 11*S*,12*S*, 16*R*/8*S*, 11*S*,12*S*, 16*S* stereoisomers; purity in excess of 95%) was obtained from AmProst Pharmaceuticals (New York). Dry methylene chloride was prepared by refluxing over molecular sieves followed by distillation.

2.1. 2*R*,*S*-Propionoxydecanoic acid

Oleic acid (52 mg) in methanol (15 ml) containing methylene blue (5 mg) was irradiated with a 250 W halogen lamp under an atmosphere of oxygen gas at 3°C for 16.5 h. The hydroperoxide fraction isolated by SiO_2 chromatography (yield, 66%) was subjected to preparative SP-HPLC (solvent, isopropanol-hexane-acetic acid (1:99:0.02 v/v)) to afford 10*R*,*S*-hydroperoxy-8*E*-octadecenoic acid (effluent volume, 14.8–16.0 ml) and 9*R*,*S*-hydroperoxy-10*E*-octadecenoic acid (17.0–18.4 ml) in a ratio of 1:1. The two compounds were reduced with sodium borohydride and esterified by treatment with diazomethane. In this way were obtained methyl 10*R*,*S*-hydroxy-8*E*-octadecenoate (mass spectrum of Me_3Si derivative, *m/z* 369 (2%, M^+-CH_3), 337 (5, $M^+-(CH_3 + CH_3OH)$), 271 (100, $Me_3SiO^+=CH-CH=CH-(CH_2)_6-COO$-

CH_3), 129 (28, Me_3SiO^+=CH—CH=CH_2) and 73 (95, Me_3Si^+)), and methyl 9*R,S*-hydroxy-10*E*-octadecenoate (mass spectrum of Me_3Si derivative, *m/z* 369 (3%, M^+-CH_3), 337 (5, $M^+-(CH_3 + CH_3OH)$), 227 (100, Me_3SiO^+=CH—CH=CH—$(CH_2)_6$—CH_3), 129 (57, Me_3SiO^+=CH—CH=CH_2), and 73 (82, Me_3Si^+)). An aliquot of the 10-hydroxyoctadecenoate was treated with propionic anhydride (0.1 ml) and pyridine (0.1 ml) at 23°C for 18 h. Oxidative ozonolysis [13] performed on the propionyl derivative afforded 2*R,S*-propionoxydecanoic acid. As expected, saponification of an aliquot of this material yielded 2*R,S* hydroxydecanoic acid (mass spectrum of methyl ester-Me_3Si derivative, *m/z* 259 (47%, M^+-CH_3), 215 (100, $M^+-COOCH_3$), 159 (8), 129 (7, Me_3SiO^+=CH—CH=CH_2), 103 (26, Me_3SiO^+=CH_2), 89 (44, Me_3SiO^+), and 73 (83, Me_3Si^+)).

2.2.2 *R,S-Propionoxy-9-carbomethoxynonanoic acid*

Methyl 9*R,S*-hydroxy-10*E*-octadecenoate, obtained as described above, was propionylated and subjected to oxidative ozonolysis. The identity of the resulting 2*R,S*-propionoxy-9-carbomethoxynonanoic acid was ascertained following hydrolysis into 2*R,S*-hydroxydecanedioic acid and analysis by GC-MS (dimethyl ester – Me_3Si derivative, *m/z* 303 (2%, M^+-CH_3), 259 (100, $M^+-COOCH_3$), 243 (32), 155 (55, OHC—$(CH_2)_7$—C≡O^+), 109 (36), 89 (37, Me_3SiO^+), and 73 (69, Me_3Si^+)) [13].

2.3. *2S-Propionoxy-9-carbomethoxynonanoic acid*

9*S*-HOTω3, prepared as described [15], was treated with diazomethane and propionylated. Oxidative ozonolysis of the product afforded 2*S*-propionoxy-9-carbomethoxynonanoic acid. The identity of the compound was confirmed by GC-MS as described for the racemic compound.

2.4.2 *R,S-Propionoxy-5-carbomethoxypentanoic acid*

Methyl 5*Z*-eicosenoate (40 mg) was photooxygenated as described above. Purification by SiO_2 chromatography afforded a mixture of methyl 5*R,S*-hydroperoxy-6*E*-eicosenoate and methyl 6*R,S*-hydroperoxy-4*E*-eicosenoate (yield, 65%). Sodium borohydride reduction afforded a mixture of methyl 5*R,S*-hydroxy-6*E*-eicosenoate and methyl 6*R,S*-hydroxy-4*E*-eicosenoate (*m/z* (Me_3Si derivatives) 397 (M^+-CH_3), 365 ($M^+-(CH_3 + CH_3OH)$), 311 (Me_3SiO^+=CH—CH=CH—$(CH_2)_{12}$—CH_3; formed from the 5-hydroxyester), and *m/z* 215 (Me_3SiO^+=CH—CH=CH—$(CH_2)_2$—$COOCH_3$; formed from the 6-hydroxyester)). Following propionylation and oxidative ozonolysis, 2*R,S*-propionoxy-5-carbomethoxypentanoic acid was obtained. Saponification of an aliquot of the material afforded 2*R,S*-hydroxyhexanedioic acid, the identity of which was confirmed by GC-MS (dimethyl ester – Me_3Si derivative, *m/z* 247 (7%, M^+-CH_3), 231 (20, M^+-OCH_3), 203 (100, $M^+-COOCH_3$), 187 (78), 129 (55, Me_3SiO^+=CH—CH=CH_2), 99 (41, OHC—$(CH_2)_3$—C≡O^+), 89 (43, Me_3SiO^+), and 73 (75, Me_3Si^+)) [17].

2.5. *2S-Propionoxy-5-carbomethoxypentanoic acid*

5*S*-HPETE (1 mg; prepared by incubation of arachidonic acid with potato lipoxygenase [18]), was treated with sodium borohydride followed by diazomethane. The resulting methyl ester of 5*S*-HETE was propionylated and subjected to oxidative ozonolysis. This yielded 2*S*-propionoxy-5-carbomethoxypentanoic acid, the identity of which was confirmed by GC-MS as described above for the racemic compound.

2.6. *3R,S-Propionoxynonanoic acid*

Methyl 12-oxo-9*Z*-octadecenoate (30 mg; prepared by oxidation of methyl ricinoleate with CrO_3-pyridine complex) was reduced with sodium borohydride and propionylated. Oxidative ozonolysis afforded 3*R,S*-propionoxynonanoic acid. An aliquot was saponified to provide 3*R,S*-hydroxynonanoic acid (GC-MS analysis of methyl ester — Me_3Si derivative, *m/z* 245 (100, M^+-CH_3), 229 (4, M^+-OCH_3), 213 (7, $M^+-(CH_3 + CH_3OH)$), 187 (13, Me_3SiO^+=CH—$(CH_2)_5$—CH_3), 175 (63, Me_3SiO^+=CH—CH_2—$COOCH_3$), 159 (18, Me_2Si=O^+—CH=CH—$COOCH_3$), 133 (32), 89 (94, Me_3SiO^+), and 73 (92, Me_3Si^+)).

2.7. *3R-Propionoxynonanoic acid*

Methyl ricinoleate (3 mg) was propionylated

and subjected to oxidative ozonolysis to afford 3*R*-propionoxynonanoic acid. The identity of the compound was established by GC-MS analysis as described above.

2.8. Ethyl hydrogen 3R,S-acetoxy-3-methylglutarate (**3**)

3-Hydroxy-3-methylglutaric acid (**1**) (6.2 mmol) was refluxed with 10 ml of acetyl chloride for 3 h. Excess acetyl chloride was removed in vacuo. The solid residue due to the anhydride **2** (Fig. 1) was evaporated three times from chloroform and subsequently treated with 0.5 ml of 99.5% ethanol at 37°C for 15 h. The identity of the material obtained with the title compound was confirmed by GC-MS. The mass spectrum showed prominent ions at *m/z* 187 (2%, $M^+-OC_2H_5$), 172 (3, M^+-CH_3COOH), 154 (28, $M^+-(CH_3COOH + H_2O)$), 127 (100, $M^+-(CH_3COOH + OC_2H_5)$), 126 (79, $M^+-(CH_3COOH + C_2H_5OH)$), 98 (44), and 85 (30). As expected, treatment with diazomethane afforded the methyl-ethyl ester, the mass spectrum of which showed a base peak at *m/z* 141 due to elimination of $CH_3COOH + OC_2H_5$.

2.9. 3R,S-Hydroxy-3-methylheptanoic acid (**4a**)

Ethyl hydrogen 3*R,S*-acetoxy-3-methylglutaric acid (**3**) (5 mmol) and butanoic acid (62 mmol) were dissolved in methanol (100 ml) containing sodium methoxide (3 mmol). A current of 1.6 A was passed through the solution for 3 h. The reaction product was treated with 0.75 M NaOH in methanol-water (9:1 v/v) at 23°C for 18 h and extracted with diethyl ether. The residue obtained, following evaporation of the solvent, was esterified by treatment with diazomethane and purified by SiO_2 chromatography (column, 20 g; elution with diethyl ether-hexane [1:9 v/v]) to afford 1.7 mmol of methyl 3*R,S*-hydroxy-3-methylheptanoate (**4b**) (purity, 96%; yield, 34%); transparent in the region 200–320 nm; ν_{max} (film)/cm^{-1} 3523 (hydroxyl), 1737 (ester carbonyl); *m/z* (Me_3Si ether derivative) 231 (23%, M^+-CH_3), 215 (3, M^+-OCH_3), 199 (4, $M^+-(CH_3 + CH_3OH)$), 189 (100, $M^+-C_4H_9$), 173 (33, $Me_3SiO^+=C(CH_3)-C_4H_9$), 157 (24, $M^+-(C_4H_9 + CH_3OH)$), 131(25), 105 (33), 89 (52, Me_3SiO^+), 85 (47), and 73 (94, Me_3Si^+). Treatment of part of this material (100 mg) with 0.75 M

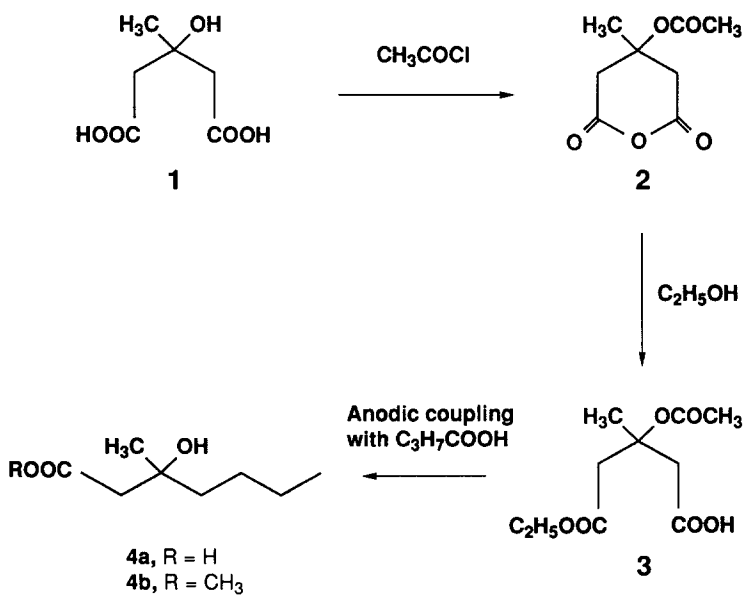

Fig. 1. Synthesis of 3*R,S*-hydroxy-3-methylheptanoic acid (**4a**) and its methyl ester (**4b**).

NaOH in methanol-water (9:1 v/v) at 23°C for 18 h yielded the free acid, 3*R,S*-hydroxy-3-methylheptanoic acid (**4a**), in virtually quantitative yield (Fig. 1). It was notable that very low concentrations of acid (**4a**) gave a distinct odor of human sweat.

2.10. Methyl hydrogen 3S-hydroxy-3-methylglutarate (**6**)

3-Hydroxy-3-methylglutaric acid (**1**) (2.7 mmol) was converted into the dimethyl ester (**5**) by treatment with diazomethane. Dimethyl ester (**5**) (2.7 mmol) was dissolved in 9 ml of 0.1 M potassium phosphate buffer pH 8.0 and stirred at 23°C for 3 h with 591 units of pig liver esterase [19]. The reaction product was subjected to SiO_2 chromatography to afford 0.8 mmol of methyl hydrogen 3*S*-hydroxy-3-methylglutarate (**6**) (yield, 30%); *m/z* (Me_3Si ester/ether derivative) 305 (16%, M^+-CH_3), 273 (12, $M^+-(CH_3 + CH_3OH)$), 247 (11, $M^+-CH_2COOCH_3$), 231 (15, M^+-Me_3SiO), 189 (33, $M^+-CH_2COOSiMe_3$), 147 (59), and 73 (100, Me_3Si^+).

2.11. 3S-Hydroxy-3-methylheptanoic acid (**7a**)

Methyl hydrogen 3*S*-hydroxy-3-methylglutarate (**6**), 0.8 mmol, was subjected to anodic coupling with butanoic acid (4 mmol) as described above for preparation of the racemic acid (**4a**). The reaction product was hydrolyzed by treatment with 0.75 M NaOH in methanol-water (9:1 v/v) at 23°C for 18 h and subjected to SiO_2 chromatography to afford the title compound. An aliquot was treated with diazomethane to provide methyl 3*S*-hydroxy-3-methylheptanoate (**7b**) (Fig. 2). Analytical data of this compound were identical with those of the corresponding racemic ester (**4b**). The optical purity of (**7a**) was 95% as determined by steric analysis of the L-phenylalanine derivative (see below).

2.12. Preparation of 3-hydroxy-3-methylheptanoic acid from misoprostol

Misoprostol (5 mg) was acetylated by treatment with acetic anhydride (0.1 ml) and pyridine (0.1 ml) at 23°C for 15 h. Oxidative ozonolysis [13] performed on the diacetate derivative followed by hydrolysis (0.75 M NaOH in methanol-water (9:1

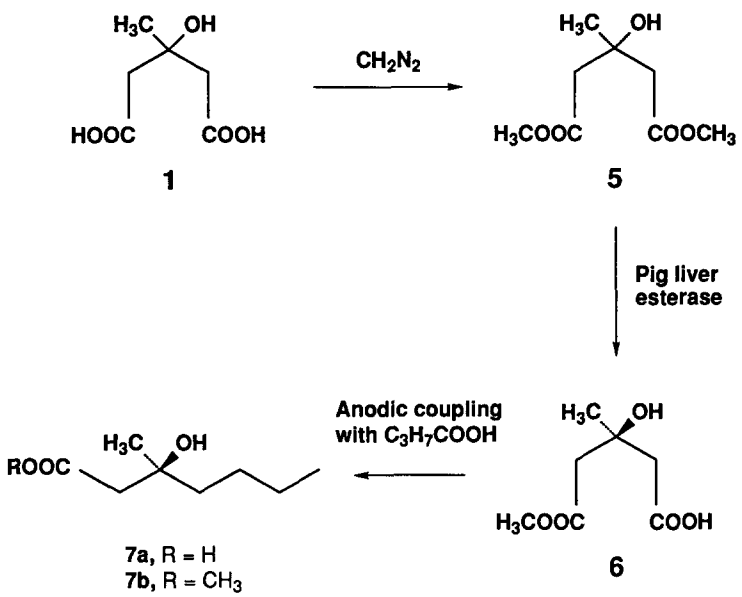

Fig. 2. Synthesis of 3*S*-hydroxy-3-methylheptanoic acid (**7a**) and its methyl ester (**7b**). The optically active intermediate, methyl hydrogen 3*S*-hydroxy-3-methylglutarate (**6**), was prepared by enzymatic hydrolysis of the prochiral dimethyl 3*R,S*-hydroxy-3-methylglutarate (**5**) using pig liver esterase.

v/v) at 23°C for 18 h) afforded 3-hydroxy-3-methylheptanoic acid. Analysis of the methyl ester/Me_3Si ether derivative by GC-MS gave results identical to those obtained for the corresponding derivative of synthetic 3*R,S*-hydroxy-3-methylheptanoic acid (**4a**).

3. Methods

GLC was carried out with a Hewlett-Packard model 5890 gas chromatograph using either a DB-210 capillary column (length, 15 m; film thickness, 0.25 μm; carrier gas, helium; flow rate, 36 or 70 cm/s as indicated) or a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm; carrier gas, helium; flow rate, 25 cm/s). GC-MS was performed with a Hewlett Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. SP-HPLC was carried out using a column of Nucleosil 50-5 (250 × 4.6 mm) purchased from Macherey-Nagel (Düren, Germany) and mixtures of isopropanol-hexane as the eluent. TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck. Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under UV light. SiO_2 column chromatography was performed with glass columns packed with silicic acid (Mallinckrodt, Paris, KY; 100 mesh, activated at 120°C). The columns were eluted under pressure with increasing concentrations of diethyl ether in hexane. FT-IR spectra were recorded with a Perkin-Elmer model 1650 instrument.

3.1. Preparation of N-(hydroxyacyl)-L-phenylalanine-methyl ester derivatives

Optimal conditions for coupling of hydroxy acids with the methyl ester of L-phenylalanine were established by experiments in which 2*R,S*-hydroxyhexanoic acid (38 μmol) and DCC (58 μmol) were treated with different amounts of L-phenylalanine methyl ester (38–381 μmol) and DMAP (39–385 μmol) in methylene chloride (1 ml) at 23°C for 0–18 h. The *N*-(2*R,S*-hydroxyhexanoyl) derivative of L-phenylalanine methyl ester was trimethylsilylated and quantitated by GLC using tetracosane (15 μmol) as internal standard. Based on these experiments, the following standard procedure was adopted for coupling of 2-hydroxy, 3-hydroxy, and 2,3-dihydroxy monocarboxylic acids with L-phenylalanine methyl ester: hydroxy acid (19 μmol) was dissolved in methylene chloride (0.5 ml) and stirred with DCC (29 μmol), L-phenylalanine methyl ester (76 μmol), and DMAP (76 μmol) at 23°C for 2 h. The same procedure was used for coupling of hydroxy dicarboxylic acids, although in these cases the amount of carboxylic acid was reduced from 19 to 9.5 μmol. Chloroform (5 ml) was added and the organic layer washed with 0.2 M hydrochloric acid followed by three portions of water. If so desired, the procedure could be scaled down by a factor of 20 (or more) to allow coupling of sub-micromolar amounts of hydroxy acid.

In a large scale preparation, 2*R,S*-hydroxyhexanoic acid (0.38 mmol) was stirred with DCC (0.58 mmol), L-phenylalanine methyl ester (1.52 mmol) and DMAP (1.52 mmol) in 10 ml of methylene chloride at 23°C for 2 h. Material obtained by extraction with chloroform was purified by silicic acid column chromatography (elution with diethyl ether-hexane (1:1 v/v)) to afford *N*-(2*R,S*-hydroxyhexanoyl)-L-phenylalanine-methyl ester as a colorless oil (0.34 mmol; yield, 89%) [λ_{max} (EtOH)/nm 252, 258, 264; ν_{max} (film/cm^{-1}) 3392 (hydroxyl), 1746 (ester carbonyl), 1657 (peptide carbonyl, 'amide I band'), 1525 (NH, 'amide II band'), and 745 and 701 (monosubstituted phenyl); *m/z* (Me_3Si ether derivative) 365 (27%, M^+), 350 (12, M^+-CH_3), 309 (26, $M^+-CH_2=CH-C_2H_5$), 278 (10), 235 (21), 162 (35, $[C_6H_5-CH=CH-COOCH_3]^+$ or its equivalent), 159 (100, $Me_3SiO^+=CH-C_4H_9$), 103 (35, $Me_3SiO^+=CH_2$), and 73 (99, Me_3Si^+)].

3.2. Preparation of N-(trimethylsilyloxyacyl)-L-phenylalanine-methyl ester (TAP) derivatives

N-(hydroxyacyl)-L-phenylalanine-methyl ester derivatives, obtained as described above, were treated with trimethylchlorosilane and hexamethyldisilazane in pyridine, and the resulting TAP derivatives were analyzed by GLC using a methyl silicone capillary column. The identity of the TAP derivatives were confirmed by GC-MS. For example, the mass spectrum of *N*-(3*R,S*-trimethylsilyloxy-3-methylheptanoyl)-L-phenylal-

anine-methyl ester showed ions of high intensity at *m/z* 393 (43%, M^+), 378 (45, M^+-CH_3), 336 (100, $M^+-C_4H_9$), 303 (26, M^+-Me_3SiOH), 215 (11, $C_4H_9-C(CH_3)(OSiMe_3)-CH_2-C\equiv O^+$), 173 (25, $Me_3SiO^+=C(CH_3)-C_4H_9$), 162 (32, $[C_6H_5-CH=CH-COOCH_3]^+$ or its equivalent), and 73 (94, Me_3Si^+).

3.3. Preparation of N-(propionoxyacyl)-L-phenylalanine-methyl ester (PAP) derivatives

N-(hydroxyacyl)-L-phenylalanine-methyl ester derivatives were treated with propionic anhydride (0.1 ml) and pyridine (0.1 ml) at 23°C for 18 h. Excess reagent was destroyed by treatment with methanol (0.1 ml) for 30 min, and the *N*-(propionoxyacyl)-L-phenylalanine-methyl ester (PAP) derivatives were isolated by extraction with diethyl ether. Alternatively, PAP derivatives were prepared by coupling of 2- and 3-propionoxy acids with the methyl ester of L-phenylalanine using the standard procedure described above. The PAP derivatives were analyzed by GLC using a DB-210 capillary column, either directly, or following purification by TLC (solvent system, ethyl acetate hexane (3.7 v/v)). GC-MS analysis confirmed the identity of the PAP derivatives. For example, the mass spectrum of *N*-(2*R*,*S*-propionoxyheptanoyl)-L-phenylalanine-methyl ester showed prominent ions at *m/z* 363 (2%, M^+), 304 (1, $M^+-COOCH_3$), 237 (3), 162 (100, $[C_6H_5-CH=CH-COOCH_3]^+$ or its equivalent), 131 (11), 120 (11), 91 (12), and 57 (85, $C_2H_5-C\equiv O^+$).

3.4. Preparation of N-(2,3-isopropylidenedioxyacyl)-L-phenylalanine-methyl ester (IAP) derivatives

N-(2,3-dihydroxyacyl)-L-phenylalanine-methyl ester derivatives, obtained by coupling of short chain 2,3-dihydroxy acids to L-phenylalanine methyl ester, were treated with acetone (2 ml) and perchloric acid (4 μl) at 23°C for 15 min. The resulting IAP derivatives were extracted with diethyl ether and subjected to GLC analysis using a methyl silicone capillary column. The identity of

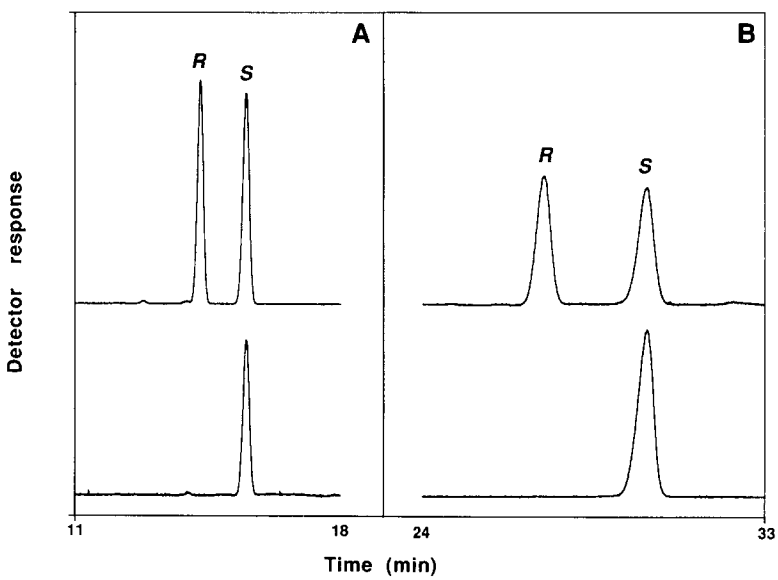

Fig. 3. (A), GLC analysis of *N*-(2-propionoxyheptanoyl)-L-phenylalanine-methyl ester derivatives. Upper panel, derivatives prepared from 2*R*,*S*-hydroxyheptanoic acid; lower panel, derivative prepared from 2*S*-hydroxyheptanoic acid. Column, DB-210; column temperature, 190°C; flow rate, 36 cm/s; (B), GLC analysis of propionyl-*bis*-L-phenylalanine-methyl ester derivatives of malic acid. Upper panel, derivatives prepared from *R*,*S*-malic acid; lower panel, derivative prepared from *S*-malic acid. Column, DB-210; column temperature, 240°C; flow rate, 70 cm/s.

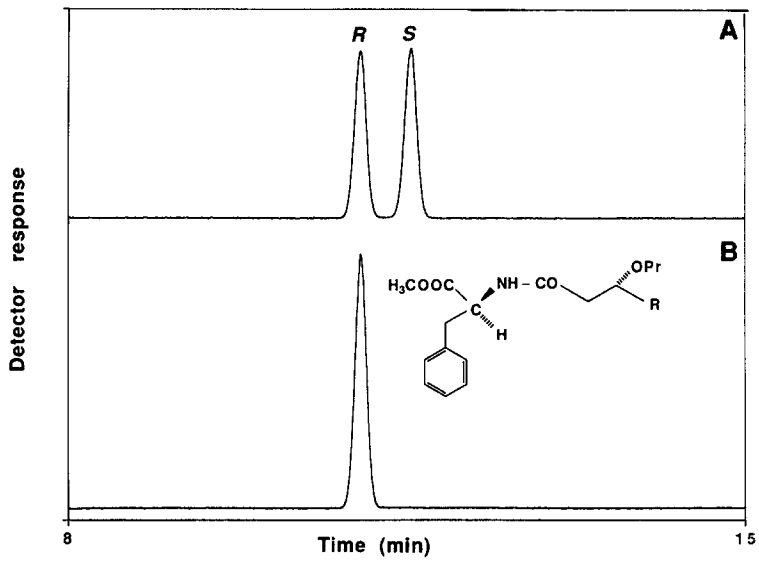

Fig. 4. GLC analysis of *N*-(3-propionoxytetradecanoyl)-L-phenylalanine-methyl ester derivatives. (A), derivatives prepared from 3*R*,*S*-hydroxytetradecanoic acid; (B), derivative prepared from 3*R*-hydroxytetradecanoic acid. Column; DB-210; column temperature, 240°C; flow rate, 36 cm/s. 'Pr', C_2H_5CO.

Table 1
Conditions and separation factors in steric analysis of 2-, 3-, and 2,3-dihydroxy acids

Compound analyzed	Derivative[a]	Column	Column temp (°C)	Flow rate (cm/s)	Separation factor[b]
2*R*,*S*-Hydroxybutanoic acid	PAP	DB-210	180	36	1.09
2*R*,*S*-Hydroxyhexanoic acid	PAP	DB-210	190	36	1.08
2*R*,*S*-Hydroxyheptanoic acid	PAP	DB-210	190	36	1.08
2*R*,*S*-Hydroxydecanoic acid	PAP	DB-210	210	36	1.07
R,*S*-Malic acid	PABP	DB-210	240	70	1.10
2*R*,*S*-Hydroxy-5-carbomethoxy-pentanoic acid	PAP	DB-210	210	36	1.05
2*R*,*S*-Hydroxy-9-carbomethoxy-nonanoic acid	PAP	DB-210	240	36	1.07
3*R*,*S*-Hydroxynonanoic acid	PAP	DB-210	210	36	1.06
3*R*,*S*-Hydroxytridecanoic acid	PAP	DB-210	230	36	1.05
3*R*,*S*-Hydroxytetradecanoic acid	PAP	DB-210	240	36	1.05
(±)-*erythro*-2,3-Dihydroxyheptanoic acid	IAP	Me-silicone	200 (1°C/min)	25	1.08
(±)-*threo*-2,3-Dihydroxyheptanoic acid	IAP	Me-silicone	200 (1°C/min)	25	1.05
(±)-Tartaric acid	IABP	DB-210	240	70	1.12
(±)-Tartaric acid	DTABP	DB-210	240	70	1.19
3*R*,*S*-Hydroxy-3-methylheptanoic acid	TAP	Me-silicone	200 (2°C/min)	25	1.02

[a]PAP, *N*-(propionoxyacyl)-L-phenylalanine-methyl ester; PABP, *N*-(propionoxyacyl)-*bis*-L-phenylalanine-methyl ester; IAP, *N*-(isopropylidenedioxyacyl)-L-phenylalanine-methyl ester; IABP, *N*-(isopropylidenedioxyacyl)-*bis*-L-phenylalanine-methyl ester; TAP, *N*-(trimethylsilyloxyacyl)-L-phenylalanine-methyl ester; DTABP, *N*-(ditrimethylsilyloxyacyl)-*bis*-L-phenylalanine-methyl ester.
[b]Monohydroxy acids, ratio between the retention times of derivatives containing the *S* and *R* hydroxy acids; *erythro*- and *threo*-dihydroxyheptanoic acids, ratio between the retention times of derivatives containing the 2*S*,3*S* and 2*R*,3*R*, and the 2*S*,3*R* and 2*R*,3*S* diols, respectively. Tartaric acid, ratio between the retention times of derivatives containing the 2*S*,3*S* and 2*R*,3*R* diols.

the IAP derivatives was ascertained by GC-MS. Thus, the mass spectrum of *N*-(*threo*-2,3-isopropylidenedioxyheptanoyl)-L-phenylalanine-methyl ester showed prominent ions at *m/z* 363 (9%, M^+), 348 (6, M^+-CH_3), 288 (16), 246 (8), 162 (100, $[C_6H_5—CH=CH—COOCH_3]^+$ or its equivalent), 157 (26), and 59 (51).

4. Results and discussion

A number of reagents, including (−)-menthyl chloroformate [3], 2*S*-phenylpropionyl chloride [4], 1*R*-phenylethyl isocyanate [5] and *S*-Trolox™ methyl ether [7], have been used for steric analysis of a variety of chiral hydroxy compounds by gas-liquid chromatography. Certain limitations have been encountered in steric analysis of hydroxy acids. For example, derivatization with (−)-menthyl chloroformate allows steric analysis of 2-hydroxy acids (but not 3-hydroxy acids), whereas use of 2*S*-phenylpropionyl chloride allows steric analysis of 3-hydroxy acids (but not 2-hydroxy acids). In the present study we have developed methodology which is generally applicable for resolution of 2- and 3-hydroxy acids, as well as for 3-hydroxy-3-methyl acids and 2,3-dihydroxy acids. In contrast to the above-mentioned reagents, which all derivatize the hydroxyl function of the hydroxy acids, the methyl ester of L-phenylalanine used in the present study derivatizes the carboxyl group of the hydroxy acids. Different ways of protecting the hydroxyl function(s) of the *N*-((di-)hydroxyacyl)-L-phenylalanine-methyl esters, including the acetyl, propionyl, trimethylacetyl, trimethylsilyl, valeryl, and isopropylidene derivatives, were tested and their effects on diastereomer separation by GLC carried out either with a non-polar stationary phase (methyl silicone) or a polar phase (DB-210) were examined.

For steric analysis of 2- and 3-monohydroxy acids, we found that the diastereomeric *N*-(propionoxyacyl)-L-phenylalanine-methyl ester (PAP) derivatives were satisfactorily resolved on a DB-210 capillary column. As shown in Fig. 3A and Fig. 4, base-line separation was achieved in separations of 2- and 3-hydroxy acids. In both cases, the derivative containing the *R*-hydroxy acid eluted first. The separation factors were 1.05–1.09 (Table 1). Also malic acid, as the propionyl derivative of the *bis*-L-phenylalanine-methyl ester conjugate, was efficiently resolved (Fig. 3B). Availability of suitable reference compounds is an important prerequisite for steric analysis by chromatographic methods. Numerous methods are available for synthesis of racemic and optically active 2- and 3-hydroxy acids. In the pre-

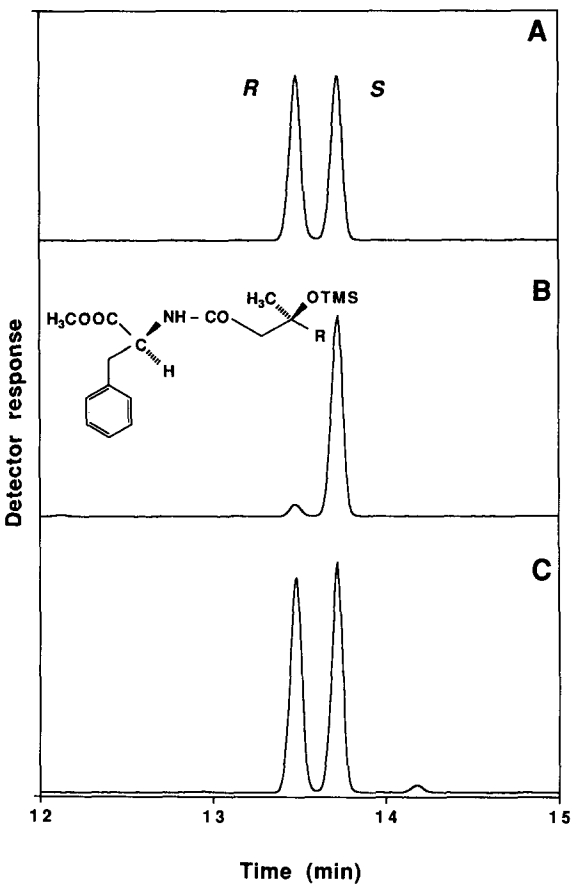

Fig. 5. GLC analysis of *N*-(3-trimethylsilyloxy-3-methylheptanoyl)-L-phenylalanine-methyl ester derivatives. (A), derivatives prepared from 3*R*,*S*-hydroxy-3-methylheptanoic acid; (B), derivative prepared from 3*S*-hydroxy-3-methylheptanoic acid; (C), derivatives prepared from 3-hydroxy-3-methylheptanoic acid generated from misoprostol by oxidative ozonolysis. Column, methyl silicone; column temperature, initially 200°C, raised at 2°C/min; flow rate, 25 cm/s. 'TMS', trimethylsilyl.

sent work, we found that photooxygenation of regioisomeric monounsaturated fatty acids into allylic hydroperoxides, followed by reduction and cleavage of the double bond by oxidative ozonolysis, offered a flexible technique for generation of a variety of racemic 2-hydroxy acids and 2-propionoxy acids:

$$R^1\text{—CH=CH—CH}_2\text{—R}^2 \xrightarrow{a,b} R^1\text{—CH(OH)—}$$

$$\text{CH=CH—R}^2 \xrightarrow{c,d} R^1\text{—CH(OCOC}_2\text{H}_5\text{)—COOH}$$

where **a** = photosensitized oxygenation, **b** = sodium borohydride reduction, **c** = propionylation, **d** = oxidative ozonolysis.

Steric analysis of 3-hydroxy-3-methylheptanoic acid using previously described techniques [3–7,20] proved unsuccessful, however, the diastereomeric *N*-(3-trimethylsilyloxy-3-methylheptanoyl)-L-phenylalanine-methyl ester (TAP) derivatives were resolved by GLC using methyl silicone as the stationary phase (Fig. 5). The separation factor was 1.02 and the derivative containing the *R*-hydroxy acid formed the first eluting peak. 3-Hydroxy-3-methylheptanoic acid is produced from the prostaglandin analogue, misoprostol ((±)-15-deoxy-(16*R*,*S*)-16-hydroxy-16-methyl-prostaglandin E_1 methyl ester) upon oxidative ozonolysis. Misoprostol is manufactured and marketed as a mixture of four stereoisomers, one of which (8*R*,11*R*,12*R*,16*S*-misoprostol) is responsible for the biological activity [21]. The availability of a sensitive method for steric analysis of 3-hydroxy-3-methylheptanoic acid, and hence of misoprostol, is a valuable complement to existing chiral HPLC techniques [22] for analysis of misoprostol isomers.

Conjugates of L-phenylalanine methyl ester proved successful also for steric analysis of 2,3-

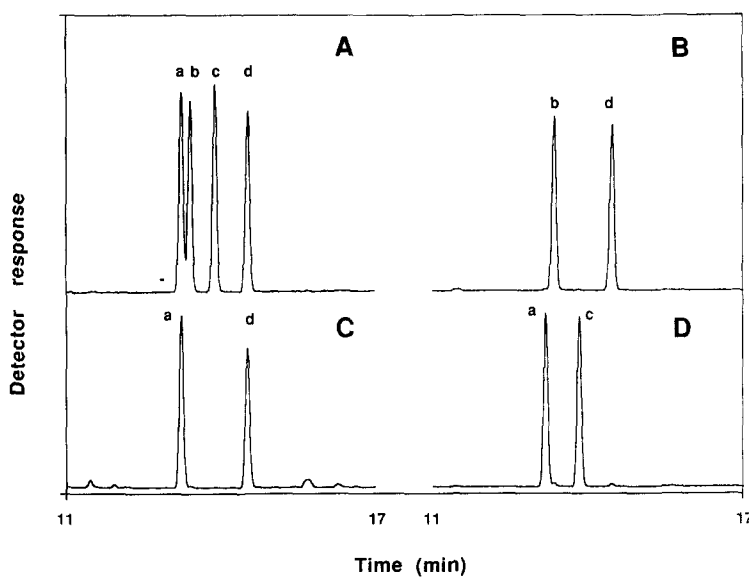

Fig. 6. GLC analysis of *N*-(2,3-isopropylidenedioxyheptanoyl)-L-phenylalanine-methyl ester derivatives. (A), derivatives prepared from a mixture of (±)-*erythro*- and (±)-*threo*-2,3-dihydroxyheptanoic acids; (B), derivatives prepared from (±)-*erythro*-2,3-dihydroxyheptanoic acids; (C), derivatives prepared from a mixture of 2*R*,3*S*- and 2*S*,3*S*-dihydroxyheptanoic acids; (D), derivatives prepared from (±)-*threo*-2,3-dihydroxyheptanoic acids. The absolute configurations of the diol structures of derivatives forming peaks **a–d** are: **a**, 2*R*,3*S*; **b**, 2*R*,3*R*; **c**, 2*S*,3*R*; **d**, 2*S*,3*S*. Column, methyl silicone; column temperature, 200°C, raised at 1°C/min; flow rate, 25 cm/s.

dihydroxy acids. In this case, separation of the diastereomeric *N*-(2,3-dihydroxyacyl)-L-phenylalanine-methyl esters was achieved either as the isopropylidene derivatives or as the ditrimethylsilyl ether derivatives. As seen in Fig. 6B,D, the derivatives of (±)-*erythro*- and (±)-*threo*-2,3-dihydroxyheptanoic acids were individually well resolved on a methyl silicone column into the 2*R*,3*R*/2*S*,3*S* and 2*R*,3*S*/2*S*,3*R* isomers, respectively, although there was partial overlapping between the peaks of the 2*R*,3*S* and 2*R*,3*R* derivatives (Fig. 6A). Also, (±)-tartaric acid (2*R*,3*R*- and 2*S*,3*S*-dihydroxysuccinic acids) were well resolved using the DB-210 column (Table 1).

The methodology described in the present study has recently been applied for determination of the absolute configurations of a number of unsaturated mono- and di-hydroperoxides formed from arachidonic acid in the presence of two lipoxygenases from rice[1]. In those analyses, the hydroperoxides (50–500 μg) were reduced into the corresponding hydroxy compounds, esterified by treatment with diazomethane, and propionylated. The 2-propionoxy acids produced on oxidative ozonolysis were coupled to L-phenylalanine methyl ester and subjected to steric analysis by GLC.

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