All (*S*) Stereoconfiguration of 7,10-Dihydroxy-8(*E*)-octadecenoic Acid from Bioconversion of Oleic Acid by *Pseudomonas aeruginosa*

Harold W. Gardner[a] and Ching T. Hou[b,*]

[a]Bioactive Agents Research and [b]Oil Chemical Research, NCAUR, ARS, USDA, Peoria, Illinois 61604

ABSTRACT: A previously established method was utilized to determine the stereoconfiguration of 7,10-dihydroxy-8(*E*)-octadecenoic acid (DHOE) from bioconversion of oleic acid by *Pseudomonas aeruginosa* NRRL strain B-18602 (PR3). The method involved formation of the (–)-menthoxycarbonyl (MCO) derivative of the two hydroxyls, oxidative cleavage of the double bond, and gas chromatography (GC) analysis of the two methyl-esterified diastereomeric fragments, methyl 2-MCO-decanoate and dimethyl 2-MCO-octanedioate. As described by previous workers, the 2(*S*)-MCO derivatives elute at earlier times by GC than the 2(*R*)-MCO derivatives. By comparing the GC analysis of the 2-MCO derivatives obtained from DHOE with that obtained from a partially racemized sample, DHOE was determined to be 7(*S*),10(*S*)-dihydroxy-8(*E*)-octadecenoic acid.

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Hou and coworkers (1,2) determined that a *Pseudomonas* sp. PR3 isolate was able to convert oleic acid into a dihydroxylated monounsaturated fatty acid [abbreviated DOD by these workers (1,2)]. Spectral methods (1–4) were used to characterize the fatty acid as 7,10-dihydroxy-8(*E*)-octadecenoic acid (DHOE). The *Pseudomonas* PR3 isolate was subsequently identified as *P. aeruginosa* NRRL strain B-18602 (PR3) (5). Knothe *et al*. (4) used circular dichroism (CD) to assign an all (*R*) stereoconfiguration to the two hydroxyls of DHOE.

Other workers have reported DHOE production by *Pseudomonas* strain 42A2 from olive oil (6,7) and from oleic acid (8,9); however, no absolute stereochemical determination was performed. In the case of strain 42A2, it appeared that 10-hydroperoxy-8(*E*)-octadecenoic acid from lipoxygenase oxygenation of oleic acid was the precursor of DHOE through intermediate 10-hydroxy-8(*E*)-octadecenoic acid (8); whereas bioconversion with *P. aeruginosa* NRRL strain B-18602 (PR3) appeared to proceed through precursor 10-hydroxy-8(*Z*)-octadecenoic acid (5).

*To whom correspondence should be addressed at NCAUR, ARS, USDA, Peoria, IL 61604. E-mail: houct@mail.ncaur.usda.gov

In the present work we used an alternative method to CD in order to determine the all (*S*) stereoconfiguration of the two hydroxyls of DHOE from *P. aeruginosa* NRRL strain B-18602 (PR3); that is, the gas chromatographic (GC) separation of diastereomeric (–)-menthoxycarbonyl (MCO) derivatives of the hydroxyls.

EXPERIMENTAL PROCEDURES

DHOE produced from oleic acid by *P. aeruginosa* NRRL strain B-18602 (PR3) was isolated as previously described (1,2). DHOE was methyl-esterified with diazomethane in diethyl ether/methanol (9:1, vol/vol). Racemic methyl 2-hydroxydecanoate was obtained from Matreya Inc. (Pleasant Gap, PA).

For chiral analysis 1 mg of the esterified DHOE was reacted with (–)-menthoxycarbonylchloride (Aldrich, Milwaukee,WI), and the MCO derivative was isolated by thin-layer chromatography (TLC) using hexane/ethyl acetate (185:15, vol/vol) development as previously described (10). This method (10) was modified to oxidatively cleave the MCO derivative with 12 mg $KMnO_4$ in 0.3 mL acetic acid for 1 h at 37°C (11). After oxidative cleavage, acetic acid was evaporated with a stream of N_2, and the products were methyl-esterified with diazomethane. The methyl 2-MCO-decanoate and dimethyl 2-MCO-octanedioate obtained by this procedure were dissolved in hexane and separated by GC monitoring by mass spectrometry (MS). As described previously, the methyl 2(*S*)-MCO-alkanoates and dimethyl 2(*S*)-MCO-alkanedioates elute before the corresponding 2(*R*)-MCO derivatives (10–17). Methyl 2(*R,S*)-MCO-decanoate was prepared from racemic methyl 2-hydroxydecanoate by the method described above (10) followed by TLC isolation.

A sample of DHOE was partially acid-racemized to serve as a basis for the separation of 2(*R*)- and 2(*S*)-MCO derivatives. For this purpose, 2 mg DHOE was treated with 150 μL tetrahydrofuran/H_2O/HCl (4:1:1, vol/vol/vol) for 17.5 h at room temperature. After reaction, the partially racemized DHOE was extracted into $CHCl_3$ with 1 mL $CHCl_3$/H_2O (1:1, vol/vol) and the $CHCl_3$ layer washed with 0.5 mL H_2O. The sample was reesterified with diazomethane.

As standards, a mixture of methyl 2-MCO-heptano-

ate 2(*S*)/2(*R*) (87:13) and dimethyl 2-MCO-decanedioate 2(*S*)/2(*R*) (97:3) was produced by applying the method to a mixture of methyl 9-hydroxy-10,12-octadecadienoate (76%) and methyl 13-hydroxy-9,11-octadecadienote (24%) obtained by oxidation of linoleic acid by tomato lipoxygenase (18) followed by $NaBH_4$ reduction of the resultant hydroperoxides. The methyl hydroxyoctadecadienoates were purified by TLC using hexane/ethyl ether (4:2, vol/vol). In order to confirm the MCO method (percentage data given above), the methyl hydroxyoctadecadienoates generated by tomato lipoxygenase were analyzed by an independent chiral analysis employing straight-phase high-performance liquid chromatography (HPLC) separation of positional and geometric isomers (19) followed by chiral-phase HPLC of collected peaks from the former by a Chiracel OB column (20). By this HPLC method, the composition of the mixture was determined to be confirmatory of analysis by the MCO method: methyl 13(*S*)- and 13(*R*)-hydroxy-9(*Z*),11(*E*) octadecadienoate, 20 and 1%, respectively; methyl 13(*R,S*)-hydroxy-9(*E*),11(*E*)-octadecadienoate, 1.5%; methyl 9(*S*)- and 9(*R*)-hydroxy-11(*E*),12(*Z*)-octadecadienoate, 76 and 1%, respectively; and methyl 9(*R,S*)-hydroxy-11(*E*),12(*E*)-octadecadienoate, 0.75%. The (*E,E*)-diene isomers are generally racemic, and thus would contribute to the (*R*)-composition by the MCO method.

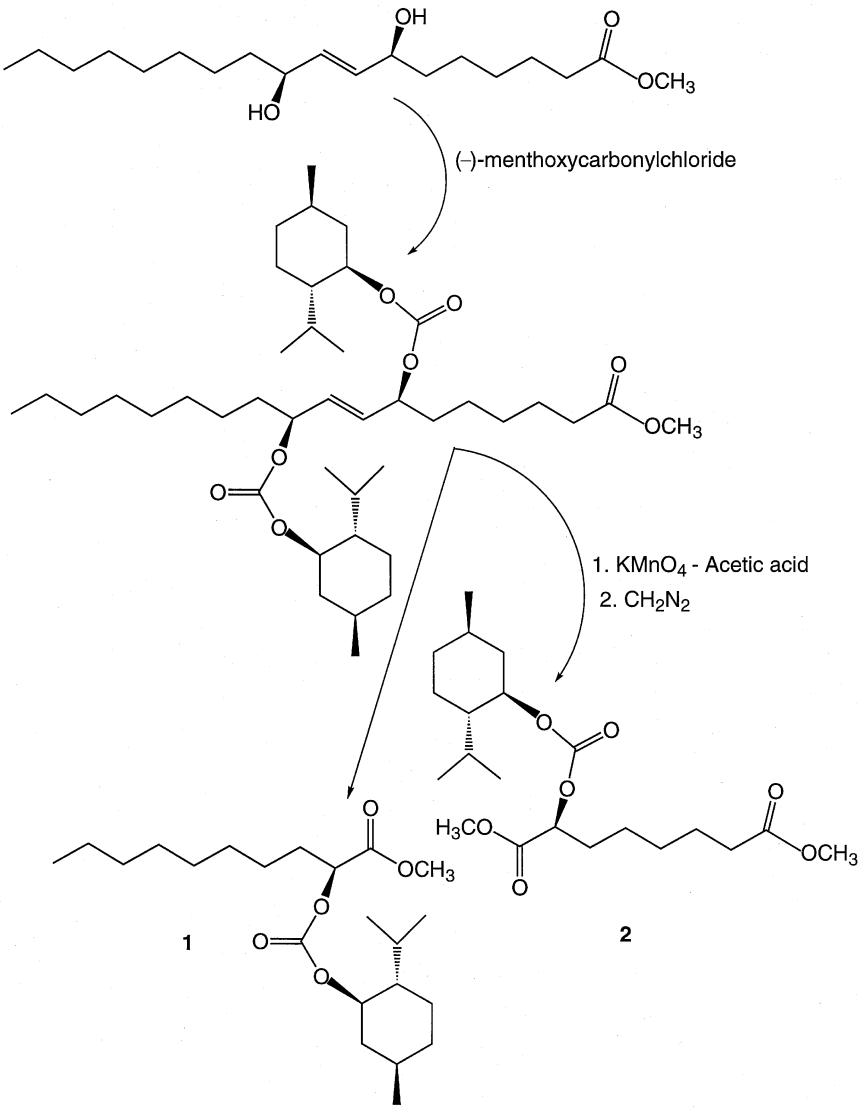
GC/MS was completed with a Hewlett-Packard Model 5890 (Palo Alto, CA) gas chromatograph interfaced with a Model 5971 mass-selective detector operating at 70 eV. The capillary column used was a Hewlett-Packard HP-5MS cross-linked 5% phenyl methyl silicone, 0.25 mm × 30 m, film thickness 0.25 µm. The methyl 2-MCO-alkanoates and dimethyl 2-MCO-alkanedioates were separated by temperature programming from 100 to 260°C at a rate of 2°C/min (He flow rate = 0.67 mL/min).

RESULTS AND DISCUSSION

Analysis of DHOE by the MCO method (10) adapted to hydroxyl fatty methyl esters would afford two fragments that would be useful for determining the stereoconfiguration of each hydroxyl (Scheme 1). As seen in Scheme 1, oxidation to the two fragments would not change the original stereoconfiguration of the hydroxyls of DHOE; that is, stereoconfiguration is based on either the double bond or carboxylic acid group of the oxidized derivative as a point of reference. The 2(*S*)-MCO-diastereomer of methyl-esterified 2-hydroxy acids and amino acids is always reported to elute by GC earlier than the 2(*R*)-MCO-diastereomer. Among the compounds previously tested were lactic acid, 3-phenyllactic acid, medium-chain 2-hydroxy iso-fatty acids, several amino acids (12), several even-numbered 2-hydroxy fatty acids from 14:0 to 26:0 (13), 2-hydroxyheptanoic acid (10), and one of the two acids characterized in this study, 2-hydroxydecanoic acid (14). After DHOE was subjected to chiral analysis by the MCO method, only two peaks were revealed by GC/MS originating from opposite ends of the fatty chain; however, no chiral (*R*) and (*S*) separation was evident (Fig. 1). The first eluting peak at 60.25 min was determined to be solely one diastereomer by comparison with standard racemic methyl 2(*R,S*)-MCO- decanoate (Fig. 1). Additionally, by coinjection of the racemate with the MCO derivative from DHOE it was shown that methyl 2-MCO-decanoate from DHOE coincided with the earlier-eluting peak, compared to racemic methyl 2(*R,S*)-MCO-decanoate, confirming that the derivative was 2(*S*) (data not shown); that is, the 10-hydroxyl was thus established as (*S*) based on elution order previously reported for this MCO derivative (14). The isomers of methyl 2-MCO-decanoate afforded virtually identical mass spectra, regardless of its origin from DHOE, 2(*S*), or the racemic methyl 2-hydroxydecanoate, 2(*R,S*), as follows: *m/z* (% relative intensity) 247 (4), 215 (1.5), 185 (3), 153 (6), 139 (55), 138 (100), 123 (29), 109 (8), 95 (52), 83 (68), 81 (51), 69 (32), 55 (41).

Based on the melting point, Knothe *et al.* (21) reported that DHOE had a *threo*-2(*E*)-1,4-diol structure. It follows from the 10(*S*)-hydroxyl and the *threo*-2(*E*)-1,4-diol that the hydroxyls are all (*S*). However, we wished to confirm the assignment of the 7(*S*)-hydroxyl by the MCO method. The elution order of dimethyl 2(*S*)- and 2(*R*)-MCO-octanedioate has not been established in the literature, but several members of the series always showed the (*S*)-isomer eluting before the (*R*)-isomer, in analogy with the series of 2-hydroxy acids. Those dimethyl MCO derivatives of dioic acids examined previously were malic acid (11,15), 2-hydroxypentanedioic acid (16), 2-hydroxynonanedioic acid (17), and 2-hydroxydecanedioic acid (10). Thus, it is unlikely that there would be a reversal of elution order for the dimethyl MCO derivative of 2-hydroxyoctanedioic acid.

Like the first GC–MS peak, the second peak was a single isomer eluting at 65.1 min (Fig. 1). Because of its chiral purity, there was no point of reference. Since standard racemic dimethyl 2-hydroxyoctanedioate was not commercially available, the experimental strategy was to partially racemize a sample of DHOE and then subject it to chiral analysis. It is known that allylic alcohols undergo methoxyl substitution in acidic methanol (22). Therefore, the structure of DHOE is ideally suited for racemization by hydroxyl substitution in acidic aqueous solution. Chiral analysis of partially racemized DHOE is shown in Figure 2, where one can see that both methyl 2-MCO-decanoate (elution time centered at about 60.4 min) and dimethyl 2-MCO-octanedioate (elution time centered at about 65.2 min) were separated into 2(*S*)- and 2(*R*)-isomers. As expected, the mass spectra of the 2(*S*) and 2(*R*) isomeric peaks of dimethyl 2-MCO-octanedioate were virtually identical as follows: *m/z* (ion structure, % relative intensity) 369 (M^+ – MeO, 0.2), 263 (0.6), 231 (7), 219 (24), 187 (23), 169 (7), 159 (35), 155 (12), 139 (40), 138 (100), 137 (64), 127 (22), 123 (38), 109 (29), 95 (70), 83 (77), 81 (87), 69 (26), 55 (51). The dimethyl 2-MCO-octanedioate derived from the original unracemized DHOE coeluted with the first peak of dimethyl 2-MCO-octanedioate from racemized DHOE, confirming the original DHOE as the 7(*S*)-hydroxyl isomer. Therefore, the complete structure of DHOE was found to be 7(*S*),10(*S*)-dihydroxy-8(*E*)-octadecenoic acid.



SCHEME 1

Because the liquid phase of our capillary column was phenyl methyl silicone, instead of the fluoropolymers generally used to separate the MCO-derivatives, we wished to determine the relative amount of separation between 2(*S*)-MCO and 2(*R*)-MCO derivatives by examining known analogous members of the series to ascertain whether the derivatives examined in this study showed similar separation. Known MCO derivatives were synthesized from a mixture of methyl hydroxyoctadecadienoates obtained from lipoxygenase oxidation of linoleic acid. Lipoxygenase oxidation typically gives predominantly the (*S*)-isomer, but the reaction is not absolutely stereospecific, giving a minor amount of the (*R*)-isomer to serve as a point of reference (see the Experimental Procedures section). As described previously (10), methyl 9(*S*)-hydroxy-10,12-octadecadienoate and methyl 13(*S*)-hydroxy-9,11-octadecadienoate were sources of dimethyl 2(*S*)-MCO-decanedioate and methyl 2(*S*)-MCO-heptanoate, respectively. Figure 3 shows a coinjection of the mixture of MCO derivatives obtained from lipoxygenase-derived products with those obtained from partially racemized DHOE. This chromatogram shows the extent of separation was similar for all derivatives examined, and that 2(*S*)-MCO derivatives eluted before the 2(*R*)-MCO derivatives.

Based on a previous determination that DHOE had a *threo*-2(*E*)-1,4-diol assignment (21) agrees with either the all-(*S*) stereoconfiguration found in this study or with an all-(*R*) assignment reported previously (4). In the previous report that disagrees with the present study, the researchers used CD to assign an all-(*R*) stereoconfiguration to DHOE (4). Based on a positive Cotton effect that reached a maximum at 188 nm, near where an olefin would be expected to absorb, Knothe *et al*. (4) concluded that DHOE was all-(*R*). This assignment by Knothe *et al.* agreed with a considerable body of evidence showing that cyclic allylic (*R*)-alcohols afforded more positive optical rotary dispersion (ORD) and positive Cotton effects, compared to the (*S*)-enantiomer (23–25). And, nonal-

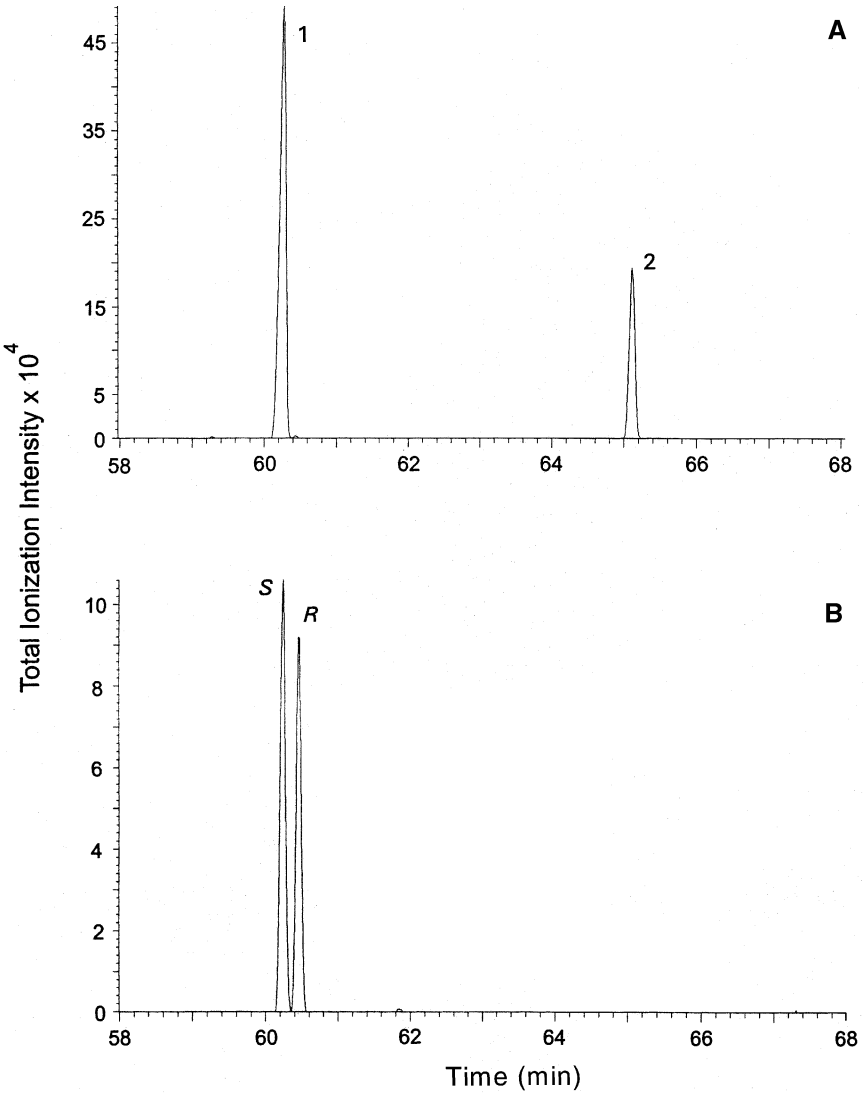


FIG. 1. (A) Total ion chromatogram of methyl 2-menthoxycarbonyl (MCO)-decanoate (peak 1) and dimethyl 2-MCO-octanedioate (peak 2) derived from 7,10-dihydroxy-8(*E*)-octadecenoic acid (DHOE). (B) Total ion chromatogram of methyl 2(*S,R*)-MCO-decanoate standard; the 2(*S*)-isomer elutes first.

lylic (*R*)-hydroxy fatty esters, such as methyl 12(*R*)-hydroxy-9(*Z*)-octadecenoate (ricinoleate) and methyl 14(*R*)-hydroxy-11(*Z*)-eicosenoate (lesquerolate), supported the assignment by Knothe *et al.*, as these fatty esters furnished positive ORD curves (reviewed in Ref. 26).

However, with the introduction of vicinal double bonds, a different result becomes apparent. Methyl 9D(*S*)-hydroxy-10(*E*),12(*E*)-octadecadienoate afforded a plain positive ORD curve (26), and methyl 13L(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoate furnished an $[\alpha]_{300}$ of +103° (27), despite the fact that their relative orientation to the ester group is opposite (D vs. L). As observed by Smith (26), the (*S*)-configuration relates to the vicinal double bonds (the chromophore), and hence the rotational direction is dependent on the vicinal diene. As expected from the data above, 13D(*R*)-enantiomer gave a plain negative ORD curve (26). Koshino *et al*. (28) isolated four different (*R*)-hydroxy fatty acids with a vicinal monoene, and with each of the methyl esters they obtained a negative specific rotation at the α_D line of Na. Therefore, it would appear that interpretation of the CD of DHOE by Knothe *et al*. (4) is suspect, and a more facile interpretation of DHOE CD might be accomplished by using the exciton-coupled CD method with hydroxyls derivatized with either *p*-bromobenzoate (29) or 2-naphthoate (30).

Finally, it should be noted that the DHOE obtained from biotransformation of olive oil by *Pseudomonas* 42A2 (6) has virtually the same melting point (1) as the DHOE studied here, suggesting that both are *threo*-2(*E*)-1,4-diols. Although the stereoconfiguration of the hydroxyls of DHOE from *Pseudomonas* 42A2 have not been studied, the present work indicates that these hydroxyls may also be all (*S*).

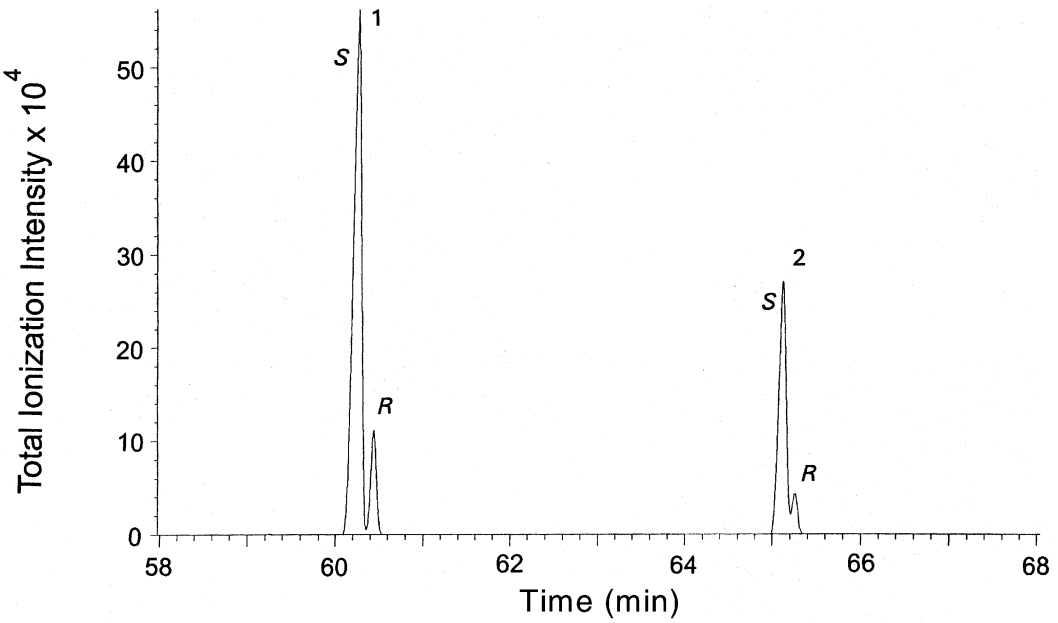


FIG. 2. Total ion chromatogram of methyl 2-MCO-decanoate (peak 1, centered at 60.5 min) and dimethyl 2-MCO-octanedioate (peak 2, centered at 65.3 min) derived from DHOE that was partially racemized by acid treatment. The 2(*S*)-isomers elute at earlier times. For abbreviations see Figure 1.

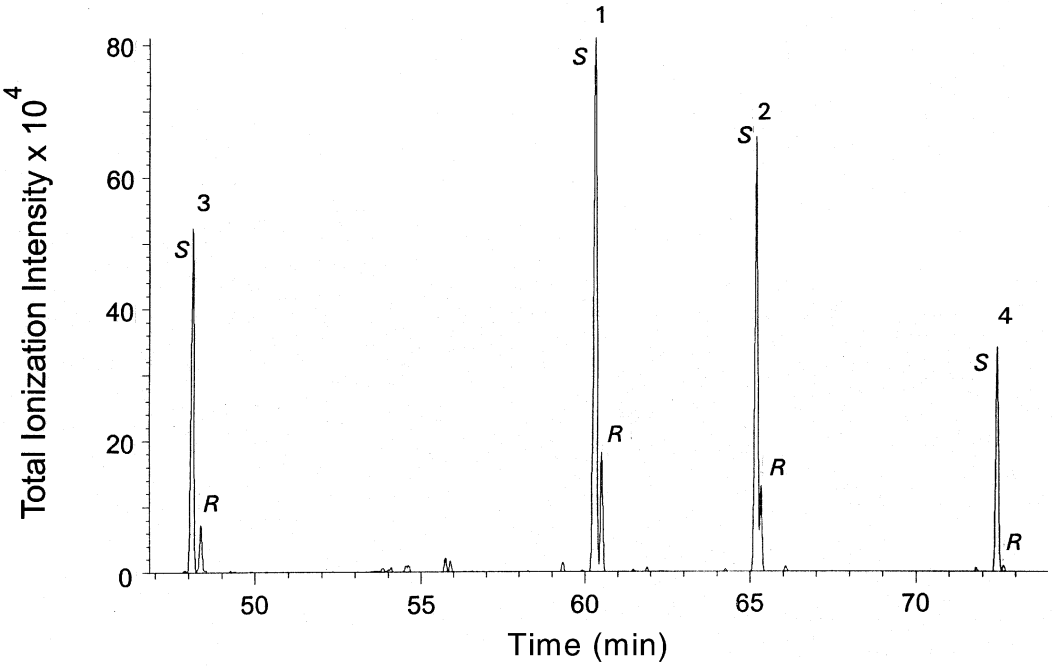


FIG. 3. Total ion chromatogram of coinjected MCO derivatives from partially racemized DHOE with MCO derivatives from lipoxygenase-oxidized linoleic acid. From racemized DHOE: methyl 2(*S,R*)-MCO-decanoate, elution time of 60.2 to 60.7 min (peak 1), and dimethyl 2(*S,R*)-MCO-octanedioate, elution time of 65.0 to 65.5 min (peak 2). From lipoxygenase-oxidized linoleic acid: methyl 2-MCO-heptanoate [2(*S*)/2(*R*) ratio of 87:13], elution time of 48.0 to 48.5 min (peak 3), and dimethyl 2-MCO-decanedioate [2(*S*)/2(*R*) ratio of 97:3], elution time of 72.4 to 73.1 min (peak 4). See Figure 1 for abbreviations.

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