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NMR and HPLC-MS/MS analysis of synthetically prepared linoleic acid diol glucuronides

Jie Yang, Martha D. Morton **, Dennis W. Hill, David F. Grant *

Department of Pharmaceutical Sciences (JY, DH, DFG) and Chemistry (MDM), University of Connecticut, Storrs, CT 06269, United States

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Abstract

Hydroxylated fatty acids are important mediators of many physiological and pathophysiological processes in a variety of human tissues. Recent evidence shows that in humans many of these are ultimately excreted in the urine as the glucuronide conjugates. In this paper we describe a general approach for the chemical synthesis of glucuronide conjugate derivatives of fatty acids. The synthesis strategy employs three steps (epoxidation, hydrolysis and glucuronidation) using methyl linoleate as a model non-hydroxylated starting compound. Hydroxylated starting compounds would require only the glucuronidation step. NMR and HPLC-MS/MS experiments were used to help determine the structure of the synthesized glucuronide conjugates and to identify fragmentation product ions useful for discriminating positional isomers in biological samples. This synthetic strategy should prove useful for generating analytical standards in order to identify and quantify glucuronide metabolites of hydroxylated fatty acids in humans.

Keywords: Linoleic acid diols; Hydroxylated fatty acids; Glucuronides; NMR; HPLC-MS/MS

1. Introduction

Glucuronides are a pharmaceutically important class of compounds (Rukhman et al., 2001). Some are antiallergenic (Takagaki et al., 1999), or have antimalarial activity (Ramu and Baker, 1995), however, they are primarily known for their importance in drug metabolism and elimination. There are also a variety of endogenous compounds which are glucuronidated and excreted in the urine. Many fatty acids and fatty acid metabolites are known to be glucuronidated prior to excretion and many of these have been shown to be associated with various pathophysiological conditions. For example, linoleic acid is metabolized in vivo and in vitro to a variety of compounds, including some which have been reported to be cytotoxic (Sevanian et al., 1979; Laethem et al., 1992; Moghaddam et al., 1996). Glucuronidation of linoleic acid and linoleic acid diols by human liver microsomes was studied earlier to investigate the role of glucuronide conjugation in the metabolism of linoleic acid diols (Jude et al., 2000). Street et al. (1996a,b) identified 16 C16 and C18 glucuronide-conjugated fatty acid diols in the urine samples of patients with generalized peroxisomal disorders by using negative ion

* Corresponding author. Tel.: +1 860 486 4265; fax: +1 860 486 4998.
** Co-corresponding author. Present address: Department of Chemistry, University of Connecticut, Storrs, CT 06269, United States. Tel.: +1 860 486 4069; fax: +1 860 486 2981.
E-mail addresses: martha.morton@uconn.edu (M.D. Morton), david.grant@uconn.edu (D.F. Grant).

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fast atom bombardment-mass spectrometry. The major compounds found were glucuronides of 9,10 and 12,13 linoleic acid diol. Glucuronides of arachidonic acid metabolites are also excreted in human urine (Costa et al., 1996; Watzer et al., 2000; Turgeon et al., 2003).

The in vivo metabolic pathway that is thought to produce linoleic and arachidonic acid glucuronides (epoxidation (Sevanian et al., 1979; Laethem et al., 1992; Moghaddam et al., 1996), hydrolysis (Watabe and Akamatsu, 1972; Westkaemper and Hanzlik, 1981; Bellucci et al., 1989; Wistuba et al., 1989) and glucuronidation (Dutton, 1980; Kirkpatrick et al., 1984; Radominska et al., 1997)) is very similar to the chemical synthesis strategy employed here using methyl linoleate as the starting compound.

2. Experimental procedures

Chemicals were purchased from either Sigma (St. Louis, MO, USA) or Aldrich Chemical Ltd. (Milwaukee, WI, USA). Solvents used for chemical synthesis were purchased from commercial sources were of analytical grade and were used without further purification unless otherwise stated. All NMR spectra were recorded on DRX-400 and Avance 500 MHz Bruker NMR spectrometers using tetramethylsilane as internal standard and $CDCl_3$ as solvent for linoleic derivatives. A solvent mixture of $CDCl_3$:CD_3OD was used for glucuronide derivatives of linoleic acid diol. HPLC purification was performed with a Shimadzu HPLC 10A VP series using a semi-preparative silica column, analytical silica column and/or reverse phase C_8 column from Varian and Agilent, respectively. HPLC-MS experiments were performed on a Waters 616/626 HPLC system coupled to a Micromass Quattro II Tandem Mass Spectrometer (Fisons Instruments, Manchester, UK) using an electrospray (ES) or atmospheric pressure chemical ionization sources (APCI). All HPLC-MS/MS analysis was performed on an HP1090 HPLC system coupled to a Micromass Q-ToF2 mass spectrometer (Micromass, Manchester, UK). All column chromatographic separations were done on silica gel 60 (230–400 mesh), whereas all TLC (silica gel) developments were performed on silica gel coated on aluminum 250 μm layer sheets (Whatman, Maidstone, UK).

2.1. Synthesis procedures

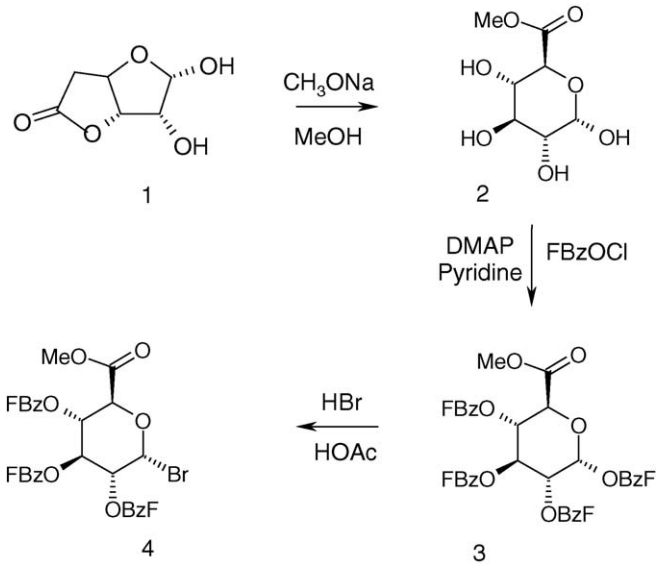
Schemes 1 and 2 show the overall synthesis strategy for synthesizing linoleic acid diol glucuronides **(9)** using methyl linoleate **(5)** as the starting fatty acid.

2.1.1. *Methyl-D-glucuronate (2)*

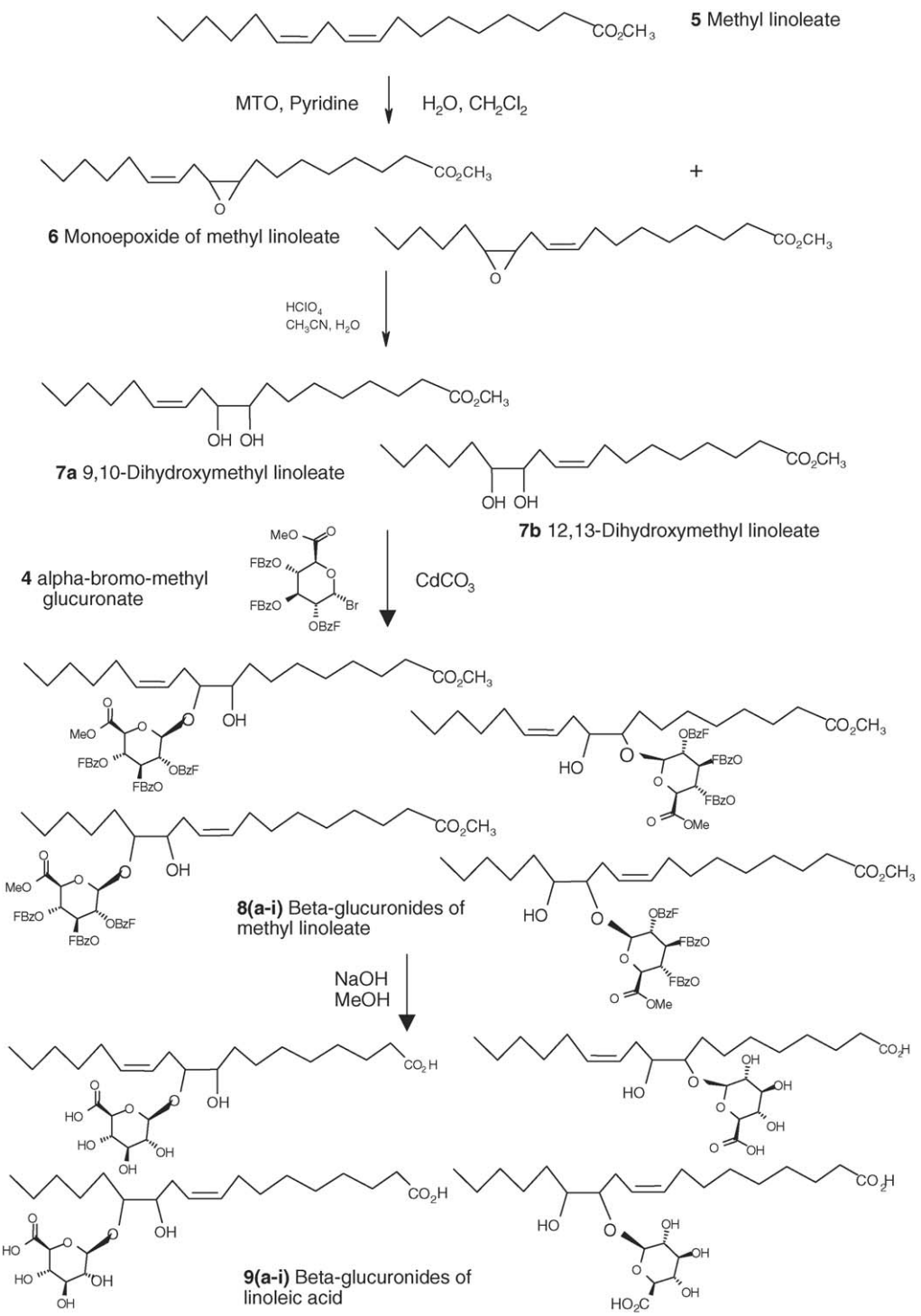
The procedure of Fehlhaber et al. (1987) was used to prepare methyl-D-glucuronate.

2.1.2. *1,2,3,4-Tetra-O-(3-fluorobenzoyl)-D-methyl glucuronate (3)*

Methyl glucuronate (**2**) (1.6 g, 7.7 mmol) and 4-dimethylamino pyridine (280 mg, 2.3 mmol) were dis-



Scheme 1.



Scheme 2.

solved in 25 mL pyridine. The mixture was cooled in an ice-water bath for 10 min. 3-Fluorobenzoyl chloride (10 g, 53 mmol) was added drop wise to this mixture over a 25 min period. The solution was stirred for 24 h and then methanol (5 mL) was added and stirred for another hour. The solution was diluted with CH_2Cl_2 (100 mL) and washed with 1N HCl (100 mL) and water (100 mL). The organic phase was dried over anhydrous Na_2SO_4 and

solvent evaporated under reduced pressure. The residual oil was purified by column chromatography over silica gel using hexane/ethyl acetate (3:1) as eluent to afford **3** (4.3 g, 81% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.71 (s, 3H, –$COOCH_3$), 4.76 (d, 1H, J = 10.0 Hz, H-5), 5.68 (dd, 1H, J = 3.67, 10.0 Hz, H-2), 5.77 (dd, 1H, J = 9.9, 9.8 Hz, H-4), 6.23 (dd, 1H, J = 9.8, 9.8 Hz, H-3), 6.90 (d, 1H, J = 3.6 Hz, H-1), 7.49 (m, 16H, Ph). ^{13}C NMR (500 MHz, $CDCl_3$): δ 171.3, 167.0, 164.7, 164.3, 164.2, 163.9, 163.8, 161.4, 130.6, 125.8, 121.0, 116.9, 90.0, 71.0, 70.2, 70.08, 70.03, 53.2.

*2.1.3. 2,3,4-Tri-O-(3-fluorobenzoyl)-α-D-methyl-glucuronide bromide (**4**)*

Hydrogen bromide in acetic acid (30 wt%, 15 mL) was added to a solution of 1,2,3,4-tetra-*O*-(3-fluorobenzoyl)-D-methyl glucuronate (**3**) (4.3 g, 6.1 mmol) in 10 mL CH_2Cl_2, and the solution was stirred overnight. It was then diluted with CH_2Cl_2 (100 mL) and washed with water (100 mL) followed by saturated aqueous $NaHCO_3$ (300 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated by evaporation. The residual oil was purified by column chromatography on silica gel using hexane/ethyl acetate (4:1) to give **4** (3.3 g, 85% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.71 (3H, s, –$COOCH_3$), 4.86 (1H, d, J = 10.2 Hz, H-5), 5.35 (1H, dd, J = 4.1, 9.9 Hz, H-2), 5.73 (1H, dd, J = 10.0, 9.8 Hz, H-4), 6.22 (1H, dd, J = 9.8, 9.8 Hz, H-3), 6.86 (1H, d, J = 4.0 Hz, H-1), 7.48 (12H, m, –Ph). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.5, 164.3, 164.3, 164.08, 164.05, 164.01, 163.7, 130.2, 125.6, 120.9, 116.7, 85.2, 72.2, 71.0, 70.3, 69.1, 53.2.

*2.1.4. 9,10- and 12,13-monoepoxide of methyl linoleate (**6**)*

Methyl linoleate (**5**, 3.0 g) was dissolved in dichloromethane (13 mL), followed by the addition of methyl trioxorhenium (VII, 15 mg) and pyridine (0.1 mL). Hydrogen peroxide (30 wt%, 1.8 mL) was then added drop wise over a 30 min period and the mixture was stirred for 1–2 h. After completion of the reaction (monitored by TLC), the aqueous phase was discarded. Excess H_2O_2 in the yellow organic phase was decomposed by the addition of a catalytic amount of MnO_2 until the yellow color totally disappeared. The solution was filtered and dried over anhydrous Na_2SO_4. The solvent was removed using a rotary evaporator. The crude product which contained diepoxides and some unreacted methyl linoleate was purified by column chromatography over silica gel using ether:dichloromethane (2:98) as eluent to afford 1.37 g (44% yield) of the title compounds as a mixture. This mixture of epoxides was then directly taken for hydrolysis.

*2.1.5. 9,10- and 12,13-dihydroxy methyl linoleate (**7a** and **7b**)*

A mixture of monoepoxides of methyl linoleate (**6**, 1.37g), acetonitrile (60 mL) and perchloric acid (70%, 0.7 mL) in water (55 mL) were stirred at room temperature overnight. The reaction mixture was then neutralized with sodium bicarbonate and concentrated under vacuum. The product was extracted in ethyl ether. The ether layer was then washed with saturated sodium chloride solution. The organic layer was dried over anhydrous sodium sulphate and evaporated using a rotary evaporator to afford an oil which contained both diols. The two regioisomeric diols were then purified by normal phase HPLC using isopropanol:hexane (3:97) as the mobile phase to obtain pure **7a** (450 mg, 31% yield) and **7b** (432 mg, 30% yield).

*2.1.6. 9,10-Dihydroxy methyl linoleate (**7a**)*

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (t, 3H, J = 6.5 Hz, –CH_3), 1.46 (m, 18H, H-3 to H-7, H-14 to H-17), 2.04 (m, 2H, H-2), 2.30 (m, 4H, H-8, H-11), 2.80 (bs, 2H, –OH), 3.45 (bs, 2H, H-9, H-10), 3.66 (s, 3H, –$COOCH_3$), 5.48 (m, 2H, H-12, H-13). ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.4, 133.3, 124.9, 74.0, 73.8, 51.5, 34.1, 33.6, 31.9, 31.7, 31.6, 29.5, 28.9, 27.4, 25.7, 25.4, 24.9, 22.6, 14.1.

*2.1.7. 12,13-Dihydroxy methyl linoleate (**7b**)*

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.87 (t, 3H, J = 7.0 Hz, –CH_3), 1.28 (bs, 12H, H-4 to H-7 and H-16 to H-17), 1.32 (bs, 2H, H-15), 1.46 (bs, 2H, H-4), 1.60 (t, 2H, J = 7.0 Hz, H-3), 2.02 (q, 2H, J = 7.2 Hz, H-8), 2.25 (bs, 2H, H-11), 2.27 (t, 2H, J = 7.3 Hz, H-2), 2.62 (bs, 2H, –OH), 3.42 (bs, 2H, H-12, H-13), 3.64 (s, 3H, –$COOCH_3$), 5.42 (m, 1H, H-10), 5.52 (m, 1H, H-9). ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.2, 132.9, 124.8, 73.8, 73.7, 51.3, 33.9, 33.5, 31.7, 31.5, 29.3, 28.9, 27.2, 25.4, 25.2, 24.7, 22.4, 13.8.

*2.1.8. 2,3,4-Tri-O-(3-fluorobenzoyl)-β-D-glucuronides of 9,10- and 12,13-dihydroxy methyl linoleate (**8**)*

Pure 9,10- or 12,13-diol methyl linoleates (1.0 g ~3 mmol) and $CdCO_3$ (780 mg, 4.5 mmol) in toluene (50 mL) were heated to reflux using a Dean–Stark water separator while stirring under nitrogen atmosphere. A solution of **4** (3.15 g, ~4.5 mmol) in toluene (20 mL) was

Table 1
^{13}C NMR chemical shift assignment of the 2,3,4-tri-*O*-(3-fluorobenzoyl)-D-glucuronides of 9,10- and 12,13-dihydroxy methyl linoleates (in $CDCl_3$)[a]

Assignment	8a	8b	8c	8d	8e	8f	8g	8h	8i
1′	101.6	101.6	101.2	100.9	100.4	101.5	100.8	100.5	100.8
2′	72.1	72.1	71.9	72.2	71.9	72.2	71.9	71.7	72.0
3′	72.7	72.6	72.6	72.8	72.5	72.8	72.4	72.6	72.5
4′	70.3	70.3	70.3	70.4	70.2	70.3	70.3	70.3	70.1
5′	72.6	72.5	72.4	72.5	72.4	72.4	72.4	72.3	72.4
6′	166.8	166.8	166.7	166.9	163.8	166.8	166.7	166.7	166.8
1	174.3	174.3	174.4	174.4	174.3	174.3	174.3	174.3	174.8
2	34.0	34.0	34.1	34.0	34.0	34.0	34.1	34.0	34.0
8	32.6	27.4	32.6	32.5	32.4	31.4	32.4	25	32.4
9	133.9	132.7	133.5	134.3	134.2	72.0	72.6	72.5	72.4
10	125.0	124.2	125.6	125.3	124.0	85.1	86.3	85.9	84.2
11	34.9	29.6	35.9	36.3	34.2	34.9	29.0	34.2	35.9
12	85.1	84.8	72.3	72.4	85.6	125.1	123.5	124.3	125.1
13	72.0	72.1	86.4	83.7	72.0	134.5	132.7	134.6	133.9
18	13.9	14.0	13.9	14.0	14.0	14.1	14.1	13.9	13.9

[a] All FBz ^{13}C resonate between 130.3 and 116.6 ppm. All methyl protecting groups at 6′ resonate at 53.0 ± 0.2 ppm. All methyl protecting groups at 1 resonate at 51.4 ± 0.1 ppm.

added drop wise over a 30 min period. The resulting mixture was refluxed for about 24 h. After completion of the reaction (monitored by TLC with the aid of orcinol ferric chloride spray reagent), it was cooled to room temperature. The resulting solids were filtered and washed with toluene. The combined organic solvents were washed with water and dried over anhydrous sodium sulphate. The solvent was removed by rotary evaporation. The

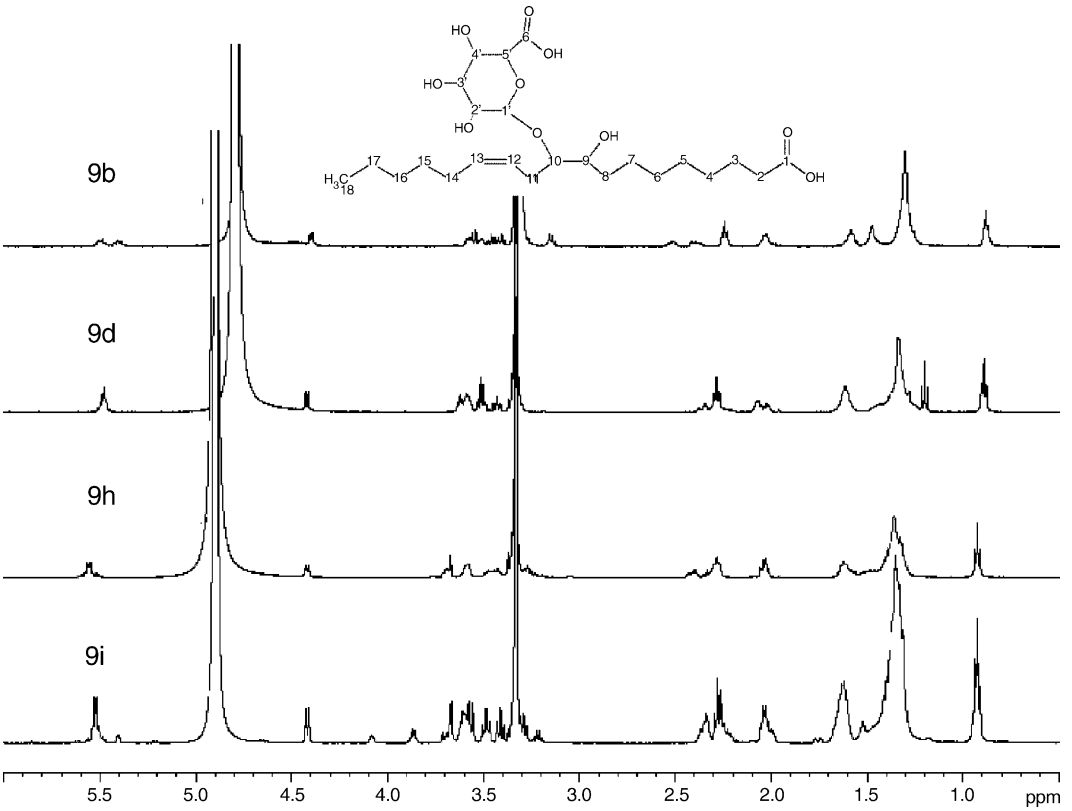


Fig. 1. ^{1}H NMR spectra of deprotected final products. **9b** is 13-glucuronide substituted dihydroxy linoleic acid. **9d** is 12-glucuronide substituted dihydroxy linoleic acid. **9h** is 10-glucuronide substituted dihydroxy linoleic acid. **9i** is 9-glucuronide substituted dihydroxy linoleic acid.

Table 2
^{1}H NMR chemical shift assignment and coupling-constants of the 2,3,4-tri-*O*-(3-fluorobenzoyl)-D-glucuronides of 9,10- and 12,13-dihydroxy methyl linoleates (in $CDCl_3$)[a]

Assignment	12Ss13S or 12Rs3S **8a**	12Ss13R or 12Rs13R **8b**	12S13Ss **8c**	12R13Ss **8d**
1′	5.00 (d, 1H, *J* = 7.8)	5.01 (d, 1H, *J* = 7.8)	4.91 (d, 1H, *J* = 7.8)	5.04 (d, 1H, *J* = 7.6)
2′	5.55 (dd, 1H, *J* = 9.8, 7.8)	5.55 (dd, 1H, *J* = 9.5, 7.8)	5.54 (dd, 1H, *J* = 9.5, 7.8)	5.55 (dd, 1H, *J* = 9.8, 7.6)
3′	5.89 (t, 1H, *J* = 9.5)	5.90 (dd, 1H, *J* = 9.8, 9.5)	5.88 (dd, 1H, *J* = 9.7, 9.4)	5.88 (dd, 1H, *J* = 9.8, 9.5)
4′	5.70 (t, 1H, *J* = 9.8)	5.70 (t, 1H, *J* = 9.8)	5.66 (dd, 1H, *J* = 9.7, 9.4)	5.70 (dd, 1H, *J* = 9.8, 9.5)
5′	4.35 (d, 1H, *J* = 9.8)	4.35 (d, 1H, *J* = 9.8)	4.35 (d, 1H, *J* = 9.8)	4.34 (d, 1H, *J* = 9.8)
2	2.32 (t, 2H, *J* = 7.3)	2.33 (t, 2H, *J* = 7.3)	2.30 (t, 2H, *J* = 7)	2.32 (t, 2H, *J* = 7.6)
8	1.98 (q, 2H, *J* = 6.6)	2.06 (q, 2H, *J* = 7.0)	1.99 (m, 1H)	1.92 (q, 2H, *J* = 6.2)
9	5.50 (m, 1H)	5.46 (m, 1H)	5.48 (m, 1H)	5.33 (m, 1H)
10	5.47 (m, 1H)	5.43 (m, 1H)	5.48 (m, 1H)	5.18 (m, 1H)
11a	2.51 (m, 1H)	2.59 (m, 1H)	2.09 (m, 1H)	2.00 (m, 1H)
11b	2.41 (m, 1H)	2.47 (m, 1H)	2.30 (m, 1H)	2.12 (m, 1H)
12	3.54 (q, 1H, *J* = 5.4)	3.56 (m, 1H)	3.61 (m, 1H)	3.49 (m, 1H)
13	3.48 (bs, 1H)	3.46 (m, 1H)	3.52 (m, 1H)	3.63 (m, 1H)
18	0.81 (t, 3H, *J* = 7.3)	0.81 (t, 3H, *J* = 7.6)	0.68 (t, 3H, *J* = 7)	0.90 (t, 3H, *J* = 6.9)

	12R13Rs **8e**	9R10Rs or 9R10Ss **8f**	9R10Ss or 9S10Rs **8g**	9R10Ss or 9S10Rs **8h**	9s **8i**
1′	4.96 (d, 1H, *J* = 7.6)	4.98 (d, 1H, *J* = 7.5)	4.98 (d, 1H, *J* = 7.8)	4.95 (d, 1H, *J* = 7.7)	5.02 (d, 1H, *J* = 7.7)
2′	5.55 (dd, 1H, *J* = 9.6, 7.8)	5.56 (dd, H, *J* = 9.8, 9.5)	5.55 (dd, 1H, *J* = 9.5, 7.8)	5.56 (t, 1H, *J* = 9.4)	5.54 (t, 1H, *J* = 9.8, 7.9)
3′	5.90 (t, 1H, *J* = 9.6)	5.89 (dd, 1H, *J* = 9.8,9.5)	5.89 (dd, 1H, *J* = 9.8, 9.5)	5.9 (t, 1H, *J* = 9.2)	5.88 (t, 1H, *J* = 9.6)
4′	5.70 (t, 1H, *J* = 9.5)	5.69 (t, 1H, *J* = 9.5)	5.70 (t, 1H, *J* = 9.5)	5.70 (t, 1H, *J* = 9.4)	5.68 (t, 1H, *J* = 9.4)
5′	4.37 (d, 1H, *J* = 9.8)	4.35 (d, 1H, *J* = 10)	4.37 (d, 1H, *J* = 9.8)	4.36 (d, 1H, *J* = 9.6)	4.32 (d, 1H, *J* = 9.6)
2	2.33 (t, 2H, *J* = 7.4)	2.32 (m, 2H)	2.32 (t, 2H, *J* = 7.4)	2.32 (t, 2H, *J* = 7.5)	2.32 (t, 2H, *J* = 7.7)
8	1.74 (m, 2H)	1.40 (m, 2H)	1.40 (m, 2H)	1.48 (m, 2H)	1.67 (m, 2H)
9	5.31 (m, 1H)	3.48 (m, 1H)	3.57 (m, 1H)	3.57 (m, 1H)	3.65 (m, 1H)
10	5.21 (m, 1H)	3.54 (m, 1H)	3.58 (m, 1H)	3.57 (m, 1H)	3.48 (m, 1H)
11a	2.13 (m, 1H)	2.44 (m, 1H)	2.16 (m, 1H)	2.19 (m, 1H)	2.07(m, 1H)
11b	2.28 (m, 1H)	2.48 (m, 1H)	2.24 (m, 1H)	2.19 (m, 1H)	1.97(m, 1H)
12	3.57 (m, 1H)	5.46 (m, 1H)	5.21 (m, 1H)	5.23 (m, 1H)	5.16 (m, 1H)
13	3.57 (m, 1H)	5.49 (m, 1H)	5.18 (m, 1H)	5.30 (m, 1H)	5.34 (m, 1H)
18	0.90 (t, 3H, *J* = 7.4)	0.91 (m, 3H)	0.90 (m, 3H)	0.89 (m, 3H)	0.89 (m, 3H)

[a] All protons of FBz protect group resonate between 7.78 and 7.14 ppm. All protons on carbon 3–7 and 14–17 resonate from 1.68 to 1.14 ppm. All protons in methyl protecting groups at 1 resonate at 3.68 ± 0.01 ppm. All protons in methyl protecting groups at 6′ resonate at 3.73 ± 0.02 ppm.

residue was purified by column chromatography using hexane:ethyl acetate (4:1) as an eluent for the silica gel column. This was followed by normal phase HPLC using 1% isopropanol in hexane for raw purification and 0.6% isopropanol in hexane for re-purification to obtain **8** as pure compounds where possible (**8a–i**). ^{13}C NMR and ^{1}H NMR data are listed in Tables 1 and 2, respectively. The labels of the atoms are given in Fig. 1.

2.1.9. β-Glucuronides of linoleic acid diol (*9*)

Each of the nine purified 2,3,4-tri-*O*-(3-fluorobenzoyl)-β-D-glucuronides of 9,10- or 12,13-dihydroxy methyl linoleate (**8a–i**) (50 mg) were hydrolyzed individually with ∼3% aq. NaOH in 2 mL methanol while stirring at room temperature for 5–8 h. After completion, as monitored by TLC, the methanol was evaporated under reduced pressure and 1 mL saturated sodium chloride solution was added. The pH of the solution was adjusted to 3–4 by adding formic acid, and then the product was extracted by ethyl ether. After removing ethyl ether by rotary evaporator, the residue was purified by reverse phase HPLC with Zorbax RX-C_8 column, fitted with a UV detector (202 nm) and using a mobile phase of 33% acetonitrile, 0.01% formic acid in water to isolate a pure final product. Reverse phase HPLC conditions and peaks of the corresponding compounds were confirmed by simultaneous detection using a photodiode array detector (PAD) and mass spectrometer. To ensure that peaks were being simultaneously detected by the PAD and mass spectrometer, a splitter was used and the tube length and speed of elution were adjusted to a suitable value using the by-product of the reaction (3-fluorobenzoic acid) as a standard. ^{13}C NMR and ^{1}H

Table 3
^{13}C MR chemical shift assignment of the β-glucuronides of 9,10- and 12,13-dihydroxy linoleic acid (in D-methanol and $CDCl_3$)

Assignment	9a	9b	9c	9d	9e	9f	9g	9h	9i
1′	105.1	105.3		104.7	103.0	105.5	102.0	101.6	105.3
2′	74.8	75.2	Limited isolate no carbon data	75.1	75.2	75.4	74.9	74.0	75.7
3′	76.9	76.7		77.1	77.7	77.5	77.5	76.7	77.9
4′	72.4	73.4		73.0	73.8	73.6	73.4	72.6	73.9
5′	75.8	75.4		76.1	73.3	76.4	76.2	75.3	74.4
6′	165.9	169.9					169.0	168.7	
1	178	177.6		177	171.0	173.4	174.2	177.5	178.8
2	34.8	35.6		35.0	36.3	35.3	35.3	34	36.1
3–7, 14–17	23.0–33.2	23.6–34.0		23.2–33.6	23.6–33.7	23.6–34.0	23.6–32.8	22.2–33.0	23.8–33.9
8	33.2	28.3		33.4	33.7	33.7	28.4	29.5	33.9
9	133.7	132.4		126.2	127.4	73.2	82.6	71.8	84.0
10	126.4	126.4		132.7	134.6	84.8	73.2	81.2	74.4
11	36.1	30.6		35.0	36.4	36.8	29.3	34.0	37.5
12	84.2	84.5		74.4	76.3	127.4	126.6	126.0	127.8
13	72.7	73.6		84.3	82.8	134.2	133.4	133.5	134.3
18	14.3	14.5		14.6	14.6	14.6	14.6	13.7	14.5

NMR data of **9** are listed in Tables 3 and 4, respectively. The labels of the atoms are given in Fig. 1.

The analysis of synthesized pure products or mixtures by HPLC/MS was performed using a 3.5 μm Eclipse XDB-C_{18} (Zorbax) analytical column (2.1 mm × 150 mm) with the flow rate of 0.1 mL/min. The isocratic mobile phase consisted of 25% B (100% acetonitrile) in A (0.01 mM ammonium acetate, pH 6). For each injection, a 200 μL sample was loaded onto a pre-column (10 μm Zorbax XDB-C_8) with mobile phase A, then washed at flow rate of 1.5 mL/min for 1 min. The trap was then back flashed onto the analytical column using 25% B in order to introduce the sample onto the Q-TOF mass spectrometer for analysis as described above. Spectra were acquired at 3 s per scan using negative ion mode.

3. Results and discussion

3.1. Theoretical consideration of structural isomers and stereoisomers

During the chemical synthesis described above (Scheme 2), one of the *cis* double bonds in methyl linoleate is epoxidized using aqueous hydrogen peroxide and catalytic methyl trioxorhenium/pyridine. This epoxidation yields four different monoepoxide products with 9R10S, 9S10R, 12S13R and 12R13S configurations. The positional isomers were separated by HPLC. When these monoepoxides are subsequently hydrolyzed to their corresponding diols by perchloric acid, the stereoselectivity is lost and all the 4 × 2 possible isomers are theoretically obtained: two positional isomers (9,10- and 12,13-diol) and four stereoisomers (RR/SS, RS/SR) for each positional isomer. The RR and SS, as well as the RS and SR are enantiomers that theoretically cannot be separated without chiral column HPLC. However, since there are additional chiral centers added, once the glucuronide group is attached, some of these may be separated by HPLC as the diastereomers (RR/SS with RS/SR). This is consistent with our observation of peak tailing when the 9,10- and 12,13-diols were analyzed by normal phase HPLC (3% isopropanol in hexane). Therefore, following the reaction of the 9,10- and 12,13-diol with bromo sugar (**4**), theoretically 16 possible isomers will be obtained for both the β and α glucuronide. The final products we obtained agree with this prediction since nine pure isomers were isolated as final products.

3.2. Characterization of glucuronide derivatives of dihydroxy methyl linoleate (*8*) and glucuronide derivatives of dihydroxy linoleic acid (*9*)

The structure and relative stereochemistry of these products were determined by extensive analysis of NMR data (including ^1H, ^{13}C, gHMQC, gHMBC, gCOSY and NOESY for each sample) as well as by molecular modeling, except for compounds **8e**, **8f**, **8g**, **8h** and **8i** where COSY and NOESY data were used for these assignments and compared to minimized models. Absolute assignments of stereochemistry are dependent on an X-ray crystal structure, which is not possible for these compounds. Circular dichroism would be useful, but beyond

Table 4
^{1}H NMR chemical shift assignment and coupling-constants of the β-glucuronides of 9,10- and 12,13-dihydroxy linoleic acid (in D-methanol and $CDCl_3$)

Assignment	12Ss13S and 12Rs13S **9a**	12Ss13R and 12Rs13R **9b**	12Ss13S **9c**	12R13Ss **9d**
1′	4.40 (d, 1H, J = 7.6)	4.40 (d, 1H, J = 7.6)	4.30	4.42 (d, 1H, J = 7.6)
2′	3.26 (m, 1H)	3.30 (m, 1H)	3.18 (m, 1H)	3.31 (m, 1H)
3′	3.41 (t, 1H, J = 9.0)	3.40 (t, 1H, J = 9.1)	3.41 (m, 1H)	3.43 (t, 1H, J = 9.1)
4′	3.52 (m, 1H)	3.46 (t, 1H, J = 9.1)	3.45 (m, 1H)	3.51 (t, 1H, J = 7.3)
5′	3.65 (d, 1H, J = 9.6)	3.55 (d, 1H, J = 9.8)	3.52 (m, 1H)	3.62 (m, 1H)
OH at 6′	7.79	8.52	8.51	8.41 (s, 1H)
OH at 1			4.4	
2	2.26 (t, 2H, J = 7.2)	2.24 (t, 2H, J = 7.2)	2.00 (m, 2H)	2.28 (t, 2H, J = 7.9)
3–7, 14–17	1.58, 1.48, 1.30	1.58, 1.48, 1.30, . . .	1.58, 1.44, 1.29, 1.24	1.61, 1.43, 1.30
8	1.98 (m, 2H)	2.03 (m, 2H)	2.00 (m, 1H)	2.06 (m, 2H)
9	5.47 (m, 1H)	5.42 (m, 1H)	5.48 (m, 1H)	5.48 (m, 1H)
10	5.47 (m, 1H)	5.50 (m, 1H)	5.48 (m, 1H)	5.48 (m, 1H)
11a	2.44 (m, 1H)	2.40 (m, 1H)	2.25 (m, 2H)	2.34 (m, 1H)
11b	2.32 (m, 1H)	2.52 (m, 1H)	2.25 (m, 2H)	2.20 (m, 1H)
12	3.52 (m, 1H)	3.57 (p, 1H, J = 5.0)	3.52 (m, 1H)	3.58 (dd, 1H, J = 8.5, 4.7)
13	3.52 (m, 1H)	3.51 (p, 1H, J = 5.0)	3.52 (m, 1H)	3.58 (dd, 1H, J = 8.5, 4.7)
18	0.88 (t, 3H, J = 7.3)	0.88 (t, 3H, J = 7.3)	0.84 (t, 3H)	0.89 (t, 3H, J = 7.9)

	12R13Rs	9R10Rs or 9R10Ss	9Ss10R		
	9e	**9f**	**9g**	**9h**	**9i**
1′	4.39 (d, 1H, J = 7.6)	4.40 (d, 1H, J = 7.9)	4.39 (d, 1H, J = 7.2)	4.42 (d, 1H, J = 7.3)	4.41 (d, 1H, J = 7.9)
2′	3.24 (t, 1H, J = 8.5)	3.25 (m, 1H)	3.25 (t, 1H, J = 8.5)	3.27 (t, 1H, J = 8.1)	3.28 (t, 1H, J = 9.0)
3′	3.41 (t, 1H, J = 8.8)	3.41 (t, 1H, J = 8.5)	3.41 (t, 1H, J = 9.1)	3.43 (m, 1H)	3.41 (t, 1H, J = 9.0)
4′	3.45 (t, 1H, J = 8.5)	3.48 (t, 1H, J = 9.8)	3.46 (t, 1H, J = 9.5)	3.48 (m, 1H)	3.48 (t, 1H, J = 9.0)
5′	3.56 (d, 1H, J = 8.5)	3.60 (d, 1H, J = 9.4)	3.56 (m, 1H)	3.59 (m, 1H)	3.56 (d, 1H, J = 9.0)
COOH at 6′ and 1	8.44 and 4.59	8.44 and 4.52	8.44	8.53	8.53
2	2.24 (m, 2H)	2.27 (t, 2H, J = 6.6)	2.26 (t, 2H, J = 7.2)	2.29 (t, 2H, J = 7.3)	2.28 (t, 2H, J = 8.1)
3–7, 14–17	1.60, 1.39, 1.30	1.60, 1.50, 1.28	1.60, 1.48, 1.32	1.62, 1.36	1.62, 1.52, 1.35
8	2.02 (q, 2H, J = 6.6)	1.98 (dd, 2H, J = 9.8, 5.7)	2.06 (q, 2H, J = 7.1)	2.04 (q, 2H, J = 6.5)	2.04 (q, 2H, J = 6.5)
9	5.51 (m, 1H)	3.54 (m, 1H)	3.67 (m, 1H)	3.69 (q, 1H, J = 6.5)	3.60 (m, 1H)
10	5.53 (m, 1H)	3.54 (m, 1H)	3.56 (m, 1H)	3.59 (m, 1H)	3.60 (m, 1H)
11a	2.38 (m, 1H)	2.47 (m, 1H)	2.41 (m, 1H)	2.40 (m, 1H)	2.34 (m, 1H)
11b	2.25 (m, 1H)	2.37 (m, 1H)	2.33 (m, 1H)	2.32 (m, 1H)	2.28 (m, 1H)
12	3.67 (m, 1H)	5.49 (m, 1H)	5.53 (m, 1H)	5.55 (m, 1H)	5.52 (m, 1H)
13	3.56 (d, 1H, J = 8.5)	5.48 (m, 1H)	5.46 (m, 1H)	5.54 (m, 1H)	5.52 (m, 1H)
18	0.91 (t, 3H, J = 6.3)	0.89 (t, 3H, J = 6.3)	0.90 (m, 3H)	0.92 (t, 3H, J = 7.3)	0.93 (t, 3H, J = 6.5)

the scope of this work. Molecular mechanics optimizations were carried out to obtain low energy conformation structures which further helped to identify isomers.

3.2.1. α-Glucuronide or β-glucuronide

The α- and β-glucuronide can usually be distinguished by the chemical shift and especially by the coupling constant of proton H-1 on the sugar portion of the molecule (Smith and Benet, 1986). The H-1 is a doublet with J_{1-2} around 8 Hz for β-glucuronide while H-1 is a doublet with J_{1-2} around 4 Hz for the α-glucuronide. For all products within the **8** and **9** series, the ^{1}H NMR spectra shows the H-1 proton as a doublet, therefore suggesting that all the glucuronide substituted products are β. A small amount of inversion of the stereochemistry at the anomeric carbon to give the α anomer was seen, but these products were not studied.

*3.3. Positional isomer assignment and stereochemistry for β-glucuronides of methyl linoleate (**8**)*

The analysis of stereochemistry in these epoxidized methyl linoleate glucuronide metabolites is complicated greatly by molecular flexibility. Molecular modeling (Schrodinger's Maestro) was used to build and mini-

mize each enantiomer of the 9, 10, 12 and 13 substituted derivatives. This gave the approximate low energy structure for each of the 16 possible derivatives. Four diastereomers of 9,10-dihydroxylinoleic acid were isolated. One of these (**8f**) is either 9R10R substituted or 9R10S substituted. The minimized structure for these two show the H9 orthogonal to H11 and H11′ and therefore no NOE should be observed between these protons, which is supported by the NMR data. This gave us the ability to establish stereochemistry for the free hydroxy carbon of the linoleic derivative, however, molecular rotation leading to transient NOEs reduced the chances of establishing stereochemistry for the glucuronide substituted carbon. A strong NOE is observed from the anomeric proton of the glucuronide to the proton of the substituted carbon in each isolated fraction. From modeling, all S-substituted glucuronide linoleates have a minimized structure with a small dihedral angle between the anomeric proton and the hydrogen on the substituted carbon. This should lead to a large NOE between these two protons. All R-substituted glucuronide linoleates have a minimized structure with a large dihedral angle between the anomeric proton and the hydrogen on the substituted carbon. Rotation about this bond could lead to an NOE. Rotation of $C_{anomeric}$–$O_{anomeric}$–$C_{linoleic}$ bond, leading to a NOE between $H_{anomeric}$ and H9, may be stabilized by a hydrogen bond to the methoxyester of the glucuronide or by the steric hindrance caused by the 3-fluorophenoxy protecting groups on the glucuronide.

Two other compounds (**8g** and **8h**), where the glucuronide is substituted onto carbon-10 were isolated. Both of these show a high degree of proton overlap at 3.57 ppm of the H-9 and H-10. Neither *J*-couplings nor NOEs can be used to identify these compounds unequivocally. There are differences in the shifts and couplings of the H11, H11′ and H8 protons. These are probably 9R10S substituted and 9S10R substituted. Again, hydrogen bonding may explain why these two are separable by chromatography. Due to overlap, one of these compounds may also be the second 9-substituted diastereomer.

8i is a 9-substituted diastereomer. Discerning the stereochemistry, however, is problematic. Molecular modeling showed NOE correlations from H10 to H11, H11′ and H12. These are expected due to the flexibility of the molecule. Each diastereomer has an energy minimum with this possibility (Fig. 2).

8a and **8b** are diastereomers, which are substituted at C-12 of the linoleate. **8a** has two possibilities: 12Ssub13S or 12Rsub13S. H13Has medium NOE correlations to H11 and H11′. From modeling data this happens only in the 13S conformers. 12Ssub13S fits both modeling and NMR data better, but conformational flexibility again can give transient NOE. However, a large coupling constant would be expected for H12–H13, which is not present in the NMR data. Potential hydrogen bonding was not accounted for in the modeling data and might help to determine the correct stereochemistry.

8b is either 12Ssub13R or 12Rsub13R. The 13R conformer shows a potential NOE from H13 to H11 in the modeling data. A small NOE from H13 (3.48 ppm) to H11 (2.59 ppm) was observed in the NMR data. It is not possible, with the current data, to determine the stereochemistry at C12.

8d and **8c** are diastereomers substituted at C-13 of the linoleate. The assignment of **8d** is likely 12R13Ssub. There are NOEs from H13 to H11 and H11′. H12 also has NOEs to H11 and H11′ as well as to H10. There is a probable hydrogen bond between the 13OH and the 2-OPh3F. **8c** is likely to be 12S13Ssub. H12 and H13 have NOEs to H10. H13 does not have any NOE correlations to H11 or H11′.

8e is a third diastereomer substituted at C13 of the linoleate. This appears to be 12R13Rsub with weak NOEs from H11 and H11′ to the anomeric proton. H13Has an NOE to H10. We believe we are seeing three diastereomers due to restricted rotation. Rotation around the anomeric oxygen is probably restricted by hydrogen bonding. The steric bulk of the FBzO protecting groups may also have an impact on an unexpected number of diastereomers.

3.4. HPLC-MS/MS investigation of synthesized compounds

Electrospray ionization was used in the HPLC-MS/MS analysis because of its compatibility with on-line HPLC separation and its sensitivity and specificity (Murphy, 1995). Mass spectrometer parameters were optimized by direct injection of standard compounds. The cone voltage was optimized to obtain the maximum signal for the pseudomolecular ion $(M–H)^-$ 489 for each compound. This was determined to be 70 V for all isomers. The collision energy was optimized to obtain unique fragment ions for each positional isomer. Fig. 3 shows the collision induced (CID) mass spectra of the positional isomers. In A, the cone voltage was 70 V and collision energy was 25 eV, and the mass spectra of these isomers are very similar. Fragmentation occurred at the bond between the glucuronide and the diol oxygen to give the $m/z = 313$ (linoleic acid fragment) and $m/z = 175$ (glucuronide fragment) ions. When the cone voltage was kept constant and the collision energy was increased to 35 eV, isomer-specific fragmentation ions

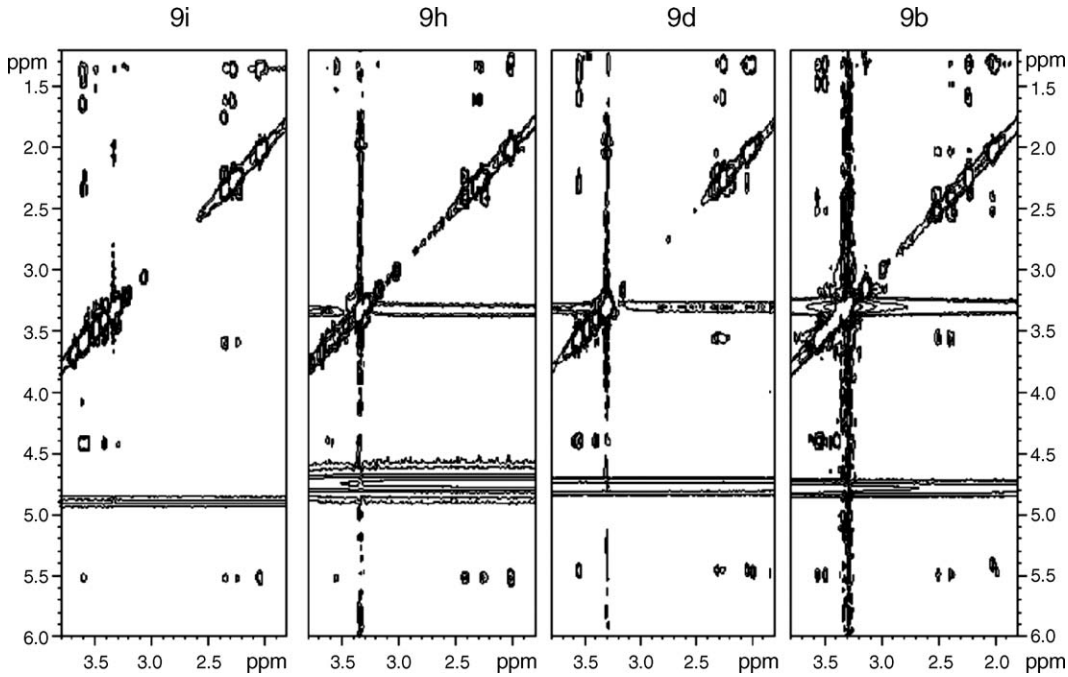


Fig. 2. NOESY data from **9i**, **9h**, **9d** and **9b**. The crosspeaks from the olefinic region to those peaks around 2 ppm and from 3.5 to 4.4 ppm and 2 ppm are used to determine stereochemistry.

were observed between the 9- or 10-glucuronides and the 12- or 13-glucuronides (B–E in Fig. 3). Fig. 4 illustrates the proposed mechanism for the formation of the *m/z* 313 ion in the CID mass spectrum of 9-glucuronide-10-hydroxy-12-linoleic acid. A similar cleavage of the glucuronide ether bond in the CID ionization of 10-glucuronide-9-hydroxy-12-linoleic acid results in the generation of the same ion structure. This fragmentation

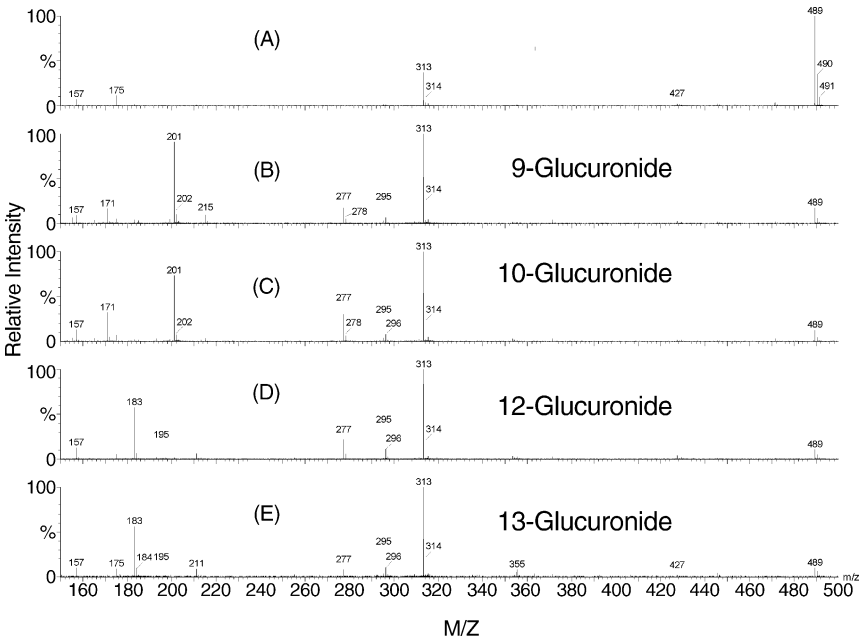


Fig. 3. A typical mass spectrum of glucuronide positional isomers. (A) All isomers, the cone voltage is 70 V and the collision energy is 25 V, (B) the 9-glucuronide, (C) the 10-glucuronide, (D) the 12-glucuronide and (E) the 13-glucuronide. The cone voltage and collision energy for B–E are 70 and 35 V, respectively.

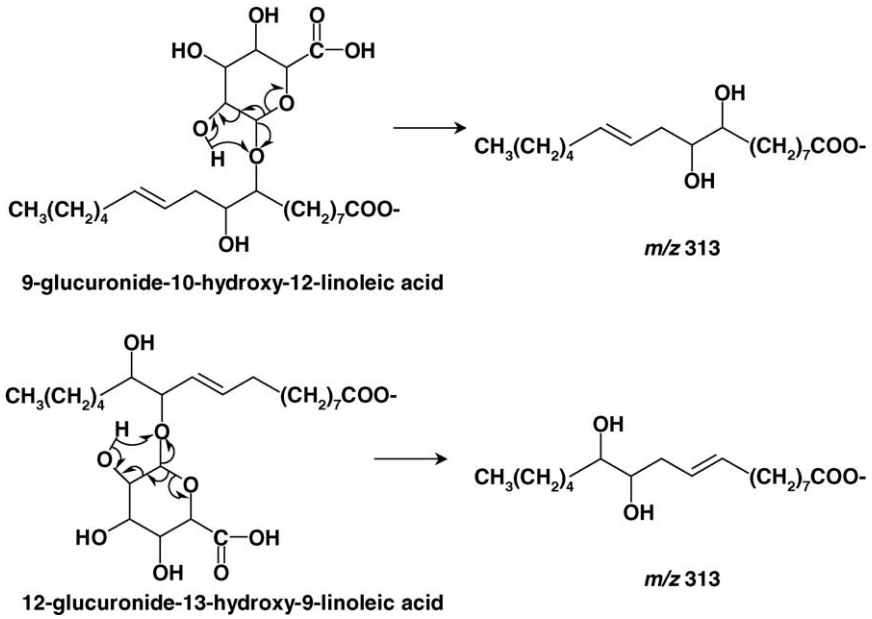


Fig. 4. Proposed fragmentation mechanism for the formation of the *m/z* 313 ion in the CID spectrum of 9-glucuronide-10-hydroxy-12-linoleic acid and 12-glucuronide-13-hydroxy-9-linoleic acid.

mechanism in the CID ionization of 12-glucuronide-13-hydroxy-9-linoleic acid (Fig. 4) and 13-glucuronide-12-hydroxy-9-linoleic acid leads to a fragment ion of the same mass but different structure. Subsequent fragmentation of the respective *m/z* 313 ions (Figs. 5 and 6) thus lead to isomer-specific ions that could be used to distinguish the 9/10 glucuronides from the 12/13 glucuronides. Previous work has shown similar patterns of fragmentation for these types of fatty acids (Wheelan et al., 1996).

Preliminary analysis (data not shown) showed that these positional isomer-specific fragment ions could be used to identify isomers with close retention times such as the 13-glucuronide **9e** (retention time 11.35 min) and the 10-glucuronide **9g** (retention time 11.21 min).

In conclusion, we have developed a general strategy for the synthesis and purification of the glucuronides of linoleic acid diols along with analytical techniques useful for isomer-specific analysis. This general synthetic scheme should also prove useful for the synthesis and analysis of these and other fatty acid diols in biological samples.

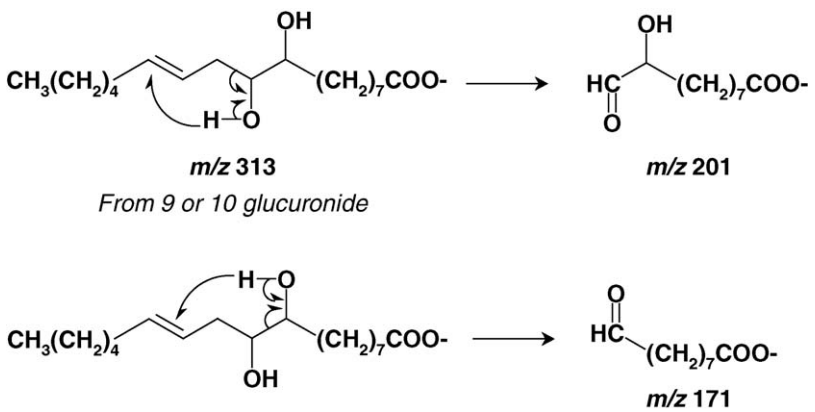


Fig. 5. Proposed fragmentation mechanism for the formation of the *m/z* 201 and *m/z* 171 ion from the *m/z* 313 ion fragment in the CID spectrum of 9-glucuronide-10-hydroxy-12-linoleic acid and 10-glucuronide-9-hydroxy-12-linoleic acid. (Charge-remote allylic fragmentation as described Wheelan et al., 1996.)

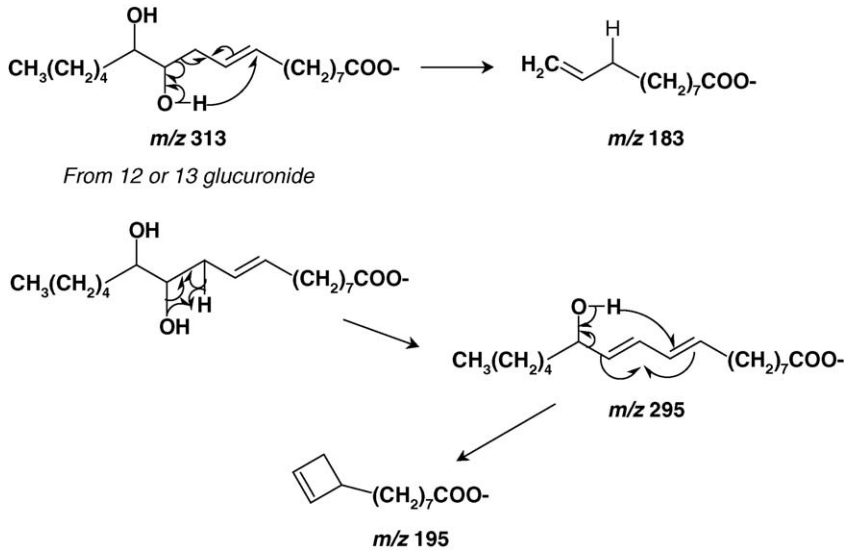


Fig. 6. Proposed fragmentation mechanism for the formation of the *m*/*z* 201 and *m*/*z* 171 ion from the *m*/*z* 313 ion fragment in the CID spectrum of 12-glucuronide-13-hydroxy-9-linoleic acid and 13-glucuronide-12-hydroxy-9-linoleic acid. (Charge-remote allylic fragmentation as described Wheelan et al., 1996.)

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