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Synthesis and Characterization of Stigmasterol Oxidation Products

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The synthesis and structural characterization of a series of oxides of stigmasterol is described providing a valuable series of reference standards for these oxides, analogous to the cholesterol oxidation products (COPs) which have been shown to have detrimental biological effects. Biological evaluation of the oxides of phytosterols is significant in the context of increased dietary use of phytosterols in the drive to reduce cholesterol absorption.

KEYWORDS: Phytosterol oxidation products; stigmasterol oxides

INTRODUCTION

Phytosterols are lipid compounds found in plant foods and are analogous to cholesterol in their structure. Much work has been conducted on the negative health effects of cholesterol and its oxidation products (COPs), which include atherosclerosis, cytotoxicity and mutagenicity (*1*). As a result of the drive to reduce cholesterol absorption, recent trends have shown an increase in the use of phytosterols as cholesterol reducing agents, with esters of phytosterols being incorporated into an ever expanding number of foods, such as margarine and salad dressings (*2*). This is expected to bring about a rise in dietary intake, and some reports have predicted that the average daily consumption may exceed 8.6 g (*3*). One study analyzed the phytosterol oxidation products (POPs) in a commercially available margarine which was enriched with phytosterol esters, and results showed that 0.1% of the phytosterols were oxidized (*4*). This compares with studies where the amount of COPs in foods could frequently reach 1% of total cholesterol and occasionally 10% or more (*5*, *6*).

The structural connection between cholesterol and the plant sterols means that these compounds can undergo oxidation by similar free radical mediated pathways to those documented for cholesterol (*7*). As with other unsaturated lipids, phytosterols may undergo oxidation upon exposure to air. This oxidation process may be enhanced by heating, by exposure to light and by chemicals or enzymes, which can occur during cooking, during food production or upon long-term storage.

In contrast to COPs very little research has been conducted on the oxidation of plant sterols to their phytosterol oxidation products, which in turn has led to a lack of data on the biological effects of these compounds. One of the challenges associated with the analysis of POPs is the fact that much of the studies to date have been carried out on phytosterol blends, principally because

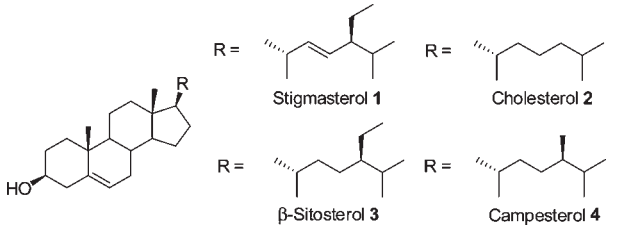

Figure 1. Stigmasterol, cholesterol, β-sitosterol and campesterol.

the phytosterols are most readily available as mixtures (*8*, *9*), containing a combination of plant sterols (β-sitosterol **3**, campesterol **4**, and stigmasterol **1, Figure 1**). Existing reports refer to nonselective oxidations, for example through thermal exposure of the blend. Inevitably this results in enormous complexity, with mixtures of various oxidation products of each of the phytosterols (>30 major compounds and numerous minor components), which must then be separated and quantified. This problem is magnified further when analysis is required of POPs in foodstuffs. Evaluation of POPs in foodstuffs has proved a difficult task due to the lack of pure compounds as standards for analysis. Little work has been carried out on the selective chemical synthesis of POPs as single compounds. Recently the first reports of the synthesis of reference samples of β-sitosterol and its oxides, and their biological evaluation, have appeared from our group (*10*, *11*). This was followed by a later report on the purification of β-sitosterol by column chromatography and the synthesis of its oxidation products (*12*).

Contrasting the structure of stigmasterol **1** with cholesterol **2** shows significant structural similarity, with the addition of an ethyl group at the 24 position as well as unsaturation between carbons 22 and 23 (**Figure 1**). In relation to the synthesis of stigmasterol oxides, most of the literature to date has concentrated on thermal oxidation (*13*−*16*) or photo-oxidation (*17*, *18*) of this phytosterol. These usually involve difficult separation of

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the resulting oxidation products, using HPLC (high performance liquid chromatography) or GC (gas chromatography) methods, and are therefore not suitable for the production of useful quantities of the oxides for use as reference standards. In many instances the oxides were simply detected and characterized by mass spectrometry. However, in recent years some reports of the synthetic formation of stigmasterol oxides have emerged (*19*, *20*).

In this paper the selective synthesis of stigmasterol oxides is described, along with full structural characterization of all compounds, thus providing access to reference standards for analysis and identification of POPs.

MATERIALS AND METHODS

General Procedures. Reagents were purchased from the Aldrich company, except stigmasterol (Fluka, 90%). Specific rotations were recorded on a Perkin-Elmer 341 polarimeter at 20 °C in the solvents indicated. The sodium D line (589 nm) was used unless otherwise indicated. All NMR (nuclear magnetic resonance) spectra are recorded at 20 °C, using tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in parts per million (ppm) and coupling constants (*J*) in hertz (Hz). Infrared spectra were measured as potassium bromide (KBr) disks for solids or as thin films on sodium chloride plates for oils. Thin layer chromatography (TLC) was carried out on precoated silica gel plates (Merck 60 PF_{254}). Column chromatography was performed using Merck silica gel 60. Visualization of compounds on TLC plates was achieved by ultraviolet (UV) (254 nm) light detection and vanillin staining. Abbreviations used: *m*-CPBA, 3-chloroperbenzoic acid; NMO, 4-methylmorpholine *N*-oxide; 4-DMAP, 4-(dimethylamino)pyridine; DHQD PHN, dihydroquinidine 9′-phenanthryl ether; DMSO, dimethyl sulfoxide; LC−MS, liquid chromatography mass spectometry.

5α,6α-Epoxystigmast-22-en-3β-ol 5. Stigmasterol **1** (1.50 g, 3.64 mmol) was dissolved in dichloromethane (90 mL), and the resulting solution was cooled to 0 °C while stirring under nitrogen. *m*-CPBA (77.1%, 696 mg, 3.11 mmol) in dichloromethane (30 mL) was added dropwise, and the reaction mixture was stirred for 3 h while warming to room temperature. The reaction mixture was then washed with saturated aqueous sodium bicarbonate (3 × 50 mL), water (2 × 50 mL), and brine (50 mL), the organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure to give the crude product as a white solid. This was then purified by chromatography on silica gel using 40% ethyl acetate−hexane to produce a white solid **5** (1.08 g, 77%). NMR analysis showed this to be a mixture of the α- and β-epoxides in a ratio of 5:1, which could not be separated by further chromatography. α-Epoxide **5**: mp (mixture) 141−143 °C; found C 80.85, H, 11.13, $C_{29}H_{48}O_2$ requires C 81.25, H 11.29%; ν_{max}/cm^{-1} 3484, 2940, 2868, 1458, 1374; δ_H (300 MHz; $CDCl_3$) 0.54−2.15 [44H, m, containing 0.63 (3H, s, 18-CH_3), 0.79−0.84 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.99 (3H, d, 21-CH_3), 1.06 (3H, s, 19-CH_3)], 2.90 (1H, d, *J* 4.4, 6-H), 3.82−3.99 (1H, m, 3α-H), 5.00 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.13 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H); δ_C (75.5 MHz; $CDCl_3$) 12.25 (CH_3), 12.43 (CH_3), 16.13 (CH_3), 19.17 (CH_3), 20.83 (CH_2), 21.31 (CH_3), 21.37 (CH_3), 24.32 (CH_2), 25.61 (CH_2), 28.97 (CH_2), 29.00 (CH_2), 30.07 (CH), 31.27 (CH_2), 32.07 (CH), 32.60 (CH_2), 35.06 (quaternary C), 39.49 (CH_2), 40.06 (CH_2), 40.69 (CH), 42.41 (quaternary C), 42.77 (CH), 51.41 (CH), 55.80 (CH), 57.15 (CH), 59.50 (CH, C-6), 65.93 (quaternary C, C-5), 68.91 (CH, C-3), 129.50 (CH), 138.44 (CH); *m*/*z* (ESI^+) 429.3747 ($M^+ + H$, $C_{29}H_{49}O_2$ requires 429.3733), 429 ($M^+ + H$, 42%), 412 (100), 391 (70), 371 (22). Characteristic β-epoxide **6** signals also present.

5β,6β-Epoxystigmast-22-en-3β-ol 6. A suspension of 5β,6β-epoxystigmast-22-en-3β-ol acetate **22** (200 mg, 0.43 mmol) in methanol (16 mL) was stirred for 5 min at room temperature under nitrogen. Sodium carbonate (97 mg, 0.91 mmol) was then added, and the reaction mixture was stirred for 18 h. The solvent was then removed under reduced pressure to give the crude epoxide as a white solid. The product was then purified by column chromatography on silica gel using 20% ethyl acetate−hexane, to give the epoxide **6** (167 mg, 92%) as a white solid. NMR analysis showed this to be a mixture of the β- and α-epoxides in a ratio of 7.5:1, which could not be separated by further chromatography. β-Epoxide **6**: mp (mixture) 155−158 °C; ν_{max}/cm^{-1} 3431, 2956, 2867, 1456, 1384; δ_H (300 MHz; $CDCl_3$) 0.54−2.15 [44H, m, containing 0.77 (3H, s, 18-CH_3), 0.81−0.85 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.99 (3H, d, 21-CH_3), 1.00 (3H, s, 19-CH_3)], 3.06 (1H, d, *J* 2.2, 6-H), 3.61−3.78 (1H, m, 3α-H), 5.00 (1H, dd, *J* 15.2, 8.5), 5.13 (1H, dd, *J* 15.2, 8.4); δ_C (75.5 MHz; $CDCl_3$) 12.12 (CH_3), 12.43 (CH_3), 17.23 (CH_3), 19.14 (CH_3), 21.27 (CH_3), 21.35 (CH_3), 22.15 (CH_2), 24.43 (CH_2), 25.58 (CH_2), 29.04 (CH_2), 29.93 (CH), 31.19 (CH_2), 32.04 (CH), 32.75 (CH_2), 35.03 (quaternary C), 37.40 (CH_2), 39.89 (CH_2), 40.65 (CH), 42.34 (quaternary C), 42.38 (CH_2), 51.41 (CH), 51.52 (CH), 56.15 (CH), 56.49 (CH), 63.15 (quaternary C), 63.92 (CH), 69.59 (CH), 129.50 (CH), 138.39 (CH); *m*/*z* (ESI^+) 429.3744 ($M^+ + H$, $C_{29}H_{49}O_2$ requires 429.3733), 429 ($M^+ + H$, 18%), 412 (100), 394 (21), 317 (5), 273 (8), 203 (4), 192 (8), 143 (14), 131 (15), 89 (22), 83 (24), 64 (30), 42 (63). Characteristic α-epoxide **5** signals also present.

Stigmasta-5,22-dien-7-on-3β-ol 7. A suspension of the acetate of stigmasta-5,22-dien-7-on-3β-ol **15** (299 mg, 0.64 mmol) in methanol (35 mL) was stirred at room temperature for 5 min. Potassium carbonate (176 mg, 1.28 mmol) dissolved in water (5 mL) was added to the suspension and the mixture stirred at room temperature for 50 h. The volatiles were removed under reduced pressure. The residue was taken up in ethyl acetate (50 mL), washed with water (3 × 50 mL) and brine (50 mL), and dried over magnesium sulfate, and the solution was concentrated under reduced pressure to give the crude product as a white solid, which was then purified by column chromatography on silica gel, eluting with gradient ethyl acetate−hexane (20%−40%), to give stigmasta-5,22-dien-7-on-3β-ol **7** (356 mg, 66%) as a white solid: mp 160−162 °C [lit. (*21*) 160−162 °C]; ν_{max}/cm^{-1} 3435, 2954, 1675, 1465; δ_H (300 MHz; $CDCl_3$) 0.70 (3H, s, 18-CH_3), 0.73−0.93 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 1.03 (3H, d, *J* 6.6, 21-CH_3), 1.08−2.57 [27H, m, containing 1.20 (3H, s, 19-CH_3)], 3.59−3.78 (1H, m, 3α-H), 5.02 (1H, dd, *J* 15.2, 8.6, one of 22-H or 23-H), 5.17 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.69 (1H, s, 6-H); δ_C (75.5 MHz; $CDCl_3$) 12.19 (CH_3), 12.26 (CH_3), 17.31 (CH_3), 19.00 (CH_3), 21.07 (CH_3), 21.19 (CH_2), 21.41 (CH_3), 25.37 (CH_2), 26.40 (CH_2), 29.06 (CH_2), 31.14 (CH_2), 31.88 (CH), 36.34 (CH_2), 38.29 (quaternary C), 38.56 (CH_2), 40.27 (CH), 41.82 (CH_2), 42.97 (quaternary C), 45.37 (CH), 49.91 (CH), 50.01 (CH), 51.20 (CH), 54.65 (CH), 70.47 (CH, C-3), 126.04 (CH), 129.45 (CH), 138.09 (CH), 165.26 (quaternary C, C-5), 202.35 (quaternary C, C=O); *m*/*z* (ESI^+) 427.3587 ($M^+ + H$, $C_{29}H_{47}O_2$ requires 427.3576), 427 ($M^+ + H$, 100%), 105 (15), 64 (4).

Stigmasta-5,22-diene-3β,7β-diol 8. A suspension of stigmasta-5,22-dien-7-on-3β-ol **7** (133 mg, 0.31 mmol) and cerium chloride heptahydrate (174 mg, 0.47 mmol) in methanol (5 mL) was stirred at room temperature for 15 min. Sodium borohydride (39 mg, 1.03 mmol) was added to the suspension and the mixture stirred at room temperature for 72 h. The reaction mixture was partitioned between water (30 mL) and ethyl acetate (30 mL). The organic layer was washed with water (2 × 20 mL) and brine (3 × 20 mL), dried over magnesium sulfate and concentrated under reduced pressure to yield the crude product as a white solid. This was then purified by chromatography on silica gel eluting with 40% ethyl acetate−hexane, yielding stigmasta-5,22-diene-3β,7β-diol **8** (72 mg, 54%) as a white solid: mp 154−156 °C [lit. (*22*) 154 °C]; ν_{max}/cm^{-1} 3413, 2958, 1633, 1460; δ_H (300 MHz; $CDCl_3$) 0.71 (3H, s, 18-CH_3), 0.73−2.41 [40H, m, containing 1.02−1.07 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 1.15 (3H, d, 21-CH_3), 1.17 (3H, s, 19-CH_3)], 3.44−3.62 (1H, m, 3α-H), 3.85 (1H, d, *J* 7.9, 7-H), 5.03 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.16 (1H, dd, *J* 15.2, 8.4, one of 22-H or 23-H), 5.29 (1H, br s, 6-H); δ_C (75.5 MHz; $CDCl_3$) 12.22 (CH_3), 12.46 (CH_3), 19.18 (CH_3), 19.34 (CH_3), 21.24 (CH_2), 21.29 (CH_3), 21.49 (CH_3), 25.58 (CH_2), 26.65 (CH_2), 29.37 (CH_2), 31.70 (CH_2), 32.06 (CH), 36.62 (quaternary C), 37.12 (CH_2), 39.63 (CH_2), 40.57 (CH), 41.00 (CH), 41.89 (CH_2), 42.99 (quaternary C), 48.43 (CH), 51.42 (CH), 55.45 (CH), 56.21 (CH), 71.57 (CH), 73.52 (CH), 125.60 (CH), 129.63 (CH), 138.33 (CH), 143.61 (quaternary C); *m*/*z* (ESI^+) 411.3624 ($M^+ + H - H_2O$, $C_{29}H_{47}O$ requires 411.3627), 411 ($M^+ + H - H_2O$, 20%), 395 (4), 394 (30), 393 (100).

5α-Stigmast-22-ene-3β,5α,6β-triol 9. To a mixture of **5** and **6** (**5**:**6** = 5:1, 276 mg, 0.64 mmol) in acetone (15 mL) and water (3 mL) was added 3 drops of concentrated sulfuric acid. The reaction mixture was stirred at room temperature for 2 h and was then concentrated. Ethyl acetate (25 mL) was added to the residue, which was then washed with water (2 × 6 mL) and brine (10 mL) and dried over magnesium sulfate. The solvent was then evaporated under reduced pressure to give the crude product as a white solid. The product was purified by column

chromatography on silica gel using ethyl acetate to give the pure triol **9** (93 mg, 32%) as a white solid: mp 252−254 °C (from ethyl acetate) [lit. (*23*) 256−257 °C]; $[\alpha]_D^{20}$ −16.7 (*c* 0.096 in $CHCl_3$) [lit. (*23*) −8]; found C 77.57, H 11.08; $C_{29}H_{50}O_3$ requires C 77.97, H 11.28%; ν_{max}/cm^{-1} 3432, 2941, 1654; δ_H (300 MHz; DMSO-d_6) 0.66 (3H, s, 18-CH_3), 0.71−2.12 [41H, m, containing 0.77−0.82 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.98 (3H, d, 21-CH_3), 1.00 (3H, s, 19-CH_3)], 3.65 (1H, br s, 6-H), 3.73−3.91 (1H, m, 3α-H), 4.20 (1H, d, *J* 5.6, OH), 4.41 (1H, d, *J* 4.0, OH), 5.02 (1H, dd, *J* 15.1, 8.5, one of 22-H or 23-H), 5.16 (1H, dd, *J* 15.1, 8.5 one of 22-H or 23-H); δ_C (75.5 MHz; DMSO-d_6) 12.46 (CH_3), 12.49 (CH_3), 16.63 (CH_3), 19.20 (CH_3), 21.08 (CH_2), 21.33 (CH_3), 21.47 (CH_3), 24.28 (CH_2), 25.27 (CH_2), 29.01 (CH_2), 30.35 (CH), 31.44 (CH_2), 31.71 (CH), 32.37 (CH_2), 34.83 (CH_2), 38.12 (quaternary C), 40.02 (CH_2), 40.49 (CH), 41.25 (CH_2), 42.50 (quaternary C), 44.93 (CH), 50.98 (CH), 55.82 (CH), 56.28 (CH), 66.10 (CH, CHOH), 74.46 (CH, CHOH), 74.65 (quaternary C, C-5), 129.05 (CH), 138.56 (CH).

3,6,-di-TMS Ether of 5α-Stigmasterol-3*β*,5α,6*β*-triol 23. 5α-Stigmasterol-3*β*,5α,6*β*-triol **9** (40 mg, 0.09 mmol) and pyridine (1 mL) were added under nitrogen, followed by hexamethyldisilizane (0.30 mL) and chlorotrimethylsilane (0.15 mL). The reaction mixture was stirred for 18 h at room temperature. Water (15 mL) was then added, and the sample was extracted into hexane (2 × 30 mL). The organic layer was washed with water (2 × 15 mL) and brine (2 × 15 mL) and dried over magnesium sulfate. The solvent was then removed under reduced pressure to yield the TMS ether **23** (32 mg, 55%) as a brown solid: mp 110−113 °C; ν_{max}/cm^{-1} 3435, 2956, 1637, 1251, 1080; δ_H (300 MHz; $CDCl_3$) 0.07 [9H, s, $Si(CH_3)_3$], 0.12 [9H, s, $Si(CH_3)_3$], 0.57 (3H, s, 18-CH_3), 0.69−1.81 [38H, m, containing 0.62−0.67 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.94 (3H, d, 21-CH_3), 0.98 (3H, s, 19-CH_3)], 1.88−2.17 (3H, m), 3.44 (1H, br s), 4.04 (1H, septet, *J* 5.4, 3α-H), 5.01 (1H, dd, *J* 15.1, 8.5, one of 22-H or 23-H), 5.15 (1H, dd, *J* 15.1, 8.5, one of 22-H or 23-H); δ_C (75.5 MHz; $CDCl_3$) 0.44 (3 × CH_3), 0.53 (3 × CH_3), 12.48 (CH_3), 12.53 (CH_3), 17.10 (CH_3), 19.19 (CH_3), 21.32 (CH_3), 21.37 (CH_2), 21.39 (CH_3), 24.44 (CH_2), 25.64 (CH_2), 29.12 (CH_2), 30.37 (CH), 31.28 (CH_2), 32.11 (CH), 32.78 (CH_2), 34.92 (CH_2), 38.71 (quaternary C), 40.11 (CH_2), 40.73 (CH), 41.67 (CH_2), 42.83 (quaternary C), 46.46 (CH), 51.45 (CH), 56.14 (CH), 56.24 (CH), 68.58 (CH), 76.21 (CH), 77.44 (quaternary C), 129.46 (CH), 138.55 (CH); *m*/*z* (ESI^+) 501.4106 (M^+ − TMSOH, $C_{32}H_{57}O_2Si$ requires 501.4128), 501 (M^+ − TMSOH, 38%), 429 (54), 411 (100).

5,6,22,23-Diepoxystigmastane 10. Stigmasterol **1** (2.00 g, 4.85 mmol) was dissolved in dichloromethane (35 mL), and the solution was cooled to 0 °C. *m*-CPBA (77.1%, 4.38 g, 17.83 mmol) in dichloromethane (50 mL) was added dropwise, and the reaction mixture was stirred for 48 h at room temperature. The reaction mixture was worked up by washing with 5% sodium thiosulfate (3 × 60 mL), saturated aqueous sodium bicarbonate solution (2 × 60 mL), and brine (60 mL), dried over magnesium sulfate and concentrated under reduced pressure to give the crude product. This was purified by chromatography on silica gel using 60% ethyl acetate−hexane to yield the bisepoxide as a mixture of diastereomers, **10a**, **10b**, **10c**, and **10d**, in a ratio of 4.5:4.5:1:1 (1.06 g, 54%) as a white solid: mp 135−148 °C (as a mixture of diastereomers); ν_{max}/cm^{-1} 3435, 2960, 2871, 1466, 1379; δ_H (300 MHz; $CDCl_3$) 5α,6α isomers **10a**, **10b**, 0.53−2.16 [44H, m, containing 0.61 (3H, m, 18-CH_3), 0.80−0.85 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.95 (3H, m, 21-CH_3), 1.05 (3H, s, 19-CH_3), 2.08 (1H, dd, *J* 12.7, 11.4, one of 4-H)], 2.40−2.55 (3H, m, 22α-H, 22*β*-H, one of 23-H), 2.73 (1H, dd, *J* 7.1, 2.4, one of 23-H), 2.91 (1H, d, *J* 4.3, 6-H), 3.81−3.99 (1H, m, 3α-H), 5*β*,6*β* isomers **10c**, **10d** distinguishable signals, 2.40−2.55 (3H, m, 22α-H, 22*β*-H, one of 23-H), 2.73 (1H, dd, *J* 7.1, 2.4, one of 23-H), 3.06 (1H, d, *J* 2.1, 6-H), 3.61−3.78 (1H, m, 3α-H); δ_C (75.5 MHz; $CDCl_3$) signals for both diastereomers of the 5α,6α-bisepoxide **10a**, **10b**, 12.01, 12.15 (2 × CH_3), 12.53, 12.66 (2 × CH_3), 16.08 (2 × CH_3), 16.36, 16.45 (2 × CH_3), 19.51, 19.59 (2 × CH_3), 19.68, 20.38 (2 × CH_3), 20.75, 20.80 (2 × CH_2), 20.98, 21.07 (2 × CH_2), 24.42, 24.27 (2 × CH_2), 27.04, 27.94 (2 × CH_2), 28.91 (2 × CH_2), 29.29, 29.46 (2 × CH), 30.03 (2 × CH), 31.17 (2 × CH_2), 32.56 (2 × CH_2), 35.01 (2 × quaternary C), 38.89, 39.01 (2 × CH), 39.34, 39.35 (2 × CH_2), 39.96 (2 × CH_2), 42.70, 42.81 (2 × CH), 42.81, 42.83 (2 × quaternary C), 48.43, 48.91 (2 × CH), 53.26, 55.91 (2 × CH), 56.58, 56.61 (2 × CH), 58.74 (CH, one of C-23), 59.41, 59.48 (2 × CH, C-6), 62.26 (CH, one of C-23), 62.42, 63.28 (2 × CH, C-22), 65.96 (2 × quaternary C, C-5), 68.74 (2 × CH, C-3), distinguishable signals for both diastereomers of the 5*β*,6*β* isomer **10c**, **10d**, 11.90 (CH_3), 12.61 (CH_3), 16.25 (CH_3), 17.22 (CH_3), 19.75 (CH_3), 20.33 (CH_3), 22.09 (CH_2), 22.14 (CH_2), 24.60 (CH_2), 27.19 (CH_2), 29.94 (CH), 32.72 (CH_2), 37.41 (CH_2), 38.70 (CH), 42.33 (CH_2), 51.44, 51.53 (2 × CH), 53.62 (CH), 55.97, 56.22 (2 × CH), 63.18 (quaternary C), 63.85 (CH), 69.48 (CH); *m*/*z* (ESI^+) 445.3690 (M^+ + H, $C_{29}H_{49}O_3$ requires 445.3682), 445 (M^+ + H, 94%), 427 (100), 409 (9), 347 (36), 329 (8), 224 (5), 127 (16).

(22*S*,23*S*)-Stigmast-5-ene-3*β*,22,23-triol 12. (22*S*,23*S*)-Diol **19** (284 mg, 0.62 mmol) was dissolved in THF−water (9:1, 10 mL), and 5 M H_2SO_4 (0.20 mL) was added. The reaction mixture was stirred for 24 h at room temperature. The solvent was removed, and the residue was dissolved in ethyl acetate (20 mL), washed with saturated aqueous sodium bicarbonate (7 mL) and brine (10 mL) and dried over magnesium sulfate. Removal of the solvent under reduced pressure gave the crude product as a white solid. Purification by column chromatography on silica gel, eluted with 40% ethyl acetate−hexane, produced the (22*S*,23*S*)-triol **12** (240 mg, 87%) as a white solid: mp 139−141 °C; $[\alpha]_D^{20}$ −22.7 (*c* 0.198 in MeOH); ν_{max}/cm^{-1} 3391, 2954, 1630; δ_H (300 MHz; DMSO-d_6) 0.63−2.24 [45H, m, containing 0.63 (3H, s, 18-CH_3), 0.73 (3H, d, 21-CH_3), 0.77−0.80 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.80 (3H, s, 19-CH_3)], 3.11−3.42 (1H, m, 3α-H), 3.76 (1H, d, *J* 7.9), 4.21 (1H, d, *J* 6.2), 4.63 (1H, d, *J* 4.1), 5.27 (1H, br s, 6-H); δ_C (75.5 MHz; DMSO-d_6) 11.93 (CH_3), 14.65 (CH_3), 14.73 (CH_3), 17.85 (CH_3), 18.60 (CH_2), 19.48 (CH_3), 20.99 (CH_2), 22.22 (CH_3), 24.53 (CH_2), 26.53 (CH), 27.89 (CH_2), 31.69 (CH_2), 31.76 (CH_2), 31.84 (CH), 36.42 (quaternary C), 37.28 (CH_2), 39.57 (CH_2), 42.31 (CH), 42.66 (CH_2), 49.06 (CH), 49.96 (CH), 52.53 (CH), 56.22 (CH), 69.84 (CH) 70.35 (CH), 71.42 (CH), 120.76 (CH, C-6), 141.57 (quaternary C, C-5), one of the quaternary carbon obscured by DMSO signals; *m*/*z* (ESI^+) 429.3741 (M^++H − H_2O, $C_{29}H_{49}O_2$ requires 429.3733), 470 (M^++Na, 5%), 429 (M^++ H − H_2O, 4), 325 (4), 115 (9), 105 (78), 64 (100), 56 (14).

(22*R*,23*R*)-Stigmast-5-ene-3*β*,22,23-triol 13. (22*R*,23*R*)-Diol **20** (210 mg, 0.46 mmol) was dissolved in THF−water (9:1, 20 mL), and 5 M H_2SO_4 (0.65 mL) was added. The reaction mixture was stirred for 42 h at room temperature. The volatiles were removed under reduced pressure, and the residue was taken up in ethyl acetate (50 mL), washed with saturated aqueous sodium bicarbonate (40 mL) and brine (40 mL) and dried over magnesium sulfate. Removal of the solvent gave the crude product as a yellow solid. Purification by column chromatography on silica gel, eluted with 40% ethyl acetate−hexane, produced the (22*R*,23*R*)-triol **13** (137 mg, 67%) as a white solid: mp 210−212 °C; ν_{max}/cm^{-1} 3401, 2936, 1640, 1470; δ_H (300 MHz, MeOD) 0.74 (3H, s, 18-CH_3), 0.82−2.31 [42H, m, containing 0.88−0.93 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.95 (3H, d, 21-CH_3), 1.06 (3H, s, 19-CH_3)], 3.23−3.48 (2H, m, containing 3α-H), 3.56 (1H, d, *J* 8.4), 3.67 (1H, dd, *J* 8.7, 1.2), 5.34 (1H, d, *J* 4.8, 6-H); δ_C (75.5 MHz, MeOD) 12.37 (CH_3), 12.80 (CH_3), 14.39 (CH_3), 20.02 (CH_3), 20.31 (CH_3 and CH_2), 21.80 (CH_3), 22.38 (CH_2), 25.36 (CH_2), 28.99 (CH_2), 30.56 (CH), 32.45 (CH_2), 33.15 (CH_2), 33.49 (CH), 37.82 (quaternary C), 38.70 (CH_2), 38.83 (CH), 41.38 (CH_2), 43.17 (CH_2), 43.46 (quaternary C), 48.40 (CH), 51.87 (CH), 54.07 (CH), 58.33 (CH), 72.58 (CH), 73.83 (CH), 75.81 (CH), 122.54 (CH), 142.40 (quaternary C); *m*/*z* (ESI^+) 429.3741 (M^++H − H_2O, $C_{29}H_{49}O_2$ requires 429.3733), 430 (M^++ H − H_2O, 13%), 412 (10), 326 (6), 116 (4), 63.8, 115 (84),74 (100).

Stigmasterol Acetate 14. Acetic anhydride (22.9 mL, 242.3 mmol) and pyridine (19.5 mL, 242.3 mmol) were combined, and stigmasterol **1** (2.00 g, 4.85 mmol) was then added portionwise. The reaction mixture was stirred under nitrogen for 5 days at room temperature. The reaction mixture was then poured onto 10% aqueous HCl (200 mL) and was extracted with ethyl acetate (4 × 50 mL). The organic layer was then washed with water (100 mL), saturated aqueous sodium bicarbonate (100 mL), and brine (100 mL) and dried over magnesium sulfate. The solvent was removed under reduced pressure to give stigmasterol acetate **14** as a white solid, which was used without further purification (1.95 g, 89%): mp 141−143 °C (from ethyl acetate−hexane) [lit. (*24*) 141 °C]; found C 82.24, H 10.95, $C_{31}H_{50}O_2$ requires C 81.88, H 11.08%; ν_{max}/cm^{-1} 2961, 1730, 1459; δ_H (300 MHz; $CDCl_3$) 0.70 (3H, s, 18-CH_3), 0.74−0.89 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.89−2.14 [32H, m, including 1.01 (3H, d, 21-CH_3), 1.03 (3H, s, 19-CH_3) 2.03 (3H, s, CO_2CH_3)], 2.31 (2H, d, *J* 7.9), 4.50−4.68 (1H, m, 3α-H), 5.01 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.16 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.37 (1H, d, *J* 4.3, 6-H); δ_C (75.5 MHz; $CDCl_3$) 12.26 (CH_3), 12.47 (CH_3), 19.20 (CH_3), 19.52 (CH_3), 21.22 (CH_2), 21.31 (CH_3), 21.44 (CH_3), 21.65 (CH_3), 24.57 (CH_2), 25.63 (CH_2), 27.98 (CH_2), 29.13 (CH_2), 32.07 (CH), 32.10 (CH_2), 32.10

(CH), 36.81 (quaternary C), 37.21 (CH_2), 38.33 (CH_2), 39.83 (CH_2), 40.72 (CH), 42.41 (quaternary C), 50.26 (CH), 51.45 (CH), 56.14 (CH), 56.99 (CH), 74.18 (CH), 122.85 (CH), 129.48 (CH), 138.53 (CH), 139.85 (quaternary C), 170.73 (quaternary C, C=O); *m/z* (ESI^+) 395.3664 ($M^+ + H - C_2H_4O_2$, $C_{29}H_{47}$ requires 395.3678), 395 ($M^+ + H - C_2H_4O_2$, 4%), 177 (28), 130 (29), 89 (100), 61 (18).

Acetate of Stigmasta-5,22-dien-7-on-3*β*-ol 15. Chromium trioxide (1.72 g, 17.2 mmol) was suspended in dry dichloromethane (50 mL) and stirred for 30 min at −25 °C. Dimethylpyrazole (1.66 g, 17.2 mmol) was added in one portion, and the reaction mixture was stirred for 30 min at −20 °C. Stigmasterol acetate **14** (0.52 g, 1.15 mmol) was added, and the mixture was stirred at −20 °C for 1 h and then allowed to warm to room temperature over 2 h. The reaction mixture was then stirred for a further 36 h. Ethyl acetate (200 mL) was then added, and the brown suspension was filtered through Celite. The filtrate was then concentrated under reduced pressure to give a brown residue. This residue was purified by chromatography on silica gel using 40% ethyl acetate−hexane, yielding the acetate of stigmasta-5,22-dien-7-on-3*β*-ol **15** (1.76 g, 52%) as a white solid: mp 184−186 °C [lit. (*25*) 187 °C]; ν_{max}/cm^{-1} 2958, 2871, 1732, 1679, 1632, 1460; δ_H (300 MHz; $CDCl_3$) 0.70 (3H, s, 18-CH_3), 0.74−0.93 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 1.03 (3H, d, *J* 6.0, 21-CH_3), 1.08−2.64 [29H, m, containing 1.09 (3H, s, 19-CH_3), 2.03 (3H, s, $COOCH_3$)], 4.62−4.82 (1H, m, 3α-H), 5.02 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.28 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.70 (1H, s, 6-H); δ_C (75.5 MHz; $CDCl_3$) 12.40 (CH_3), 12.47 (CH_3),17.46 (CH_3), 19.20 (CH_3), 21.27 (CH_3), 21.35 (CH_2), 21.49 (CH_3), 21.63 (CH_3), 25.58 (CH_2), 26.60 (CH_2), 27.55 (CH_2), 29.26 (CH_2), 32.09 (CH), 36.20 (CH_2), 37.95 (CH_2), 38.53 (quaternary C), 38.73 (CH_2), 40.48 (CH), 43.19 (quaternary C), 45.60 (CH), 50.00 (CH), 50.21 (CH), 51.41 (CH), 54.85 (CH), 71.41 (CH), 126.90 (CH), 129.67 (CH), 138.30 (CH), 164.04 (quaternary C, C-5), 170.51 (quaternary C, C=O), 202.12 (quaternary C, C-7); *m/z* (ESI^+) 469.3694 ($M^+ + H$, $C_{31}H_{49}O_3$ requires 469.3682), 469 ($M^+ + H$, 100%), 411 (19), 409 (68), 105 (9).

Stigmasteryl Tosylate 16. A solution of stigmasterol **1** (5.00 g, 12.12 mmol), 4-DMAP (0.13 g, 10 mol %) and *p*-toluenesulfonyl chloride (4.36 g, 22.9 mmol) in pyridine (50 mL) was stirred at room temperature for 22 h. The reaction mixture was poured into 10% aqueous sodium bicarbonate (200 mL), and the precipitate was filtered, washed with water (20 mL), and recrystallized from acetone to give the tosylate **16** (5.80 g, 94%) as white needles: mp 142−144 °C (from ethyl acetate) [lit. (*10*) 142−144 °C]; ν_{max}/cm^{-1} 2957, 1598, 1464, 1354; δ_H (300 MHz; $CDCl_3$) 0.67 (3H, s, 18-CH_3), 0.73−2.12 [38H, m, containing 0.78−0.85 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 1.01 (3H, s, 19-CH_3), 1.03 (3H, d, 21-CH_3)], 2.21−2.32 (1H, m), 2.36−2.52 [4H, m, containing 2.45 (3H, s, $TsCH_3$)], 4.21−4.41 (1H, m, 3α-H), 5.00 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.14 (1H, dd, *J* 15.1, 8.4), 5.30 (1H, d, *J* 5.3, 6-H), 7.33 (2H, d, *J* 8.3, ArH), 7.80 (2H, d, *J* 8.3, ArH); δ_C (75.5 MHz; $CDCl_3$) 12.22 (CH_3), 12.47 (CH_3), 19.18 (CH_3), 19.35 (CH_3), 21.17 (CH_2), 21.31 (CH_3), 21.41 (CH_3), 21.85 (CH_3), 24.51 (CH_2), 25.61 (CH_2), 28.82 (CH_2), 29.10 (CH_2), 31.93 (CH), 32.03 (CH_2), 32.08 (CH), 36.55 (quaternary C), 37.08 (CH_2), 39.05 (CH_2), 39.74 (CH_2), 40.69 (CH), 42.37 (quaternary C), 50.11 (CH), 51.43 (CH), 56.08 (CH), 56.93 (CH), 82.59 (CH), 123.71 (CH), 127.84 (CH, 2 × ArCH), 129.49 (CH), 129.95 (CH, 2 × ArCH), 134.85 (quaternary C), 138.46 (CH), 139.04 (quaternary C), 144.61 (quaternary C).

***i*-Stigmasterol Methyl Ether 17.** The tosylate **16** (5.80 g, 10.20 mmol) and pyridine (2.48 mL) were dissolved in anhydrous methanol and refluxed for 6 h. The solution was evaporated and extracted into ethyl acetate (150 mL) and washed with water (2 × 120 mL). The organic layer was washed with brine (2 × 100 mL) and dried over magnesium sulfate to give the crude product as an oily solid, as a mixture (4:1) of *i*-stigmasterol methyl ether **17** and stigmasterol methyl ether **18** (3.70 g, 85%). The residue was then purified on silica gel using 5% ethyl acetate−hexane to give *i*-stigmasterol methyl ether **17** (2.62 g, 60%) as a clear oil: ν_{max}/cm^{-1} 2956, 2906, 1454; δ_H (300 MHz; $CDCl_3$) 0.43 (1H, dd, *J* 7.9, 5.1), 0.56−2.21 [43H, m, containing 0.74 (3H, s, 18-CH_3), 0.78−0.85 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.96 (3H, s, 19-CH_3), 1.00 (3H, d, 21-CH_3)], 2.77 (1H, t, *J* 2.7, CHOMe), 3.33 (3H, s, OCH_3), 5.01 (1H, dd, *J* 15.1, 8.5, one of 22-H or 23-H), 5.15 (1H, dd, *J* 15.3, 8.5, one of 22-H or 23-H); δ_C (75.5 MHz; $CDCl_3$) 12.48 (CH_3), 12.66 (CH_3), 13.30 (CH_2), 19.21 (CH_3), 19.51 (CH_3), 21.33 (CH_3), 21.44 (CH_3), 21.68 (CH), 22.98 (CH_2), 24.48 (CH_2), 25.18 (CH_2), 25.64 (CH_2), 29.25 (CH_2), 30.70 (CH), 32.11 (CH), 33.57 (CH_2), 35.30 (CH_2), 35.47 (quaternary C), 40.41 (CH_2), 40.78 (CH), 42.88 (quaternary C), 43.61 (quaternary C), 48.28 (CH), 51.47 (CH), 56.33 (CH), 56.78 (CH_3, OCH_3), 56.85 (CH), 82.63 (CH, C-6), 129.41 (CH), 138.63 (CH); *m/z* (ESI^+) 428 ($M^+ + H$, 50%), 412 (34), 391 (14), 327 (16), 253 (26), 192 (22), 151 (100), 105 (48), 84 (80), 83 (59), 64 (41).

Stigmasterol methyl ether **18** (white solid obtained from previous silica gel purification, 0.46 g, 11%): mp 117−119 °C (from ethyl acetate−hexane) [lit. (*10*) 116−117 °C]; ν_{max}/cm^{-1} 2938, 2865, 1461, 1381; δ_H (300 MHz; $CDCl_3$) 0.62−2.23 [42H, m, containing 0.62 (3H, s, 18-CH_3), 0.69−0.74 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.89 (3H, s, 19-CH_3), 0.92 (3H, d, 21-CH_3)], 2.39 (1H, ddd, *J* 13.2, 4.7, 2.1), 2.98−3.14 (1H, m, 3α-H), 3.36 (3H, s, OCH_3), 5.01 (1H, dd, *J* 15.1, 8.5, one of 22-H or 23-H), 5.15 (1H, dd, *J* 15.3, 8.5, one of 22-H or 23-H), 5.35 (1H, d, *J* 5.3, 6-H); δ_C (75.5 MHz; $CDCl_3$) 12.26 (CH_3), 12.48 (CH_3), 19.20 (CH_3), 19.59 (CH_3), 21.27 (CH_2), 21.32 (CH_3), 21.44 (CH_3), 24.58 (CH_2), 25.63 (CH_2), 28.22 (CH_2), 29.16 (CH_2), 32.10 (CH), 32.10 (CH), 32.14 (CH_2), 37.11 (quaternary C), 37.39 (CH_2), 38.90 (CH_2), 39.89 (CH_2), 40.74 (CH), 42.42 (quaternary C), 50.42 (CH), 51.45 (CH), 55.83 (CH_3, OCH_3), 56.15 (CH), 57.09 (CH), 80.56 (CH, C-3), 121.80 (CH), 129.46 (CH), 138.56 (CH), 141.08 (quaternary C).

(22*S*,23*S*)-3α,5-Cyclo-5α-stigmasta-3*β*,22,23-triol 19. *i*-Stigmasterol methyl ether **17** was dissolved in a THF−*t*-butanol−water mixture (21.6 mL:12.3 mL:3.3 mL), and NMO (9.20 g, 78.53 mmol) was added. The resulting suspension was stirred for 5 min, and osmium tetroxide (1.60 mL, 4% in water) was added. The yellow suspension was stirred at room temperature for 11 days. Sodium sulfite (6 g) was added, and the mixture was stirred overnight. The volatiles were removed under reduced pressure, the residue was taken up in ethyl acetate (100 mL) and was then washed with water (2 × 60 mL) and brine (60 mL) and dried over magnesium sulfate, and the solvent was removed to give the crude product as a pale yellow oil. The product was purified by column chromatography on silica gel using 40% ethyl acetate−hexane to give the pure product **19** (564 mg, 47%) as white solid: mp 61−62 °C [lit. (*26*) 122−123 °C]; $[\alpha]_D^{20}$ +33.2 (*c* 0.19 in $CHCl_3$) [lit. (*27*) +32.7]; ν_{max}/cm^{-1} 3436, 2955, 2870, 1471, 1384; δ_H (300 MHz; $CDCl_3$) 0.44 (1H, dd, *J* 7.9, 5.1), 0.61−0.69 (1H, m), 0.76 (3H, s, 18-CH_3), 0.80−2.19 [40H, m, containing 0.88−0.99 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 1.02 (3H, s, 19-CH_3), 1.03 (3H, d, *J* 7.0, 21-CH_3)], 2.26 (1H, d, *J* 6.3), 2.77 (1H, t, *J* 2.7, 6-H), 3.33 (3H, s, OCH_3), 3.56−3.69 (2H, m, 22-H and 23-H); δ_C (75.5 MHz; $CDCl_3$) 12.35 (CH_3), 13.30 (CH_2), 14.25 (CH_3), 14.71 (CH_3), 17.91 (CH_3), 18.69 (CH_2), 19.47 (CH_3), 21.64 (CH), 22.00 (CH_3), 22.97 (CH_2), 24.62 (CH_2), 25.16 (CH_2), 27.09 (CH), 28.30 (CH_2), 30.70 (CH), 33.55 (CH_2), 35.27 (CH_2), 35.40 (quaternary C), 40.43 (CH_2), 42.72 (CH), 43.50 (quaternary C), 43.57 (quaternary C), 48.17 (CH), 49.85 (CH), 52.97 (CH), 56.36 (CH), 56.78 (CH_3, OCH_3), 70.71 (CH), 72.35 (CH), 82.55 (CH); *m/z* (ESI^+) 429.3739 ($M^+ + H$ - CH_3OH, $C_{29}H_{49}O_2$ requires 429.3733), 429 ($M^+ + H - CH_3OH$, 100%), 411 (32), 332 (18), 313 (19), 192 (13) 127 (10).

(22*R*,23*R*)-3α,5-Cyclo-5α-stigmasta-3*β*,22,23-triol 20 (*28*). To a stirring solution of NMO (3.29 g, 28.12 mmol) and DHQD PHN (0.94 g, 1.88 mmol) in THF−*t*-butanol−water (14.4 mL:8 mL:2.2 mL) at 0 °C, was added *i*-stigmasterol methyl ether **17** (400 mg, 0.94 mmol) in THF−*t*-butanol−water (7.2 mL:4 mL:1.1 mL) over 1 h. The solution was maintained at 0 °C until the addition was complete and was then stirred at room temperature in the dark for 5 days. Dichloromethane (100 mL) and 5 M HCl (60 mL) was added to the reaction mixture and the organic layer was washed with saturated aqueous sodium metabisulfite solution (100 mL), water (100 mL), and brine (100 mL). The organic layer was dried over magnesium sulfate to give the crude product as a brown solid. The product was purified by column chromatography on silica gel using 20% ethyl acetate−hexane to give the pure product **20** (129 mg, 30%) as a white solid: mp 68−70 °C; $[\alpha]_D^{20}$ +30.1 (*c* 1.38 in $CHCl_3$) [lit. (*27*) +69.1]; ν_{max}/cm^{-1} 3435, 2957, 2870, 1470, 1384; δ_H (300 MHz; $CDCl_3$) 0.44 (1H, dd, *J* 8.1, 5.1), 0.58−2.03 [43H, m, containing 0.73 (3H, s, 18-CH_3), 0.88−0.98 (12H, m, 26-CH_3, 27-CH_3, 29-CH_3 and 21-CH_3), 1.03 (3H, s, 19-CH_3)], 2.15 (2H, br s, 22-OH and 23-OH), 2.78 (1H, t, *J* 2.7, 6α-H), 3.33 (3H, s, OCH_3), 3.61 (1H, d, *J* 8.9, one of 22-H or 23-H), 3.72 (1H, d, *J* 8.9, one of 22-H or 23-H); δ_C (75.5 MHz; $CDCl_3$) 11.93 (CH_3), 12.09 (CH_3), 13.07 (CH_2), 13.46 (CH_3), 18.86 (CH_2), 19.27 (CH_3), 19.43 (CH_3), 21.25 (CH_3), 21.44 (CH), 22.78 (CH_2), 24.03 (CH_2), 24.92 (CH_2), 27.88 (CH_2), 28.82 (CH), 30.55 (CH), 33.32 (CH_2), 35.02 (CH_2), 35.17 (quaternary C), 36.94 (CH), 40.25 (CH_2), 42.61 (quaternary C), 43.33

Table 1. LC−MS Analysis of Individual Stigmasterol Oxides

phytosterol	RT/min	obsd ions *m*/*z*	exact mass *m*/*z* (molecular formulas)
epoxy diol	3.4	409, 427, 445, 463	463.3777 ($C_{29}H_{51}O_4$) M^+
3,22*R*,23*R*-triol	4.1	393, 411, 429	429.3727 ($C_{29}H_{49}O_2$) M^+ ($-H_2O$)
bisepoxide	5.7, 6.6	409, 427, 429, 445	445.3697 ($C_{29}H_{49}O_3$) M^+
7-ketostigmasterol	7.1	427	427.3570 ($C_{29}H_{47}O_2$) M^+
7β-OH stigmasterol	7.6	393, 411	411.3635 ($C_{29}H_{47}O$) M^+ ($-H_2O$)
5,6-β-epoxide	9.4	427, 411, 409	411.3631 ($C_{29}H_{47}O$) M^+ ($-H_2O$)
3,5,6-triol	10.6	393, 411, 429	411.3650 ($C_{29}H_{47}O$) M^+ ($-2H_2O$)
5,6-α-epoxide	11.3	393, 411, 429	411.3627 ($C_{29}H_{47}O$) M^+ ($-H_2O$)
stigmasterol	14.8	395, 433, 488	395.3691 ($C_{29}H_{47}$) M^+ ($-H_2O$)

(quaternary C), 46.20 (CH), 47.93 (CH), 52.68 (CH), 56.38 (CH), 56.55 (CH_3, OCH_3), 72.66 (CH), 74.72 (CH), 82.36 (CH, C-6); *m*/*z* (ESI^+) 429.3731 ($M^+ + H - CH_3OH$, $C_{29}H_{49}O_2$ requires 429.3733), 429 ($M^+ + H - CH_3OH$, 100%), 411 (70), 393 (51), 285 (20), 127 (8), 64 (7).

(22*S*,23*S*)-5,6-Epoxystigmasta-22,23-diol 21. (22*S*,23*S*)-Stigmast-5-ene-3β,22,23-triol **12** (381 mg, 0.85 mmol) was dissolved in dichloromethane (20 mL) and cooled to 0 °C. *m*-CPBA (75.1%, 162 mg, 0.71 mmol) was dissolved in dichloromethane (10 mL) and added dropwise. The resulting suspension was stirred for 24 h at room temperature. The reaction mixture was worked up by washing with saturated sodium bicarbonate (3 × 10 mL), water (15 mL) and brine (15 mL). The organic layer was dried over magnesium sulfate and the solvent removed under reduced pressure to give the crude product. This was purified by chromatography on silica gel using 40% ethyl acetate−hexane to yield the epoxy diol **21**, as a mixture of diastereomers **21a** and **21b** in a ratio of 5:1 (178 mg, 45%) as a white solid: mp (mixture) 80−86 °C; ν_{max}/cm^{-1} 3421, 2947, 1469, 1384; δ_H (300 MHz; $CDCl_3$) 0.65 (3H, s, 18-CH_3), 0.78−2.18 [41H, m, containing 0.88−0.99 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 1.01 (3H, d, 21-CH_3), 1.06 (3H, s, 19-CH_3)], 2.22−2.54 (1H, m), 2.92 (1H, d, *J* 4.4, 6-H), 3.40−3.76 [3H, m, including 3.59 (2H, s, 22-H and 23-H)], 3.79−3.98 (1H, m, 3α-H); δ_C (75.5 MHz; $CDCl_3$) 11.95 (CH_3), 14.23 (CH_3), 14.68 (CH_3), 16.10 (CH_3), 17.90 (CH_3), 18.70 (CH_2), 20.83 (CH_2), 22.00 (CH_3), 24.48 (CH_2), 27.06 (CH), 28.03 (CH_2), 28.93 (CH_2), 30.07 (CH), 31.25 (CH_2), 32.58 (CH_2), 35.03 (quaternary C), 39.57 (CH_2), 40.01 (CH_2), 42.58 (CH), 42.72 (CH), 43.06 (quaternary C), 49.80 (CH), 52.55 (CH), 56.70 (CH), 59.44 (CH), 65.94 (quaternary C), 68.86 (CH), 70.66 (CH), 72.33 (CH); *m*/*z* (ESI^+) 463.3778 ($M^+ + H$, $C_{29}H_{51}O_4$ requires 463.3787), 463 ($M^+ + H$, 100%), 445 (78), 427 (18), 333 (4), 325 (4), 87 (5). Distinguishable signals for the minor isomer **21b**: δ_H (300 MHz; $CDCl_3$) 3.07 (1H, d, *J* 2.1, 6-H); δ_C (75.5 MHz; $CDCl_3$) 11.84 (CH_3), 14.30 (CH_3), 22.16 (CH_2), 24.62 (CH_2), 32.75 (CH_2), 51.45 (CH), 52.83 (CH), 60.61 (CH), 69.58 (CH), 70.76 (CH).

5β,6β-Epoxystigmast-22-en-3β-ol Acetate 22. Copper sulfate pentahydrate (12.20 g) and potassium permanganate (5.40 g) were ground together into a fine powder with a mortar and pestle, to which water (1.5 mL) was added. The resulting moist solid was then transferred to a 100 mL round bottomed flask that contained the acetate **14** (1.17 g, 2.57 mmol), dissolved in dichloromethane (30 mL). *t*-Butanol (1.5 mL) was then added, and the reaction mixture was stirred at room temperature for 16 h. The reaction mixture was then filtered through a plug of silica and was eluted with dichloromethane. This was then concentrated to give the crude product as a brown solid, which was purified by column chromatography on silica gel using 10% ethyl acetate−hexane. This produced the protected epoxide **22** (596 mg, 49%) as a white solid. NMR analysis showed this to be a mixture of the β- and α-epoxides in a ratio of 6.8:1, which could not be separated by further chromatography. β-Epoxide: mp (mixture) 143−145 °C; ν_{max}/cm^{-1} 2867, 1732, 1457, 1370, 1266, 1252; δ_H (300 MHz; $CDCl_3$) 0.53−2.28 [46H, m, containing 0.66 (3H, s, 18-CH_3), 0.77−0.85 (9H, m, 26-CH_3, 27-CH_3 and 29-CH_3), 0.98 (3H, d, 21-CH_3), 0.99 (3H, s, 19-CH_3), 2.03 (3H, s, $COOCH_3$)], 3.07 (1H, d, *J* 2.1, 6-H), 4.67−4.84 (1H, m, 3α-H), 5.00 (1H, dd, *J* 15.2, 8.5, one of 22-H or 23-H), 5.14 (1H, dd, *J* 15.2, 8.4, one of 22-H or 23-H); δ_C (75.5 MHz; $CDCl_3$) 12.15 (CH_3), 12.28 (CH_3), 17.24 (CH_3), 19.18 (CH_3), 21.30 (CH_3), 21.38 (CH_3), 21.53 (CH_3), 22.10 (CH_2), 24.45 (CH_2), 25.60 (CH_2), 27.40 (CH_2), 29.06 (CH_2), 29.91 (CH), 32.07 (CH), 32.65 (CH_2), 35.23 (quaternary C), 36.86 (CH_2), 38.20 (CH_2), 39.87 (CH_2), 40.67 (CH), 42.36 (quaternary C), 51.19 (CH), 51.43 (CH), 56.17 (CH), 56.46 (CH), 62.72 (quaternary C), 63.78 (CH), 71.54 (CH), 129.55 (CH), 138.41 (CH), 170.77 (quaternary C, C=O); *m*/*z* (ESI^+) 471.3832 ($M^+ + H$, $C_{31}H_{51}O_3$ requires 471.3838), 471 ($M^+ + H$, 74%), 453 (100), 393 (64), 192 (48), 139 (21), 64 (22).

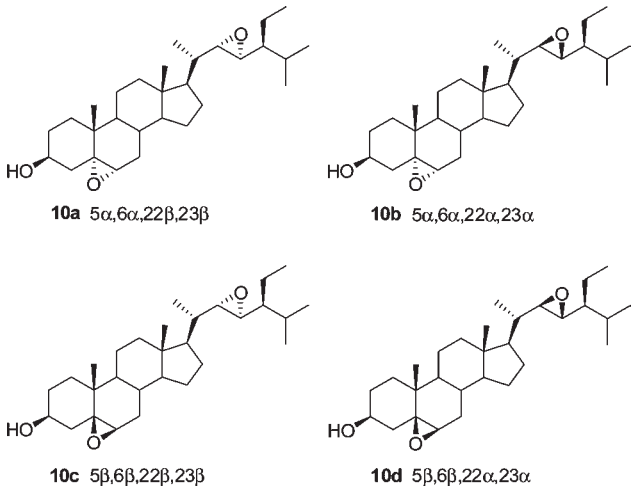

Figure 2. Bisepoxide oxidation products of stigmasterol.

LC−MS of Stigmasterol Oxides. LC was performed with a Waters (Milford, MA) Alliance 2695 equipped with an autosampler, a degasser, a column heater and an in-line 996 PDA detector. Mass spectrometry was performed with an LCT Premier time-of-flight instrument from Waters-Micromass (Manchester, U.K.). Data was collected by use of Masslynx 4.1 software. Compounds were separated on a Waters Atlantis 5 μm particle column 150 mm × 4.6 mm i.d. The column temperature was set at 20 °C, and the run time was 33 min. The mobile phase was a gradient prepared from acetonitrile + 0.1% formic acid (A) and chloroform (B). The column was equilibrated with 80:20 A:B at a flow rate of 1 mL min^{-1} for 20 min, and consequently the following gradient was used: A:B at 0 min 80:20, at 15 min 80:20, at 18 min 60:40, at 30 min 60:40 and at 33 min back to 80:20.

Analytes were detected by the use of ESI in positive mode with the following parameter set: capillary voltage 3.6 kV; cone voltage 30 V; source temperature 130 °C; desolvation temperature 350 °C; nebulizing gas 450 L/h; cone gas 10 L/h. Ions were detected in positive mode, and extracted ion chromatograms were consequently generated from the software for each of the relevant ions.

A table of retention times for each of the analytes tested by this method is shown in **Table 1**. Critically each of the stigmasterol oxides can be detected and identified using this method.

RESULTS AND DISCUSSION

As in our earlier study based on β-sitosterol (*10*), the stigmasterol oxides targeted in this study, namely, the 5α,6α- **5** and 5β,6β-epoxides **6**, the 3β,5α,6β-triol **9**, 7-keto **7** and 7β-hydroxide **8** (Figure **3**) were chosen for comparison with the most toxic of the cholesterol oxidation products. Taking into account the additional sites for oxidation, due to the presence of the second alkene bond on the side chain of stigmasterol **1**, the 3β,22,23-triols **12**, **13** and the pentol **11** (**Figure 4**) were included as targets. In contrast to the β-sitosterol study, the commercial availability of relatively pure samples of stigmasterol **1** allowed us to use this material

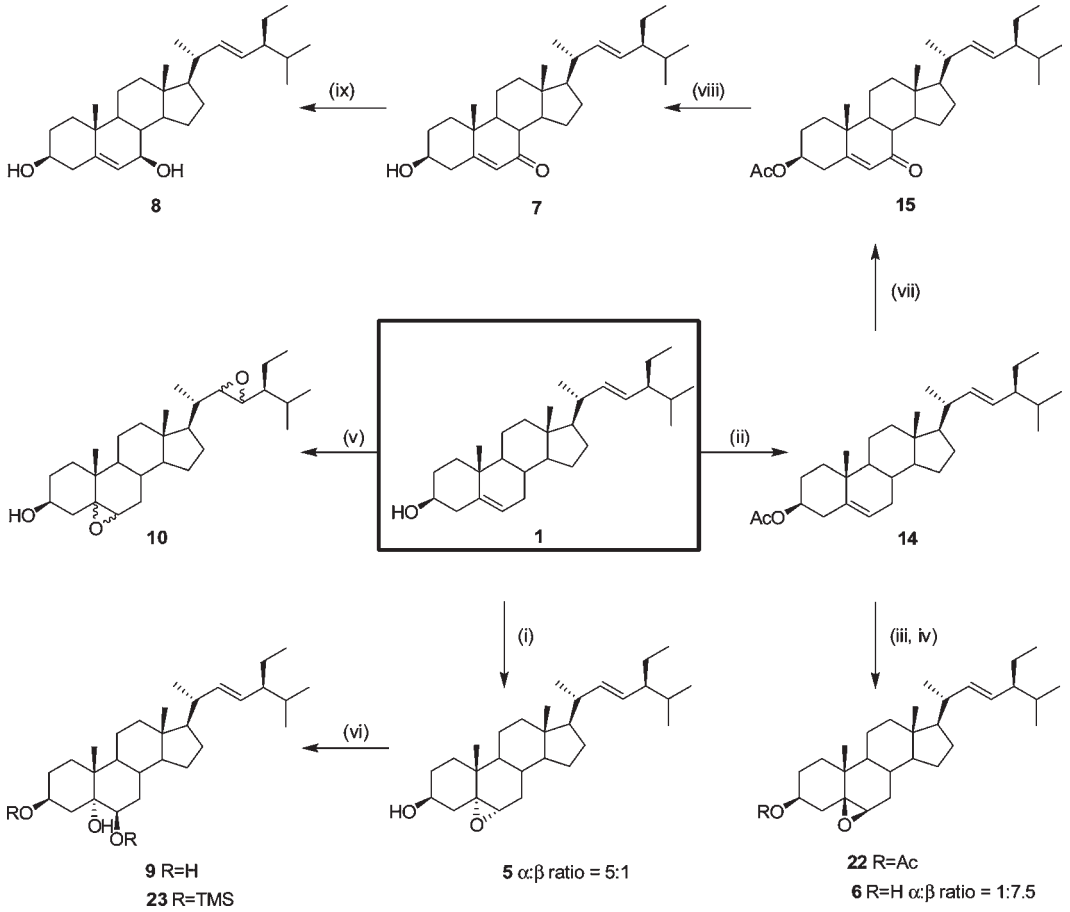

Figure 3. Synthesis of stigmasterol oxidation products.

(i) 0.95 equiv of *m*-CPBA, CH_2Cl_2 (77%); (ii) Ac_2O, pyridine (89%); (iii) $KMnO_4-CuSO_4 \cdot 5H_2O$, *t*-BuOH, H_2O (49%); (iv) Na_2CO_3, MeOH (92%); (v) 3.7 equiv of *m*-CPBA, CH_2Cl_2 (54%); (vi) H_2SO_4, THF$-H_2O$ (32%); (vii) CrO_3, dimethylpyrazole, DCM, -20 to 5 °C (66%); (viii) K_2CO_3, MeOH (59%); (ix) $CeCl_3 \cdot 7H_2O$, $NaBH_4$, MeOH (54%).

directly to synthesize the various oxidation products of this member of the phytosterol family. Initial attempts concentrated on the alkene bond between carbons 5 and 6. Stigmasterol oxidation products which feature oxygenation at this position include the α-epoxide **5**, the *β*-epoxide **6** and the 3*β*,5α,6*β*-triol **9**.

Stigmasterol **1** was exposed to standard epoxidation conditions using 0.95 equiv of *m*-CPBA, to achieve the selective oxidation of the Δ^5-alkene and to prevent formation of the bisepoxide. This procedure generated the α-epoxide **5** as a mixture of α- and *β*-epoxides in a 5:1 ratio (**Figure 3**), which could not be separated by column chromatography.

In order to achieve the stereoselective synthesis of the *β*-epoxide of stigmasterol **6**, an alternative method had to be used. There has been considerable interest in this area in the literature in recent years, much of which is concerned with oxidation of the acetate derivatives of the parent steroid (*29*−*31*). The *β*-epoxide **6** was synthesized from stigmasterol acetate **14**, using a $KMnO_4-CuSO_4 \cdot 5H_2O$ mixture in dichloromethane, in the presence of a catalytic amount of water and *t*-butanol, followed by removal of the acetate group with sodium carbonate. This resulted in the production of the *β*-epoxide of stigmasterol **6** as an inseparable mixture with its α-stereoisomer **5** in a ratio of 7.5:1 (**Figure 3**). These compounds had been synthesized previously with hydrogen peroxide in the presence of ferric acetylacetonate (*20*) and has also been used by Centurión *et al*. in the investigation of the cleavage reaction of 5*β*,6*β*-steroidal epoxides (*32*).

The use of an excess of *m*-CPBA, and an increased reaction time brought about the epoxidation of both double bonds of stigmasterol. This bisepoxide of stigmasterol **10** was produced as a complex mixture of diastereomers, due to epoxidation of each alkene on both the upper and lower faces of the molecule. The products were isolated as a mixture of 5α,6α,22*β*,23*β*- **10a**, 5α,6α,22α,23α- **10b**, 5*β*,6*β*,22*β*,23*β*- **10c** and 5*β*,6*β*,22α,23α-epoxides **10d** in a 4.5:4.5:1:1 ratio (**Figure 2**), calculated using the ^{1}H NMR spectrum of the mixture (the 4.5:1 ratio was calculated from the signals for the 6-H protons at 2.91 and 3.06 ppm respectively, and the 1:1 ratio from one of 23-H at 2.73 and 2.73 ppm respectively). The 5α,6α-isomers **10a** and **10b** could be identified by their characteristic ^{1}H NMR signals for 6-H (δ_H 2.91, d, *J* 4.3) and 3α-H (δ_H 3.81−3.99, m), compared with those for the 5*β*,6*β*-isomers **10c** and **10d**, with distinguishable resonances for 6-H (δ_H 3.06, d, *J* 2.1) and 3α-H (3.61−3.78, m). These multiple diastereomers could not be separated by column chromatography.

The synthesis of the 3*β*,5α,6*β*-triol of stigmasterol **9** was initially attempted *via* a one pot procedure featuring a three step synthesis, generating performic acid *in situ*. This method had been used previously in the synthesis of the 3*β*,5,6-triol of both cholesterol and *β*-sitosterol (*10*). This was unsuccessful in forming the required triol product, with recovered starting material being isolated from the reaction mixture, along with a minor amount of an inseparable mixture of the α- **5** and *β*-epoxides **6**. It was envisaged that epoxide ring-opening of either isomer would result

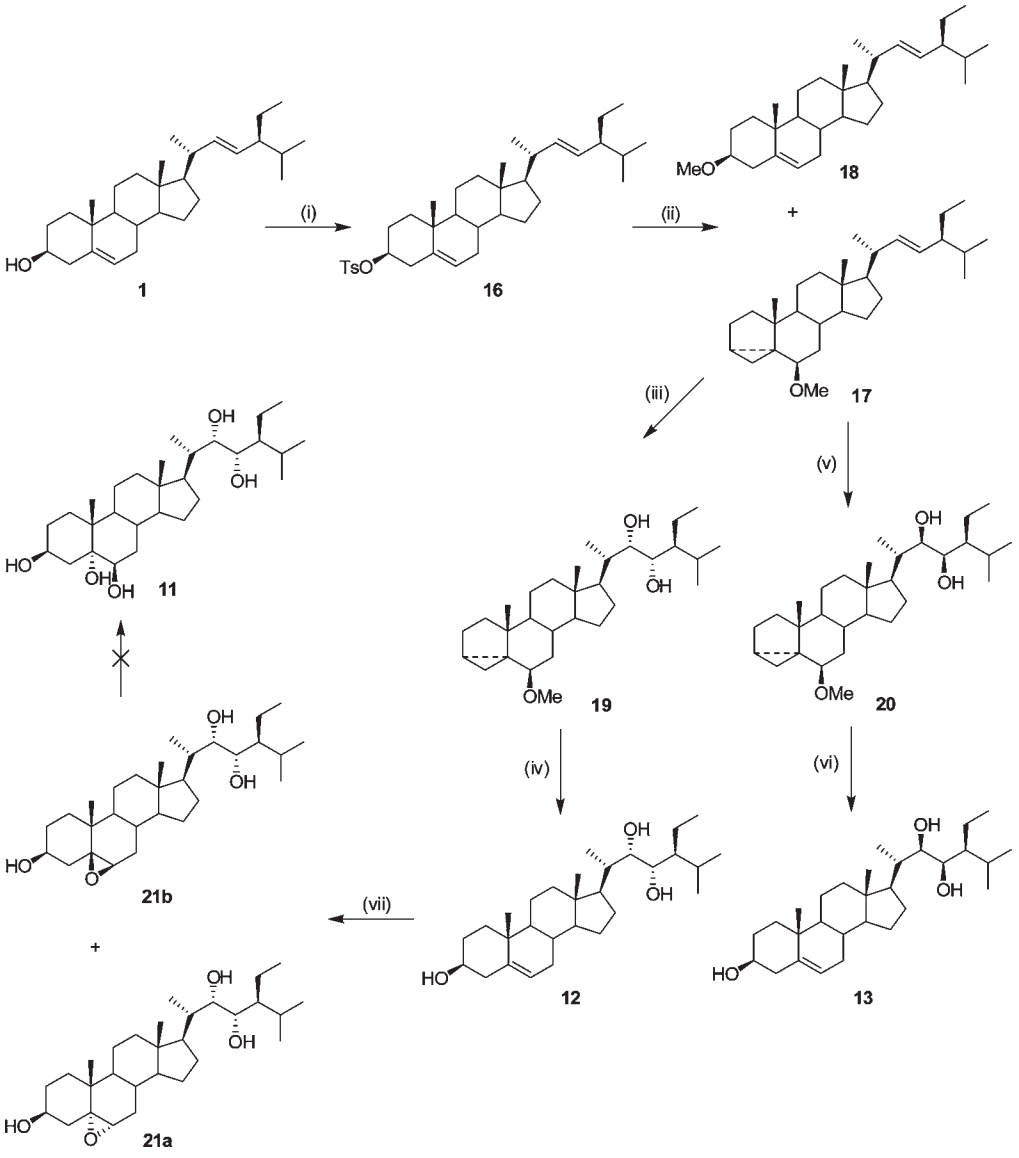

Figure 4. Oxidation of stigmasterol side chain.

(i) *p*-TsCl, DMAP, pyridine (94%); (ii) MeOH (anhydrous), pyridine (60%); (iii) OsO_4, NMO, *t*-BuOH, THF, H_2O (47%); (iv) H_2SO_4, THF, H_2O (87%); (v) OsO_4, NMO, DHQD PHN, *t*-BuOH, THF, H_2O (30%); (vi) H_2SO_4, THF, H_2O (67%); (vii) *m*-CPBA, CH_2Cl_2 (45%).

in the formation of the (5*R*,6*R*)-triol. In order to produce this mixture of α- **5** and *β*-epoxides **6** in larger quantities, stigmasterol **1** was subjected to *m*-CPBA epoxidation. Acid catalyzed ring-opening (*33*) produced the 3*β*,5α,6*β*-triol of stigmasterol **9** as a single diastereomer (**Figure 3**). This is a modified route of that described by Alvarez *et al.* who used the acetate derivative of stigmasterol as the starting material (*23*). The di-TMS ether **23** of the triol **9** was prepared in order to obtain mass spectroscopic data, as attempts to observe ionization of the triol **9** were unsuccessful.

In order to synthesize the relevant stigmasterol oxide products containing oxidation at the 7 position, stigmasterol acetate **14** underwent allylic oxidation using chromium trioxide, to the corresponding 7-keto derivative **15**. Protection of the secondary alcohol at the 3 position eliminated the possibility of oxidation to the diketone. Synthesis of stigmasta-5,22-dien-7-on-3*β*-ol **7** was achieved following removal of the acetate group. Stereoselective reduction of the carbonyl, using sodium borohydride and cerium chloride heptahydrate produced the 7-*β* hydroxyl derivative of stigmasterol **8**, whose spectral characteristics were consistent with literature data (*34*).

As outlined previously, it had been seen in the epoxidation of stigmasterol that the cyclic double bond in the B ring was oxidized before the C22−C23 double bond. The synthesis of the 3*β*,22*R*, 23*R*-triol and 3*β*,22*S*,23*S*-triol, therefore required the protection of the C5−C6 double bond, in order to achieve the selective oxidation on the side chain. Stigmasterol **1** was first converted to stigmasteryl tosylate **16** by standard conditions. The tosylate was then treated with anhydrous methanol and pyridine in a solvolysis (*35*) to give *i*-stigmasterol methyl ether **17**, as a mixture with stigmasterol methyl ether **18** in a ratio of 4:1 (**Figure 4**). These two compounds could be separated by careful column chromatography. In *i*-stigmasterol methyl ether **17** only one double bond is available for oxidation to the corresponding diols.

The dihydroxylation of the C22−C23 bond was conducted using osmium tetroxide with NMO as the co-oxidant. Under these conditions the (22*S*,23*S*)-diol of *i*-stigmasterol methyl ether **19** was the major product, along with a small amount of the opposite diastereomer, which could be separated by column chromatography. The cyclic double bond deprotection of **19** was carried out using sulfuric acid, to give the (22*S*,23*S*)-triol **12** as a single compound, following chromatography.

To synthesize the opposite diastereomer, the (22*R*,23*R*)-triol **13** in large enough quantities for analysis, a slightly varied method had to be used. Again subjecting *i*-stigmasterol methyl ether **17** to dihydroxylation conditions, this time employing the use of a chiral ligand, dihydroquinidine 9′-phenanthryl ether (DHQD PHN) (*28*), to direct the approach of the dihydroxylation to the more hindered, upper face of the molecule. This method succeeded in producing the required diastereomer **20** as the major product of the reaction, which was again purified by chromatography. The deprotection of **20** was carried out in the same fashion as for the (22*S*,23*S*)-diol, to give the (22*R*,23*R*)-triol **13** (*36*) (**Figure 4**).

The final stigmasterol oxidation product to be synthesized was the 3,5,6,22,23-pentol **11**. In an attempt to control the number of stereoisomers that may result from the direct oxidation of stigmasterol **1** itself, further oxidation of the 3,22,23-triols **12** and **13** was seen as a more viable route. As large quantities of the (22*S*,23*S*)-triol **12** could be produced without the use of an expensive chiral ligand, this compound was used as the starting material for the synthesis of the pentol. Epoxidation of the triol **12**, *via* the method described previously with *m*-CPBA, resulted in producing the epoxy triol **21**. This compound was isolated as a mixture of the α-**21a** and *β*-**21b** stereoisomers in a ratio of 5:1. The attempted ring-opening of this mixture of epoxides was conducted with sulfuric acid. This method was unsuccessful in producing the required stigmasterol pentol **11** instead giving a complex mixture.

As the availability of each of the series of stigmasterol oxides as a single compound enables use of these compounds as reference standards for the detection of these oxides in foods or *in vivo*, it is critical that definitive analytical techniques which enable their detection, identification and quantification individually within a mixture are developed. Use of LC−MS has been explored as a powerful analytical technique in this context. Each of the stigmasterol oxides could be characterized by LC−MS (see Materials and Methods for details). While the stigmasterol epoxides **5**, **6**, **10** and **21** were formed and analyzed as diastereomeric mixtures, in most cases distinct LC−MS peaks could be seen for each diastereomer.

The stigmasterol oxides—the 5α,6α- **5** and 5*β*,6*β*-epoxides **6**, the 3*β*,5α,6*β*-triol **9**, 7-keto **7**, 7*β*-hydroxide **8**, 3*β*,22*S*,23*S*-triol **12**, 3*β*,22*R*,23*R*-triol **13** and bisepoxide **10**—have been synthesized following selective synthetic routes as opposed to thermal oxidation. They have been fully characterized structurally to provide reference standards of these POPs for use in biological evaluation in relation to their potential toxicity and as standards for detection of POPs in food stuffs. Further details of these studies will appear in due course.

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