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# Synthesis, isolation and characterisation of β-sitosterol and β-sitosterol oxide derivatives†

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β-Sitosterol is the most prevalent plant cholesterol derivative (phytosterol) and can undergo similar oxidation to cholesterol, leading to β-sitosterol oxides. The biological impact of phytosterol oxides has only been evaluated in a phytosterol blend (usually of β-sitosterol, campesterol, stigmasterol and dihydrobrassicasterol). The lack of pure phytosterols, including β-sitosterol, hinders the collection of significant toxicity data on the individual β-sitosterol oxides. An efficient synthetic route to multi-gram quantities of pure β-sitosterol is described here, together with the first syntheses and characterisation of pure β-sitosterol oxides.

## Introduction

### Background

The term phytosterols refers to sterols synthesized in plants, of which the most prevalent are β-sitosterol **2** and campesterol **6** (comprising 95% of total sterols). Interest in phytosterols and β-sitosterol has increased in recent years due to the incorporation of phytosterol esters into foodstuffs, a function of which is to lower cholesterol levels.[1,2] Dietary intake of phytosterols is projected to increase in Western countries as consumers respond to health messages to increase vegetable oil consumption at the expense of animal fats.[3]

β-Sitosterol is structurally similar to cholesterol with the addition of an ethyl substituent at the 24-position and, consequently, it may undergo similar oxidation processes to cholesterol and yield similar oxidation products. Cholesterol oxidation products (COP's) have well documented adverse effects, including a harmful role in the development of atherosclerosis.[4–6] The consumption of dietary phytosterols in increased quantities has lead to the possibility of increased levels of phytosterol oxides in the blood.

Phytosterols and phytosterol oxidation products (POP's) have generally been assumed to absorb poorly from the diet and hence decrease cholesterol uptake *via* disruption of cholesterol micelle formation. Recently however, POP's have been isolated from the plasma of healthy human subjects.[7] Discussion continues as to whether these POP's are absorbed or transformed *in vivo* from the parent phytosterols.[8] One paper which describes phytosterolaemia (a disease of phytosterol storage as a dysfunctional mutation of an active cellular sterol reexporter) would suggest that phytosterols can passively enter the cellular lining of the gut before their fate is determined.[9] It appears that phytosterols and POP's can enter the bloodstream and this has a significant impact on their respective toxicity profiles.

Toxicity data on POP's is incomprehensive due to the fact that β-sitosterol and campesterol have been difficult to purify from natural sources and consequently POP's are tested as part of a phytosterol blend.[9–12] The blend of phytosterol oxidation products has inherent toxicity, however designation of toxicity to individual POP's from this blend is problematic. Compounding the problem, there is a lack of pure commercially available phytosterols hindering the development of individual phytosterol oxides as standards.

A recent comment in the British Journal of Nutrition concluded, "the development of accurate and sensitive methods for qualitative and quantitative analyses of oxysterols and oxyphytosterols in food, dietary products and biological samples has become a new challenge."[13] As an initial step towards developing a range of standard POP's, the synthesis of pure β-sitosterol and β-sitosterol oxidation products was undertaken.

### Problematic synthesis of β-sitosterol

A synthetic route to β-sitosterol **2** is attractive as the natural extract usually contains some impurities (stigmasterol **1**, campesterol **6**, dihydrobrassicasterol **7** *etc.*) in varying ratios, which are virtually inseparable by chromatography (save for HPLC which is arduous on a synthetically useful scale).[14,15]

β-Sitosterol has been synthesized previously starting from stigmasterol **1** *via* simple hydrogenation of the side chain double bond[16]. Prior to hydrogenation of the $\Delta^{22-23}$-double bond, the B-ring alkene must be protected through an *i*-stigmasterol methyl ether. Standard conditions for the subsequent hydrogenation employ Pd/C as catalyst and ethyl acetate as solvent.[16]

Recently however, it has been reported that in the synthesis of campesterol **6** (a compound similar in structure to β-sitosterol, where the side chain ethyl group is replaced by a methyl group) following similar methodology, the side chain is susceptible to isomerisation during the hydrogenation of the $\Delta^{22-23}$-double bond using $Pd/CaCO_3$ as catalyst and ethanol as solvent (Scheme 1).[17] Critically, the stereochemistry is compromised at C-24 during the hydrogenation step and a mixture of products formed (**4** and **5**). This isomerisation had not been considered previously in phytosterol synthesis. $^{13}C$ NMR data shows slight but distinct differences between the two isomers synthesised (**4** and **5**), direct precursors of campesterol **6** and dihydrobrassicasterol **7**. It is suggested this isomerisation is catalyst dependant in the case of campesterol. Spectral data showed an appreciable amount of by-product **5** present, up to 10% when $PtO_2$ was used as the catalyst and up to 25% when $Pd/CaCO_3$ was employed.[17]

The lack of $^{13}C$ data in the literature regarding reactions of this type leads us to our current investigation towards the synthesis of pure β-sitosterol.

† Electronic supplementary information (ESI) available: GCMS analysis of TMS-ethers of β-sitosterol synthesised under various conditions. See http://dx.doi.org/10.1039/b505069c

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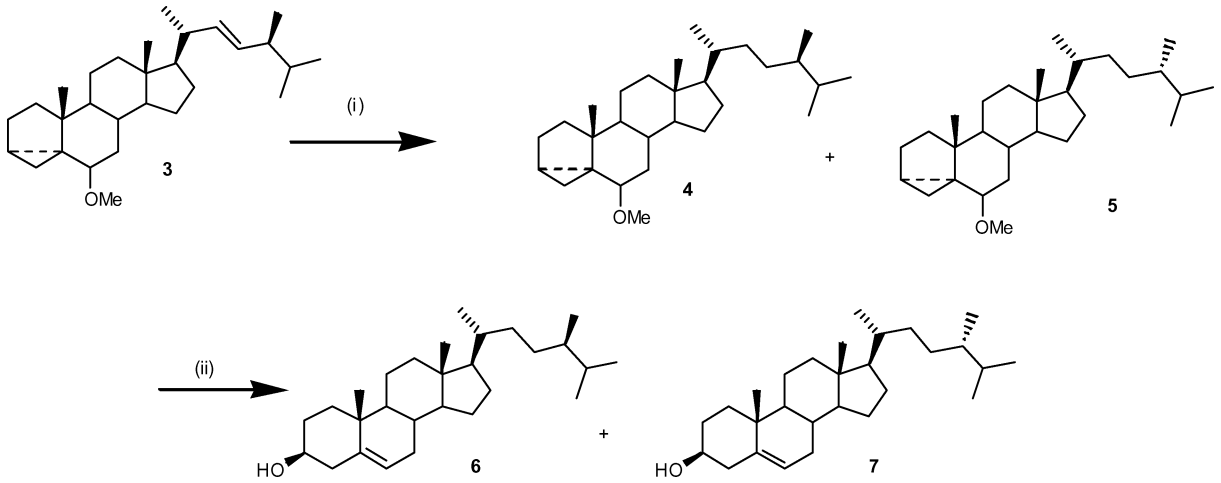



**Scheme 1** Synthesis of campesterol **6** and dihydrobrassicasterol **7** from common intermediate. Reagents and conditions: (i) $H_2$, $Pd/CaCO_3$ or $PtO_2$ in EtOH or EtOH–EtOAc (5 : 3) (ii) *p*-TsOH, aq. dioxane, 80 °C.

## Results and discussion

### Synthesis and isolation of β-sitosterol

The initial step in the synthesis of pure β-sitosterol is the preparation of the stigmasterol tosylate **8** from the readily available stigmasterol **1** (95% purity) using standard tosylation conditions (Scheme 2).[18]

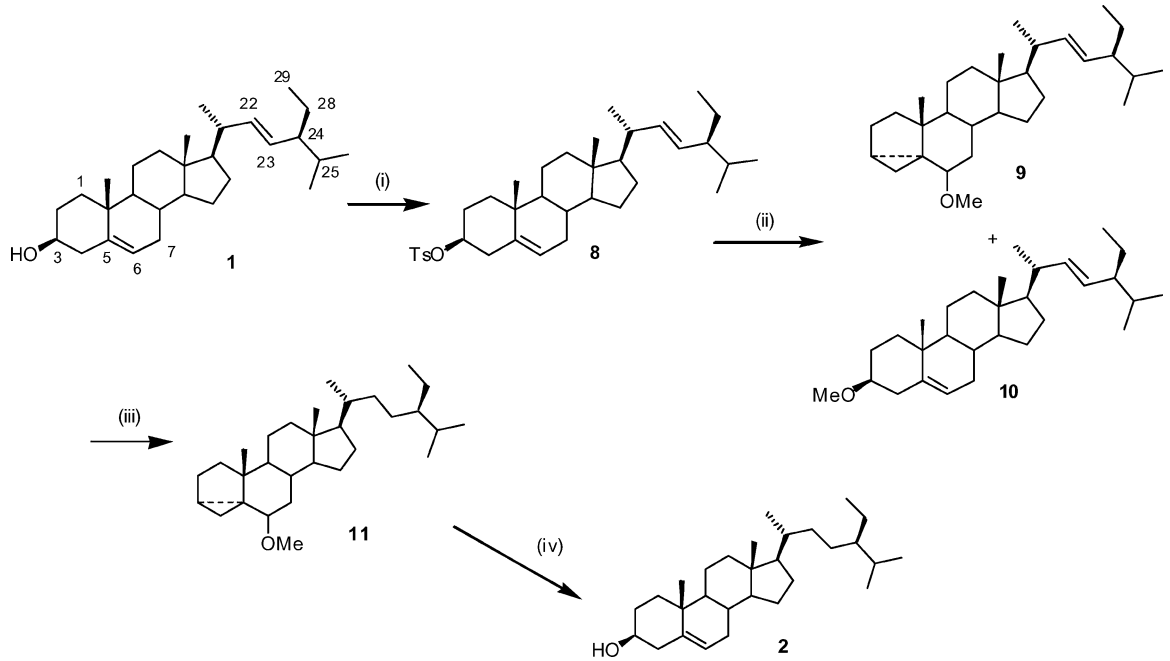
The tosylate was then treated with anhydrous MeOH and pyridine in a solvolysis to give *i*-stigmasterol methyl ether **9** as an oily solid in 74% yield, as a mixture with stigmasterol methyl ether **10** in a ratio of 5 : 1. This minor product has not been previously identified as a side product from this reaction. The mixture can be separated after repeated chromatography, however the material was used unpurified and the minor product removed by chromatography in the subsequent step.

Standard hydrogenation conditions for the conversion were developed in 1963 and use Pd/C as catalyst and ethyl acetate as solvent.[16] As mentioned earlier, a recent publication has identified isomerisation during hydrogenation in campesterol synthesis under similar conditions.[17] Therefore a range of hydrogenation catalysts were screened and the yields and purities obtained using these conditions are summarised in Table 1.

The catalysts that effect hydrogenation are $PtO_2$ and 5% Pd/C. It can be seen from Table 1 that under conditions (a) and following deprotection of the B-ring double bond, the β-sitosterol formed was of only 85.9% purity by GCMS. The use of $PtO_2$ improved the selectivity of hydrogenation in campesterol, however little if any improvement was seen when used for β-sitosterol, even on changing solvents (c and d). Other catalysts used included Raney nickel and Wilkinson's catalyst (e, f and g), however no reaction was seen in these cases even when a large excess of the catalyst and different solvents were used.[19]

Ethyl acetate was initially employed as solvent for the hydrogenation due to solubility issues and literature precedent; this however gave rise to a significant amount of an unidentified isomer with characteristic alkene signals in the $^{13}C$ NMR (a). We believe that the unidentified isomer is formed by double bond rearrangement on the side chain, catalysed by traces of acetic acid in the ethyl acetate. Subsequent hydrogenation in the presence of base yielded no reduced material (b).

Ethanol was then used as solvent even though the starting material **9** is only partially soluble in ethanol at rt. As the hydrogenation proceeded the organic material went into solution. Following deprotection of the B-ring double bond, β-sitosterol **2**




**Scheme 2** *Reagents and conditions*: (i) *p*-TsCl, DMAP, pyridine (90%); (ii) MeOH (anhydrous), pyridine (74%); (iii) $H_2$, catalyst and solvent as in Table 1; (iv) *p*-TsOH, aq. dioxane, 80 °C (55% for 2 steps).

**Table 1** Summary of hydrogenation conditions employed in hydrogenation and deprotection (compound **9** to **2**)

| Reaction[a] | Catalyst | Scale | Yield (%) | Solvent | Purity[b](%) |
|---|---|---|---|---|---|
| a | Pd/C 5% | 0.1 g | 52 | EtOAc | 85.9 |
| b | | 0.4 g | 0 | EtOAc/$NaHCO_3$ | — |
| c | $PtO_2$ | 0.3 g | 54 | EtOAc | <85 |
| d | | 0.3 g | 55 | EtOH | <85 |
| e | Raney Ni | 0.3 g | 0 | EtOAc | — |
| f | Wilkinson's | 0.2 g | 0 | EtOAc | — |
| g | | 0.2 g | 0 | THF | — |
| h | Pd/C 5% | 0.3 g | 55 | EtOH | 98.9 |
| i | | 8.0 g | 55 | EtOH | 98.9 |

[a] All reactions were carried out at 50 psi. [b] Purity of compound **2** assessed by GCMS and NMR.

was obtained in good purity by NMR with no extra alkene signals (h).

Whereas $^1$H NMR is of limited use in the synthesis of phytosterols due to the number of alkane signals, $^{13}$C NMR can be used to differentiate the stereochemistry at C-24.[20–22] The chemical shift at C-29 differs significantly between the *R* and *S* epimers in β-sitosterol [(24-*R*)-β-sitosterol, $\delta$ 12.0 and (24-*S*)-β-sitosterol (clionasterol), $\delta$ 12.3], which conclusively assigns our structure as the correct epimer (24-*R*)-β-sitosterol.

GCMS analysis of the TMS-ethers of the β-sitosterol products led to confirmation of the NMR data and assignment of purity (Table 2).

The by-product formed by conditions (a) appears to be an isomer of stigmasterol, which is slow to hydrogenate even on using forcing conditions (high catalyst loading and long reaction times). Using ethanol as solvent circumvents this isomerisation, even though the substrate solubility is poor and requires heating to dissolve. The remaining impurity appears to be campesterol, which is residual from the stigmasterol starting material.

Hydrogenation method (h) was scaled up to 8 g with excellent reproducibility of purity and yield (i). This material was used in the synthesis of phytosterol oxides as outlined in Scheme 3.

### Synthesis of β-sitosterol oxides

The initial targets in the synthesis of β-sitosterol oxides were chosen to form a comparative study with the most toxic COP's and our strategy for the synthesis of phytosterol oxides was based on a variety of methods used previously to synthesise cholesterol oxidation products. Starting from β-sitosterol, the acetate **12** was produced using standard acetylation conditions (Scheme 3).[23]

The synthesis of the β-epoxide **15** was carried out using the procedure described by Wilson *et al*., using copper sulfate, potassium permanganate and *t*-butanol in DCM, followed by cleavage of the acetate.[24] The yield of this reaction seems to be variable and modest when compared with Wilson and coworkers reports of yields in the region of 70%. The β-epoxide was obtained in 93% purity; the other 7% being the α-epoxide. The isomers are easily distinguished by $^1$H NMR (H-6 comes at $\delta$ 2.90 for the α-epoxide and $\delta$ 3.05 for the β-isomer).

In the synthesis of the 7-keto derivative **13**, the yield reported by Wilson *et al.* for cholesterol derivatives was 65% using PCC as the oxidant in benzene; however a slightly modified procedure for β-sitosterol gave a yield of 79%.[24]

Initial synthesis of 7-β-hydroxysitosterol **14** was undertaken with sodium borohydride and lithium aluminium hydride with neither proving a very selective reduction (10–20%, 7-α-hydroxysitosterol). The use of sodium borohydride and cerium chloride heptahydrate however gave the 7-β-hydroxysitosterol in excellent yield and selectivity (97 : 3). It was noted that the two isomers are separable by flash chromatography on silica gel using 40% ethyl acetate in hexane.

The α-epoxide **16** was generated through *m*CPBA oxidation of β-sitosterol yielding a mixture of α- and β-epoxide in a 6 : 1 ratio which could not be separated by chromatography on a small scale.[10]

Triol **17** is synthesised in a one-pot procedure featuring a three step synthesis generating performic acid *in situ*.

## Conclusion

As mentioned earlier, the hydrogenation step was key to the synthesis of pure β-sitosterol. By employing ethanol as the solvent we have eliminated the unidentified impurity that was produced using the standard conditions.

This synthesis of β-sitosterol **2** (98.9% purity) has lead to the synthesis of standards for toxicity testing in β-sitosterol oxides. The synthesis of β-sitosterol oxides proceeds in a facile manner as per the literature precedent for cholesterol and provides for the first time standard samples of β-sitosterol oxides. The toxicity of these compounds has been evaluated, with the 7-keto-β-sitosterol and 7-β-hydroxy-β-sitosterol being the most toxic to U937, Caco2 and HepG2 cell lines in a similar fashion to their corresponding cholesterol counterparts. This toxicology is the subject of another publication.[25]

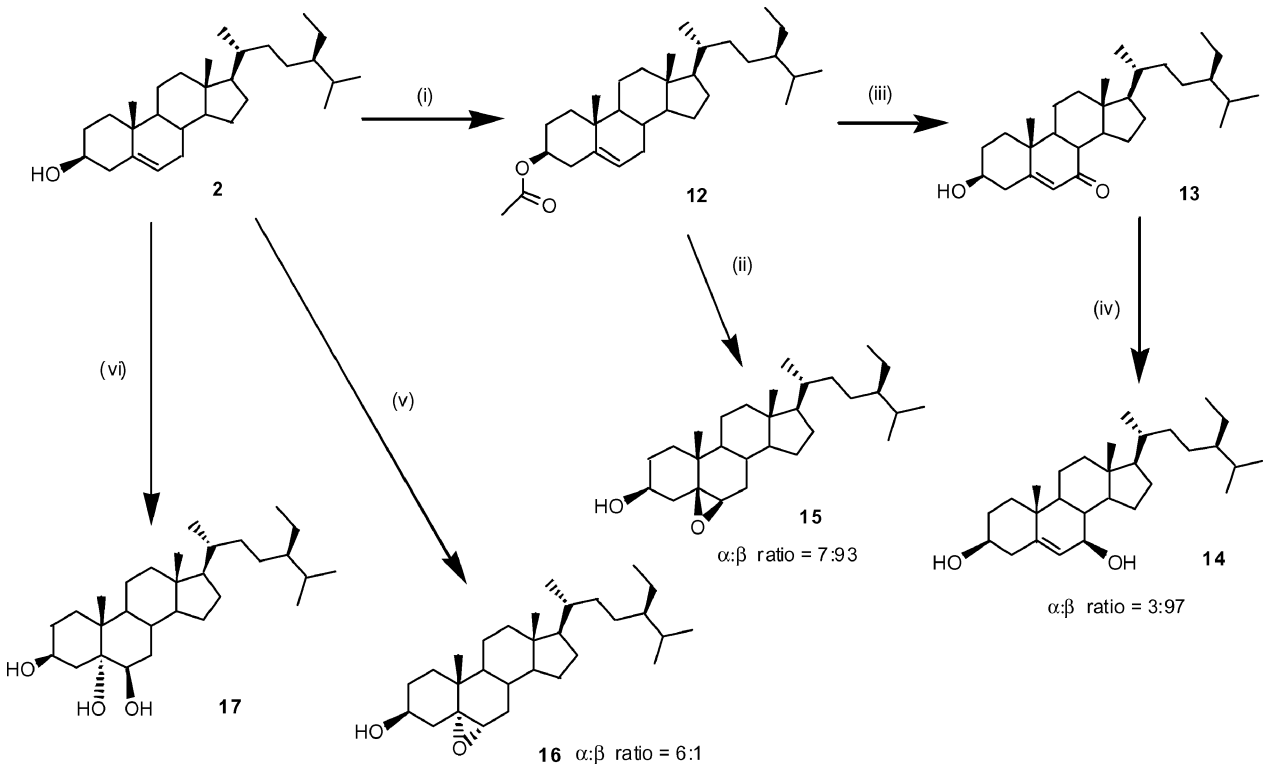
## Experimental

### Materials and methods

Reagents were purchased from the Aldrich chemical company (Poole, Dorset, UK), including β-sitosterol (>40%) and

**Table 2** GCMS analysis of hydrogenation reaction products†

| Conditions | Retention/min | Peak area | $M^+$ | Product (as TMS-ether) |
|---|---|---|---|---|
| Commercial sample ~40% purity | 9.8 | 0.4% | 471 | Brassicasterol/crinosterol or isomer |
| | 11.1 | 31.6% | 473 | Campesterol or isomer |
| | 11.7 | 13.0% | 485 | Stigmasterol or isomer |
| | 13.2 | 55.0% | 487 | β-Sitosterol |
| a | 11.1 | 1.1% | 473 | Campesterol or isomer |
| | 11.6 | 13.0% | 485 | Stigmasterol or isomer |
| | 13.2 | 85.9% | 487 | β-Sitosterol |
| h | 11.1 | 1.1% | 473 | Campesterol or isomer |
| | 13.2 | 98.9% | 487 | β-Sitosterol |




**Scheme 3** *Reagents and conditions*: (i) $Ac_2O$, pyridine (88%); (ii) $CuSO_4/KMnO_4$, *t*-BuOH, $H_2O$ (54%), followed by $K_2CO_3$, MeOH (71%); (iii) $CrO_3$, dimethylpyrazole, DCM, −20 °C to 5 °C (79%), followed by $K_2CO_3$, MeOH (91%); (iv) $CeCl_3{\cdot}7H_2O$, $NaBH_4$, (87%); (v) *m*CPBA, DCM, 0 °C (70%); (vi) HCOOH, 80 °C, 10 min, followed by $H_2O_2$, followed by MeOH, NaOH (49% for 3 steps).

stigmasterol (95%). *m*CPBA was purified by washing with phosphate buffer, followed by filtration and drying. Melting points are uncorrected. Impurities could be identified on spectra run on a 500 MHz NMR spectrometer and are detailed. All spectra were recorded at 20 °C in deuterated chloroform ($CDCl_3$) unless otherwise stated, using tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in parts per million (ppm) and coupling constants in Hertz (Hz). Infrared spectra were measured as potassium bromide (KBr) discs for solids or 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 and the fractions are reported in the order in which they eluted unless otherwise stated. Visualisation of compounds on TLC plates was achieved by UV (254 nm) light detection and vanillin or potassium permanganate staining.

### GCMS analysis

GCMS analysis was effected on a Varian CP 3800 gas chromatograph coupled to a Varian Saturn 2000 mass spectrometer. The column used was a Chrompack WCOT fused silica CP-Sil 8 CB low bleed (30 m × 0.25 mm id, film thickness 0.25 μm).

The protocol used in GCMS runs was: column oven 275 °C for 30 min, injector 350 °C (split mode), detector 300 °C, flow 30psi He. Mass spectroscopy setup was in EI, scan range 40 to 650 *m*/*z*, (transfer line 260 °C, trap 200 °C, manifold 100 °C). A Combi Pal (CTC Analytics AG, CH-4222 Zwingen, Switzerland) autosampler was used for injections and chromatographic data processing was undertaken with Saturn GC/MS Workstation Version 5.51 software.

### Representative procedure for preparation of TMS-ethers for GCMS samples

β-Sitosterol (155 mg, 0.37 mmol) and pyridine (0.9 mL) were added under nitrogen, followed by hexamethyldisilizane (0.6 mL) and chlorotrimethylsilane (0.3 mL). The reaction mixture was stirred for 12 h at rt. Water was then added (20 mL) and the sample was extracted into hexane (30 mL). The organic layer was washed with water (20 mL), saturated aq. sodium chloride (2 × 20 mL) and dried over magnesium sulfate. Solvent was then evaporated and the sample stored in an inert atmosphere until ready for GCMS analysis.

### Stigmasterol tosylate (8)

A solution of stigmasterol **1** (25.010 g, 57 mmol, 95% pure), 4-DMAP (0.7 g, 10 mol%) and tosyl chloride (23.0 g, 120 mmol) in pyridine (250 mL) was stirred at rt for 6 h. The solution was poured into 10% aq. sodium bicarbonate (1000 mL) and the precipitate was filtered, washed with water, dried and recrystallised from acetone to give the corresponding tosylate **8** as white needles (29.003 g, 90%): mp 142–144 °C (from acetone) (found: C, 76.27; H, 9.60. Calc. for $C_{36}H_{54}O_3S$: C, 76.26; H, 9.67%). $\nu_{max}/cm^{-1}$ 2956, 2867, 1716, 1598, 1464, 1354, 1192, 1172, 1098, 965, 943, 892, 871, 817, 666, 555; $\delta_H$ 0.55–2.36 (43H, m), 2.44 (3H, s, Ts–$CH_3$), 4.12–4.39 (1H, m, 3α-H), 5.01 (1H, dd, *J* 8.4, 15.2, 22-H or 23-H), 5.16 (1H, dd, *J* 8.5, 15.2, 22-H or 23-H), 5.30 (1H,d, *J* 5.2, 6-H), 7.39 (2H, d, *J* 8.3, ArH), 7.72 (2H, d, *J* 8.3, ArH); $\delta_C$ (75.5 MHz) 12.41 ($CH_3$), 12.65 ($CH_3$), 19.37 ($CH_3$), 19.54 ($CH_3$), 21.36 ($CH_2$), 21.48 ($CH_3$), 21.60 ($CH_3$), 22.03 ($CH_3$), 24.71 ($CH_2$), 25.80 ($CH_2$), 29.02 ($CH_2$), 29.27 ($CH_2$), 32.13 (CH), 32.23 (CH), 32.26 ($CH_2$), 36.75 (quaternary C), 37.27 ($CH_2$), 39.25 ($CH_2$), 39.94 ($CH_2$), 40.87 (CH), 42.57 (quaternary C), 50.31 (CH), 51.62 (CH), 56.29 (CH), 57.13 (CH), 82.77 (CH), 123.90 (CH), 128.03 (CH, 2 × ArCH), 129.70 (CH), 130.13 (CH, 2 × ArCH), 135.08 (quaternary C), 138.64 (CH), 139.25 (quaternary C), 144.79 (quaternary C).

### *i*-Stigmasterol methyl ether (9)

The tosylate **8** (14.303 g, 25.2 mmol) and pyridine (6.1 mL, 3 eq.) were dissolved in anhydrous methanol and refluxed for 6 h. The solution was evaporated and extracted into ethyl acetate

(400 mL) and washed with water (2 × 300 mL). The organic layer was washed with saturated aq. sodium chloride (2 × 200 mL) and dried over magnesium sulfate. The residue was then purified by chromatography on silica gel using 5% ethyl acetate in hexane to give the *i*-stigmasterol methyl ether **9** (8.211 g, 74%) as an oily solid as a mixture (5 : 1) with stigmasterol methyl ether **10**. An analytical sample of **9** and **10** was provided by repeated chromatography, however this mixture was used unpurified in the next step. *i*-Stigmasterol methyl ether **9**: clear oil; $\delta_H$ 0.39–0.49 (1H, m), 0.61–2.09 (43H, m), 2.77 (1H, t, *J* 3.0, CHOMe), 3.32 (3H, s, $OCH_3$), 5.01 (1H, dd, *J* 8.5, *J* 15.2), 5.15 (1H, dd, *J* 8.5, *J* 15.2); $\delta_C$ (75.5 MHz) 12.26 ($CH_3$), 12.44 ($CH_3$), 13.08 ($CH_2$), 19.07 ($CH_3$), 19.29 ($CH_3$), 21.10 ($CH_3$), 21.22 ($CH_3$), 21.48 (CH), 22.70 ($CH_2$), 24.27 ($CH_2$), 24.97 ($CH_2$), 25.42 ($CH_2$), 29.02 ($CH_2$), 30.49 (CH), 31.89 (CH), 33.36 ($CH_2$), 35.09 ($CH_2$), 35.27 (quaternary C), 40.20 ($CH_2$), 40.55 (CH), 42.68 (quaternary C), 43.40 (quaternary C), 48.08 (CH), 51.26 (CH), 56.13 (CH), 56.56 ($CH_3$), 56.65 (CH), 82.43 (CH), 129.22 (CH), 138.33 (CH). Stigmasterol methyl ether **10** (white needles): mp 116–117 °C (from EtOAc–hexane); $\delta_H$ 0.70–2.41 (43H, m), 3.04 (1H, m, 3α-H), 3.35 (3H, s, $OCH_3$), 5.01 (1H, dd, *J* 8.3, 15.3, 22-H or 23-H), 5.15 (1H, dd, *J* 8.5, 15.3, 22-H or 23-H), 5.35 (1H,d, *J* 5.1, 6-H); $\delta_C$ (75.5 MHz) 12.44 ($CH_3$), 12.66 ($CH_3$), 19.38 ($CH_3$), 19.77 ($CH_3$), 21.45 ($CH_2$), 21.51 ($CH_3$), 21.63 ($CH_3$), 24.76 ($CH_2$), 25.81 ($CH_2$), 28.41 ($CH_2$), 29.35 ($CH_2$), 32.28 (CH), 32.28 (CH), 32.32 ($CH_2$), 37.29 (quaternary C), 37.58 ($CH_2$), 39.08 ($CH_2$), 40.08 ($CH_2$), 40.93 (CH), 42.60 (quaternary C), 50.60 (CH), 51.64 (CH), 56.00 ($CH_3$), 56.33 (CH), 57.27 (CH), 80.74 (CH), 121.99 (CH), 129.64 (CH), 138.74 (CH), 141.25 (quaternary C).

### 22,23-Dihydro-*i*-stigmasterol methyl ether (11) and β-sitosterol (2)

*i*-Stigmasterol methyl ether **9** was dissolved in the appropriate solvent and the catalyst (5% by wt.) was added (see Table 1). The resulting suspension was shaken at rt for 16 h. Reaction completion was then confirmed by $^1$H NMR. The reaction mixture was then filtered through celite and concentrated to give the hydrogenated methyl ether **11**. A solution of **11** (6.820 g, 15.84 mmol), TsOH (0.3 g, 1.58 mmol) in aqueous dioxane (132 mL of dioxane and 15 mL of water) was heated at 80 °C for 3 h before the evaporation of the dioxane. The residue was taken up in $CHCl_3$ and the organic layer was washed with water (2 × 50 mL), saturated aq. sodium chloride (2 × 50 mL), dried using anhydrous magnesium sulfate, then concentrated to give the crude product, which was then purified by chromatography on silica gel using 20% ethyl acetate in hexane to give β-sitosterol **2** as a white solid in the various purities and yields outlined in Table 1. β-Sitosterol (98.9%): mp 130–134 °C (needles from EtOAc–hexane) (found: C, 82.47; H, 12.08. Calc. for $C_{29}H_{50}O\cdot(\frac{1}{2}H_2O)$: C, 82.20; H, 12.13%). $\nu_{max}$/cm$^{-1}$ 3434, 2937, 1466, 1382, 1054; $\delta_H$ 0.59–2.34 (48H, m), 3.41–3.59 (1H, m, 3α-H), 5.35 (1H, bd, *J* 5.1, H-6); $\delta_C$ (125.8 MHz) 11.99 ($CH_3$, C-29), 12.18 ($CH_3$), 18.80 ($CH_3$), 19.06 ($CH_3$), 19.40 ($CH_3$), 19.83 ($CH_3$), 21.10 ($CH_2$), 23.31 ($CH_2$), 24.13 ($CH_2$), 26.11 ($CH_2$), 28.26 ($CH_2$), 29.18 (CH), 31.66 ($CH_2$), 31.92 ($CH_2$ and overlapping CH), 33.96 ($CH_2$), 36.16 (CH), 36.52 (quaternary C), 37.28 ($CH_2$), 39.79 ($CH_2$), 42.30 ($CH_2$), 42.33 (quaternary C), 45.85 (CH), 50.15 (CH), 56.08 (CH), 56.78 (CH), 71.80 (CH), 121.70 (CH), 140.77 (quaternary C). The following peaks were distinguishable for the minor (approx. 1%) impurity; $\delta_C$ 12.24, 12.33, 18.84, 18.99, 19.45, 19.61, 21.18, 23.00, 23.03, 24.21, 24.40, 26.57, 28.04, 29.03, 29.31, 33.40, 33.93, 35.77, 36.28, 39.90, 42.46, 50.18, 56.22.

**GCMS analysis of TMS-ether.** Retention time 11.1 min: 1.1% TMS-campesterol or isomer; *m*/*z* (EIMS) 473 (21%), 383 (100), 344 (62).

Retention time 13.2 min: 98.9% TMS-sitosterol; *m*/*z* (EIMS) 487 ($M^+$, 48%), 397 (100), 358 (52), 256 (23), 130 (40).

### β-Sitosterol acetate (12)

Acetic anhydride and pyridine were combined and β-sitosterol **2** (820 mg, 2.0 mmol) was added slowly. The reaction mixture was stirred at rt for 24 h. The reaction mixture was then poured into water (200 mL) and extracted with ethyl acetate (4 × 50 ml). The organic layer was washed with water (3 × 50 mL), saturated aq. sodium chloride (2 × 50 mL), dried over magnesium sulfate and solvent concentrated under a reduced pressure to give an off-white solid **12**, which was used without further purification (801 mg, 88%): mp 118–119 °C (white needles from EtOAc–hexane) (found: C, 81.50; H, 11.32. Calc. for $C_{31}H_{52}O_2$: C, 81.52; H, 11.48%). $\nu_{max}$/cm$^{-1}$ 2938, 1731, 1467, 1368, 1251, 1039; $\delta_H$ 0.61–2.41 (50H, m), 4.55–4.66 (1H, m, 3α-H), 5.38 (1H, bd, *J* 4.4, H-6); $\delta_C$ (125.8 MHz) 11.86 ($CH_3$), 11.99 ($CH_3$), 18.80 ($CH_3$), 19.06 ($CH_3$), 19.31 ($CH_3$), 19.83 ($CH_3$), 21.04 ($CH_2$), 21.42 ($CH_3$), 23.08 ($CH_2$), 24.30 ($CH_2$), 26.11 ($CH_2$), 27.79 ($CH_2$), 28.25 ($CH_2$), 29.17 (CH), 31.88 (CH), 31.91 (CH), 33.96 ($CH_2$), 36.16 ($CH_2$), 36.60 (quaternary C), 37.01 ($CH_2$), 38.14 ($CH_2$), 39.74 ($CH_2$), 42.32 (quaternary C), 45.86 (CH), 50.05 (CH), 56.06 (CH), 56.70 (CH), 73.98 (CH), 122.33 (CH), 139.66 (quaternary C), 170.49 (quaternary C). The following peaks were distinguishable for the minor (approx. 1%) impurity; $\delta_C$ 12.25, 12.32, 18.90, 18.99, 19.22, 19.45, 19.60, 21.18, 23.00, 23.03, 24.21, 26.39, 26.57, 28.02, 28.96, 28.98, 31.74, 33.41, 35.76, 36.29, 36.68, 36.91, 39.84, 42.37, 46.00, 46.08, 49.91, 50.18, 55.74, 55.91, 56.19.

### 7-Keto-β-sitosterol (13)

Chromium trioxide (985 mg, 9.8 mmol) was suspended in dry dichloromethane (40 mL) and stirred for 30 min at −25 °C. Dimethylpyrazole (947 mg, 9.8 mmol) was added in one portion and the reaction mixture stirred for 30 min at −20 °C. β-Sitosterol acetate **12** (300 mg, 0.65 mmol) was added and the mixture stirred at −20 °C allowing to warm to 5 °C over 2.5 h. Ethyl acetate (200 mL) was then added and the brown suspension filtered through celite. The filtrate was concentrated under a reduced pressure to give a brown residue. This residue was purified by chromatography on silica gel using hexane–ethyl acetate (100 : 0 to 75 : 25), yielding 7-keto-β-sitosterol acetate (241 mg, 79%): mp 151–153 °C (needles from EtOAc–hexane) (found: C, 78.52; H, 10.47. Calc. for $C_{31}H_{50}O_3$: C, 79.1; H, 10.71%). $\nu_{max}$/cm$^{-1}$ 2959, 2873, 1731, 1673, 1466, 1375, 1264, 1044; $\delta_H$ 0.59–2.60 (48H, m), 4.61–4.79 (1H, m, 3α-H), 5.79 (1H, bs, H-6); $\delta_C$ (75.5 MHz) 11.97 (2 × $CH_3$), 17.26 ($CH_3$), 18.91 ($CH_3$), 19.02 ($CH_3$), 19.80 ($CH_3$), 21.16 ($CH_2$), 21.28 ($CH_3$), 23.02 ($CH_2$), 26.04 ($CH_2$), 26.31 ($CH_2$), 27.35 ($CH_2$), 28.56 ($CH_2$), 29.08 (CH), 33.91 ($CH_2$), 35.99 ($CH_2$), 36.09 (CH), 37.74 ($CH_2$), 38.31 (quaternary C), 38.63 ($CH_2$), 43.10 (quaternary C), 45.41 (CH), 45.79 (CH), 49.78 (CH), 49.93 (CH), 54.65 (CH), 72.21 (CH), 126.71 (CH), 163.86 (quaternary C), 170.31 (quaternary C), 202.02 (quaternary C).

A suspension of 7-keto-β-sitosterol acetate (239 mg, 0.51 mmol) in methanol (25 mL) was stirred at rt for 5 min. Potassium carbonate (77 mg, 0.56 mmol) dissolved in water (5 mL) was added to the suspension and the mixture stirred at rt over 24 h. The reaction mixture was then partitioned between water (100 mL) and ethyl acetate (50 mL). Washed with water (2 × 100 mL) and saturated aq. sodium chloride (2 × 100 mL), dried over magnesium sulfate and the solution was then concentrated under a reduced pressure to yield the crude product as a white solid (240 mg), which was purified by chromatography on silica gel eluting with ethyl acetate–hexane (0 : 100 to 100 : 0) to yield the pure product **13** as a white solid (199 mg, 91%): mp 119–121 °C (from EtOAc–hexane) (found: C, 79.50; H, 11.20. Calc. for $C_{29}H_{48}O_2\cdot(\frac{1}{2}H_2O)$: C, 79.58; H, 11.28%). $\nu_{max}$/cm$^{-1}$ 3535, 3338, 2937, 2871, 1673, 1658, 1464, 1385, 1066; $\delta_H$ 0.59–2.60 (46H, m), 3.55–3.77 (1H, m, 3α-H), 5.73 (1H, bs, H-6); $\delta_C$ (125.8 MHz) 12.03 (2 × $CH_3$), 17.37 ($CH_3$), 18.99 ($CH_3$), 19.11 ($CH_3$), 19.86 ($CH_3$), 21.28 ($CH_2$), 23.11 ($CH_2$), 26.15 ($CH_2$),

26.39 ($CH_2$), 28.61 ($CH_2$), 29.19 (CH), 31.21 ($CH_2$), 33.99 ($CH_2$), 36.13 (CH), 36.42 ($CH_2$), 38.36 ($CH_2$), 38.75 (quaternary C), 41.89 ($CH_2$), 43.16 (quaternary C), 45.48 (CH), 45.87 (CH), 49.99 (CH), 50.02 (CH), 54.76 (CH), 70.50 (CH), 126.09 (CH), 165.46 (quaternary C), 202.49 (quaternary C).

The following peaks were distinguishable for the minor (approx. 1%) impurity; $\delta_C$ 12.29, 12.37, 14.25, 15.59, 18.87, 19.04, 19.27, 19.49, 19.64, 21.28, 21.34, 23.03, 23.06, 26.30, 26.13, 28.39, 28.98, 29.00, 30.92, 32.91, 33.72, 33.73, 35.12, 35.51, 36.24, 39.74, 43.21, 46.05, 46.09, 52.01, 54.35, 54.72, 60.49, 63.11, 68.24, 68.70.

**GCMS analysis of TMS-ether.** Retention time 24.2 min: TMS-7-keto-β-sitosterol; *m/z* (EIMS) 501 ($M^+$, 100%), 414 (34), 396 (62).

### 7-β-Hydroxy-β-sitosterol (14)

A suspension of 7-keto-β-sitosterol **13** (119 mg, 0.28 mmol) and cerium chloride heptahydrate (156 mg, 0.42 mmol) in methanol (5 mL) was stirred at rt for 10 min. Sodium borohydride (12 mg, 0.3 mmol) was added to the suspension and the mixture stirred at rt over 3 h. The reaction was worked up by partitioning between water (30 mL) and ethyl acetate (30 mL). The organic layer was washed with water (2 × 20 mL) and saturated aq. sodium chloride (2 × 20 mL), dried over magnesium sulfate and concentrated under a reduced pressure to yield the crude product (104 mg, 87%, β : α-ratio 97 : 3 from NMR) as white solid. The crude product was purified by chromatography on silica gel eluting with hexane–ethyl acetate (60 : 40), the first fraction yielding the β-alcohol **14** as a single stereoisomer (50 mg, 41%) and the second fraction as a mixture of isomers (28 mg, 23%): mp 137–139 °C (from EtOAc–hexane) (found: C, 79.30; H, 11.43. Calc. for $C_{29}H_{50}O_2 \cdot (\frac{1}{2}H_2O)$: C, 79.21; H, 11.69%). $\nu_{max}/cm^{-1}$ 3400, 2959, 2871, 1465, 1384, 1056; $\delta_H$ 0.70–2.32 (47H, m), 3.51–3.58 (1H, m, 3α-H), 3.85 (1H, bd, $J = 2.6$, H-7), 5.29 (1H, bs, H-6); $\delta_C$ (75.5 MHz) 12.21 ($CH_3$), 12.37 ($CH_3$), 19.22 ($CH_3$), 19.41 ($CH_3$), 19.55 ($CH_3$), 20.22 ($CH_3$), 21.46 ($CH_2$), 23.44 ($CH_2$), 26.46 ($CH_2$), 26.78 ($CH_2$), 28.95 ($CH_2$), 29.51 (CH), 31.94 ($CH_2$), 34.35 ($CH_2$), 36.49 (CH), 36.82 (quaternary C), 37.32 ($CH_2$), 39.93 ($CH_2$), 41.28 (CH), 42.10 ($CH_2$), 43.31 (quaternary C), 46.21 (CH), 48.64 (CH), 55.74 (CH), 56.33 (CH), 71.81 (CH), 73.75 (CH), 125.82 (CH), 143.87 (quaternary C). The following peaks from the $^1$H NMR of the mixture could be attributed to the 7-α-hydroxy-β-sitosterol: $\delta_H$ 3.63–3.69 (1H, m, 3α-H), 5.69 (1H, bs, H-6).

**GCMS analysis of TMS-ether.** Retention time 13.9 min: 3% TMS-7-α-hydroxy-β-sitosterol; *m/z* (EIMS) 574 ($M^+ - 1$, 1%), 485 ($M^+ - 90$, 100%).

Retention time 15.4 min: 97% TMS-7-β-hydroxy-β-sitosterol; *m/z* (EIMS) 485 ($M^+ - 90$, 100%).

### β-Sitosterol-5,6-β-epoxide (15)

Copper sulfate pentahydrate (1.008 g) and potassium permanganate (2.280 g) were ground together into a fine powder with a mortar and pestle to which water (0.5 mL) was added. The resulting paste was transferred to a flask containing the acetate **12** (221 mg, 0.48 mmol) in dichloromethane (30 mL). *Tert*-butanol (0.3 mL) was added and the reaction mixture was refluxed for 15 min before cooling to rt. The reaction mixture was then stirred for a further 16 h at rt. The reaction mixture was then filtered through silica gel plug column eluting with dichloromethane. The product rich layer was then dried over magnesium sulfate and concentrated under a reduced pressure to yield the crude protected epoxide (123 mg, 54%) as a white solid. This crude product was used directly in the next step: $\nu_{max}/cm^{-1}$ 2959, 2868, 1732, 1468, 1376, 1265, 1242, 1044; $\delta_H$ 0.57–2.26 (50H, m), 3.00 (1H, d, $J$ 2.1, H-6), 4.64–4.75 (1H, m, 3α-H); $\delta_C$ (75.5 MHz) 10.73 ($CH_3$), 10.95 ($CH_3$), 16.02 ($CH_3$), 17.75 ($CH_3$), 17.99 ($CH_3$), 18.80 ($CH_3$), 20.31 ($CH_3$), 20.90 ($CH_2$), 22.02 ($CH_2$), 23.16 ($CH_2$), 24.98 ($CH_2$), 26.18 ($CH_2$), 27.14 ($CH_2$), 28.09 (CH), 28.70 (CH), 31.43 ($CH_2$), 32.84 ($CH_2$), 33.99 (quaternary C), 35.06 (CH), 35.64 ($CH_2$), 36.97 ($CH_2$), 38.73 ($CH_2$), 41.24 (quaternary C), 44.77 (CH), 49.94 (CH), 55.04 (CH), 55.14 (CH), 61.51 (quaternary C), 62.59 (CH), 70.33 (CH), 169.56 (quaternary C).

A suspension of β-sitosterol-5,6-β-epoxide acetate (62 mg, 0.13 mmol) in methanol (14 mL) was stirred for 5 min at rt. Sodium carbonate (30 mg, 0.28 mmol) was added and the mixture stirred at rt for 16 h. The reaction mixture was then concentrated under a reduced pressure to give the crude epoxide as a white solid. The product was purified by chromatography on silica gel using hexane–ethyl acetate (100 : 0 to 80 : 20) to give the epoxide **15** (40 mg, 71%) in a 93 : 7 mixture of the β-epoxide : α-epoxide as a white solid: $\nu_{max}/cm^{-1}$ 3424, 2937, 1465, 1382, 1063; $\delta_H$ 0.55–2.10 (48H, m), 3.05 (1H, d, $J$ 2.2, H-6), 3.60–3.78 (1H, m, 3α-H); $\delta_C$ (75.5 MHz) 11.76 ($CH_3$), 11.97 ($CH_3$), 17.05 ($CH_3$), 18.72 ($CH_3$), 19.01 ($CH_3$), 19.82 ($CH_3$), 21.99 ($CH_2$), 23.04 ($CH_2$), 24.20 ($CH_2$), 26.02 ($CH_2$), 28.17 ($CH_2$), 29.11 (CH), 29.77 (CH), 31.04 ($CH_2$), 32.60 (quaternary C), 33.87 ($CH_2$), 34.84 (quaternary C), 36.08 (CH), 37.23 ($CH_2$), 39.82 ($CH_2$), 42.22 ($CH_2$), 42.29 ($CH_2$), 45.78 (CH), 51.32 (CH), 56.09 (CH), 56.22 (CH), 62.94 (quaternary C), 63.74 (CH), 69.45 (CH). Characteristic signals for the α-epoxide were also seen (see below).

**GCMS analysis of TMS-ether.** Retention time 17.4 min: 93% TMS-β-sitosterol-5,6-β-epoxide; *m/z* (EIMS) 503 ($M^+$, 55%), 488 (48), 485 (52), 474 (73), 413 (100), 385 (79).

Retention time 17.9 min: 7% TMS-β-sitosterol-5,6-α-epoxide; *m/z* (EIMS) 503 ($M^+$, 51%), 488 (29), 485 (26), 413 (100), 385 (91).

### β-Sitosterol-5,6-α-epoxide (16)

A solution of *m*CPBA (100%, 125 mg, 0.7 mmol) in dichloromethane (10 mL) was added dropwise to a stirred ice-cold solution of β-sitosterol **2** (250 mg, 0.6 mmol) in dichloromethane (30 mL). The resulting mixture was stirred for 2 h at rt. The reaction mixture was then washed with 10% aq. sodium hydrogen sulfite solution (2 × 50 mL), 5% aq. sodium thiosulfate solution (2 × 50 mL), saturated aq. sodium bicarbonate (2 × 100 mL) and saturated aq. sodium chloride (2 × 100 mL). The dichloromethane extracts were then dried over magnesium sulfate and concentrated under a reduced pressure to produce a white solid (180 mg, 70%). NMR analysis proved this to be a mixture of the α- and β-epoxides in a ratio of 6 : 1, which could not be separated using chromatography. α-Epoxide **16**: $\nu_{max}/cm^{-1}$ 3431, 2959, 2869, 1467, 1377, 1064; $\delta_H$ 0.61–2.07 (48H, m), 2.90 (s, 1H, H-6), 3.85–3.95 (1H, m, 3α-H); $\delta_C$ (75.5 MHz) 10.84 ($CH_3$), 10.95 ($CH_3$), 14.90 ($CH_3$), 17.68 ($CH_3$), 18.00 ($CH_3$), 18.81 ($CH_3$), 19.62 ($CH_2$), 22.02 ($CH_2$), 23.04 ($CH_2$), 25.04 ($CH_2$), 27.08 ($CH_2$), 27.79 ($CH_2$), 28.09 (CH), 28.86 (CH), 30.02 ($CH_2$), 31.38 ($CH_2$), 32.86 (C quaternary), 33.83 ($CH_2$), 35.11 (CH), 38.37 ($CH_2$), 38.83 ($CH_2$), 41.31 (C quaternary), 41.52 (CH), 44.78 (CH), 54.75 (CH), 55.83 (CH), 58.32 (CH), 64.75 (C quaternary), 67.66 (CH). Characteristic β-epoxide peaks also present.

**GCMS analysis of TMS-ether.** Retention time 17.4 min: 14% TMS-β-sitosterol-5,6-β-epoxide; *m/z* (EIMS) 503 ($M^+$, 59%), 488 (44), 485 (49), 474 (67), 413 (100), 385 (77).

Retention time 17.9 min: 86% TMS-β-sitosterol-5,6-α-epoxide; *m/z* (EIMS) 503 ($M^+$, 53%), 488 (27), 485 (25), 413 (100), 394 (93).

### β-Sitosterol-3,5,6-triol (17)

A solution of formic acid (95%, 3 mL) was added to β-sitosterol **2** (149 mg, 0.36 mmol) and this suspension was stirred for 10 min at 80 °C (turns a dark colour and an oily layer forms on top of the formic acid). The reaction mixture was then cooled to 25 °C, forming a solid from the oil, and hydrogen

peroxide was then added (1 mL). The suspension was stirred at 25 °C for 16 h. Boiling water (50 mL) was then added to the suspension and extracted using dichloromethane (100 mL). The dichloromethane layer was washed with saturated aq. sodium chloride (2 × 50 mL), dried over magnesium sulfate and the solvent evaporated yielding a white solid used without purification.

This solid was dissolved in methanol (20 mL) and sodium hydroxide (25 mg) added. The suspension was stirred for 3 h at rt. The reaction was partitioned between water (50 mL) and ethyl acetate (50 mL). The organic layer was washed with water (2 × 50 mL) and saturated aq. sodium chloride (2 × 50 mL), dried over magnesium sulfate and concentrated under a reduced pressure to yield the crude product (130 mg) as white solid, which was purified by column chromatography eluting with ethyl acetate to yield the triol **17** as a white solid (79 mg, 49%): $\nu_{max}/cm^{-1}$ 3436, 2956, 2870, 1466, 1386, 1293, 1044; $\delta_H$ ($d_6$-DMSO) 0.47–1.95 (46H, m), 2.56 (s, 1H), 3.35 (broad s, 1H), 3.68 (s, 1H, H-6), 3.83–3.89 (1H, m, 3α-H), 4.22 (1H, d, $J$ = 5.5 Hz), 4.44 (1H, d, $J$ = 4.0 Hz); $\delta_C$ (75.5 MHz) 12.11 ($CH_3$), 12.27 ($CH_3$), 16.62 ($CH_3$), 18.93 ($CH_3$), 19.27 ($CH_3$), 20.06 ($CH_3$), 21.09 ($CH_2$), 22.94 ($CH_2$), 24.25 ($CH_2$), 25.87 ($CH_2$), 28.26 ($CH_2$), 29.03 (CH), 30.35 (CH), 31.44 ($CH_2$), 32.36 ($CH_2$), 33.70 ($CH_2$), 34.83 ($CH_2$), 35.98 (CH), 38.11 (C quaternary), 40.13 ($CH_2$), 41.24 ($CH_2$), 42.61 (C quaternary), 44.89 (CH), 45.47 (CH), 56.02 (CH), 56.15 (CH), 66.10 (CH), 74.48 (CH), 74.64 (C quaternary).

**GCMS analysis of TMS-ether.** Retention time 20.5 min: TMS-β-sitosterol-3,5,6-triol; $m/z$ (EIMS) 575 ($M^+ - 90$, 18%), 560 (27), 546 (24), 485 (100), 432 (70)

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