STEROIDS 73 (2008) 702–707

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Synthesis of highly pure oxyphytosterols and (oxy)phytosterol esters
Part I. Regioselective hydrogenation of stigmasterol: An easy access to oxyphytosterols

Philippe Geoffroy[a], *Diane Julien-David*[b], *Eric Marchioni*[b], *Francis Raul*[c], *Dalal Aoudé-Werner*[d], *Michel Miesch*[a,*]

[a] *Institut de Chimie, Université Louis Pasteur, UMR CNRS/ULP 7177-Laboratoire de Chimie Organique Synthétique-1, rue Blaise Pascal, BP 296/R8, 67008 Strasbourg-Cedex, France*
[b] *IPHC, UMR CNRS/ULP 7178, Laboratoire de Chimie Analytique et Sciences de l'Aliment-Université Louis Pasteur, Faculté de Pharmacie 74, route du Rhin, 67401 Strasbourg-Cedex, France*
[c] *INSERM U682, Université Louis Pasteur - IRCAD-1, place de l'Hôpital, BP406, 67091 Strasbourg-Cedex, France*
[d] *Aérial, Parc d'Innovation, rue Laurent Fries-B.P. 40443, 67412 Illkirch-Cedex, France*

ARTICLE INFO

Article history:
Received 20 December 2007
Received in revised form
29 January 2008
Accepted 10 February 2008
Published on line 17 February 2008

Keywords:
Hydrogenation
Nickel-Raney catalyst
Regioselectivity
Epoxydes
Bromohydrins

ABSTRACT

The synthesis of several oxyphytosterols is described starting from stigmasterol, the key step being the regioselective hydrogenation of the 22–23 double bond of the latter.
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1. Introduction

In the frame of a collaborative study concerning *inter alias* the antiproliferative effects of oxyphytosterol on the human colon cancer cell line Caco-2, we needed to have in our hands large amounts of highly pure (≥95%) oxyphytosterols especially 7β-hydroxysitosterol. For that purpose, we developed recently a purification method affording sitosterol on a gram-scale (≥95% purity) starting from a commercial source of a phytosterol mixture. However, this method was tedious because very time-consuming [1–4]. On the other hand, it has to be known that until so far, no pure individual phytosterols are commercially available except Δ5-stigmasterol (Sigma, purity: 95%) and sitosterol (Acros or Sigma, purity: 97%, purified from natural plant resource or synthesis), the latter being available in low amounts and being excessively expensive.

* *Corresponding author*. Tel.: +33 390241752; fax: +33 390241754.
E-mail address: miesch@chimie.u-strasbg.fr (M. Miesch).
0039-128X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2008.02.004

In the present paper, we report on an efficient and reproducible regioselective hydrogenation of the 22–23 double bond present in Δ5-stigmasterol allowing afterwards an easy access to several oxyphytosterols.

2. Experimental

2.1. General

Melting points were measured on a Stuart Scientific melting point apparatus (SMP 3) and are uncorrected. Reactions were carried out under argon with magnetic stirring and degassed solvents. Et_2O and THF were distilled from Na/benzophenone. Thin layer chromatography (TLC) was carried out on silica gel plates (Merck $60F_{254}$) and the spots were visualized under UV lamp (254 or 365 nm) and/or sprayed with a solution of vanillin (25 g) in EtOH/H_2SO_4 (98/2; 1 L) or with phosphomolybdic acid solution (25 g phosphomolybdic acid, 10 g cerium sulfate, 60 mL H_2SO_4, 940 mL H_2O) followed by heating on a hot plate. For column chromatography, silica gel (Merck Si 60 40–60 μm) was used. IR spectra were recorded on a PerkinElmer IR-881 spectrophotomer as CCl_4 or $CHCl_3$ solutions. ^{1}H NMR spectra were recorded at 300 MHz (Bruker AC-300) and ^{13}C NMR spectra at 75 MHz (Bruker AC-300) using the signal of the residual nondeuterated solvent as internal reference. Significant ^{1}H NMR data are tabulated in the following order: chemical shift (δ) expressed in ppm, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants in hertz, number of protons. Microanalysis (Elementar Vario apparatus) were performed by the Service Commun de Microanalyse, Institut de Chimie, Strasbourg.

Prior to gas chromatography (GC) analysis, the analytes were transformed to trimethylsilyl (TMS) ethers with 50 μL of pyridine and 40 μL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Sigma, Steinheim, Germany) and heated 1 h at 50 °C, then diluted with 410 μL of isooctane. Gas chromatography–mass spectrometry (GC–MS) analyses were performed on a Varian Star 3400 GC instrument equipped with an on-column SPI injector coupled to a Varian Saturn 2000 mass sensitive detector (Varian, France) operating in the electron impact (EI) ionization mode at 70 eV and monitored on the full-scan range (*m*/*z* 40–600). Data acquisition and processing, and instrumental control were performed by Varian Saturn WS software. Analytes were separated with a VF-5ms capillary column (phase stationary: 5% phenyl–95% dimethylpolysiloxane, thickness of 0.1 μm, 60 m × 0.25 mm, Varian, France). The column temperature gradient was programmed from 105 °C (hold for 2 min) to 170 °C at 20 °C/min and then, to 320 °C at 3 °C/min for analysis of the mixture β-sitosterol (**2**) and stigmastanol (**3**) and 7 °C/min for analysis of stigmasterol (**1**), 7-ketositosterol (**7**) and 3β-hydroxy-5,6β-epoxystigmastane (**11**). The injector operating conditions were as follows: injection volume 1 μL; initial injector temperature of 105 °C was increased to 320 °C at 100 °C/min (hold until the end of analysis). Helium (purity 99.9995%) was used as a carrier gas with a flow rate of 1 mL/min.

The crude Raney-Nickel hydrogenation reaction mixture as well as the Pd/C and PtO_2 hydrogenation reaction mixtures were analyzed according to the above procedure.

2.2. Preparation of Raney-Nickel W-2 catalyst

The nickel–aluminium alloy (6.0 g; VWR Prolabo No. 20935.233) was used as a starting material. The Raney-Nickel W-2 catalyst was prepared according to the procedure reported in organic syntheses [5]. At the end of the procedure, the catalyst (~3.0 g) was rapidly washed with 95% EtOH (3 × 50 mL) and with pro analysis EtOAc (3 × 50 mL).

2.3. 3β-Hydroxystigmast-5-ene (2) and 3β-hydroxystigmastane (3)

Commercially available (TCI) stigmasterol (**1**) (5.0 g, 12.11 mmol) was purified on a silica gel column (100 g SiO_2; 5% EtOAc/95% petroleum ether) leading to the following mixture of products determined by GC: 1.5% brassicasterol; 1.0% campesterol; 95.0% stigmasterol; 2.5% sitosterol. Thus, we assumed that stigmasterol has a purity ≥95%.

To a suspension of the catalyst (3.0 g) in EtOAc (10 mL) was added at room temperature a solution of stigmasterol (**1**) (1.5 g, 3.60 mmol) in EtOAc (200 mL). The reaction mixture was flushed with hydrogen (3× hydrogen–vacuum cycle) and the hydrogenation was carried under an atmospheric pressure of hydrogen. After 15 h stirring at room temperature, the reaction mixture was degassed with argon, filtered over celite and the solvent was removed (25 °C, 15 mmHg) to give a mixture of 3β-hydroxystigmast-5-ene (or sitosterol) (**2**) and 3β-hydroxystigmastane (**3**) [ratio: ~2/1 (see Table 3, entry 16), 1.5 g; yield: 100%].

2.4. 3β-Acetoxystigmast-5-ene (4) and 3β-acetoxystigmastane (5)

To a solution of sitosterol (**2**) and stigmastanol (**3**) (ratio: ~2/1, 2.75 g, 6.63 mmol) in CH_2Cl_2 (70 mL) was added at room temperature pyridine (0.78 g, 9.90 mmol) followed by a dropwise addition of a solution of acetyl chloride (0.66 g, 8.40 mmol) in CH_2Cl_2 (1 mL). After 1 h stirrring at room temperature, the solvents were removed (25 °C, 15 mmHg) and the crude product was purified on a silica gel column (100 g SiO_2, EtOAc/petroleum ether: 5/95) leading to a mixture of 3β-acetoxystigmast-5-ene (**4**) and 3β-acetoxystigmastane (**5**) (ratio: ~2/1, 2.88 g, yield: ~95%)

2.5. 3β-Acetoxystigmast-5-ene-7-one (6)

To a suspension of celite (60 g) in benzene (300 mL; *warning*: benzene has to be handled with care and used under a well-ventilated hood) was added at room temperature PCC (Aldrich no. 19.014-4, 25 g, 139 mmol) followed by the addition of 3β-acetoxystigmast-5-ene (**4**) and 3β-acetoxystigmastane (**5**) (ratio: ~2/1, 2.50 g, ~5.50 mmol). The glassware was equipped with a Dean Stark apparatus and the reaction mixture was refluxed during 24 h. After cooling down at room temperature, the reaction mixture was filtered on a fritted glass funnel and the filtrate was carefully washed with Et_2O (3 × 50 mL). The solvents were removed under reduced pressure (25 °C, 15 mmHg). The crude product was purified on a silica gel column (100 g SiO_2, EtOAc/hexane: 5/95) leading to 3β-acetoxystigmastane (**5**) (0.59 g, 1.28 mmol, yield: 23%)

and to 3β-acetoxystigmast-5-ene-7-one (**6**) (1.21 g, 2.57 mmol, yield: 47% (72%/3β-acetoxystigmast-5-ene present in the starting reaction mixture).

Spectral data: see Ref. [1].

2.6. 3β-Hydroxystigmast-5-ene-7-one (7)

To a solution of 3β-acetoxystigmast-5-ene-7-one (**6**) (1.21 g, 2.55 mmol) in MeOH (40 mL) was added at room temperature Na_2CO_3 (0.40 g, 3.85 mmol) followed by the addition of H_2O (4 mL). The reaction mixture was stirred 20 h at room temperature, hydrolyzed with water (20 mL), extracted with CH_2Cl_2 (3 × 20 mL). The organic layers were washed with a saturated aqueous NaCl solution (10 mL) and dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure (25 °C, 15 mmHg). The crude product was purified on a silica gel column (30 g SiO_2, EtOAc/petroleum ether: 20/80) leading to 3β-hydroxystigmast-5-ene-7-one (**7**) (1.11 g, 2.44 mmol, yield: 95%; purity determined by GC: ≥95%).

Spectral data: see Ref. [1].

2.7. 3β-Acetoxy-5α-bromo-6β-hydroxystigmastane (8)

To a mixture of 3β-acetoxystigmast-5-ene (**4**) and 3β-acetoxystigmastanol (**5**) (1.47 g, ∼.20 mmol) in dioxan (40 mL), water (2 mL) and three drops of perchloric acid (70–74%) was added at 0 °C freshly prepared *N*-bromoacetamide (0.532 g, 3.8 mmol) [9]. The reaction was maintained 1 h at 0 °C then 2 h at 20 °C under light exclusion. The solution was then treated with a solution of sodium thiosulfate until decoloration and extracted with Et_2O (3 × 30 mL). The organic phase was washed with brine (30 mL) and dried over magnesium sulfate. After filtration and removal of the solvents (25 °C, 15 mmHg), the crude mixture was chromatographed on a silica gel column (100 g SiO_2, EtOAc/petroleum ether: 10/90) leading to 3β-acetoxystigmastane (**5**) (0.400 g, 0.89 mmol, yield: 24%; 72%/starting material) along with an unseparable mixture of 3β-acetoxy-5α-hydroxy-6β-bromostigmastane (**9**), 3β-acetoxy-5,6α-epoxystigmastane (**10a**), 3β-acetoxy-5,6β-epoxystigmastane (**10**) (0.310 g; ratio (**9**)/(**10** + **10a**): 1.3/1 determined by NMR) and finally to 3β-acetoxy-5α-bromo-6β-hydroxystigmastane (**8**) as a pure compound. (0.864 g, 1.60 mmol, yield: 49%; 72%/starting material).

3β-Acetoxy-5α-bromo-6β-hydroxystigmastane (**8**): ^{1}H NMR ($CDCl_3$): δ 5.60–5.40 (m, 1H); 4.18 (broad s, 1H); 2.60–2.40 (m, 1H); 2.35–2.10 (m, 2H); 2.10–1.50 (m, 10H); 1.50–0.70 (m, 35H); 0.61 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 170.16, 86.44, 75.49, 71.85, 55.71, 55.43, 47.13, 45.54, 42.41, 40.06, 39.37, 38.14, 35.85, 34.82, 34.31, 33.60, 30.29, 28.84, 27.94, 26.07, 25.75, 23.79, 22.78, 21.08, 21.01, 19.53, 18.74, 18.45, 17.74, 11.90, 11.69. Analysis for $C_{31}H_{53}BrO_3$: calcd C, 67.25; H, 9.65. Found C, 67.56; H 9.71.

2.8. 3β-Acetoxy-5,6β-epoxystigmastane (10)

The mixture of 3β-acetoxy-5α-bromo-6β-hydroxystigmastane (**8**) (0.844 g, 1.50 mmol) and sodium acetate (1.55 g, 1.9 mmol) in ethanol (30 mL) was refluxed under argon during 3 h. The solvent was removed under reduced pressure (25 °C, 15 mmHg) and after water addition (20 mL), extraction with CH_2Cl_2 (3 × 20 mL), the organic phase was washed with brine (30 mL) and dried over magnesium sulfate. After filtration and removal of the solvent (25 °C, 15 mmHg), the crude mixture was chromatographed on a silica gel column (30 g SiO_2, EtOAc/petroleum ether: 5/95) leading to 3β-acetoxy-5,6β-epoxystigmastane (**10**) (0.642 g, 1.30 mmol, yield: 89%).

Spectral data: see Ref. [1].

2.9. 3β-Hydroxy-5,6β-epoxystigmastane (11)

To a solution of 3β-acetoxy-5,6β-epoxystigmastane (**10**) (0.642 g, 1.30 mmol) in methanol (70 mL) was added sodium carbonate (0.245 g, 2.30 mmol). The reaction mixture was stirred 4 h at room temperature then the solvent was removed under reduced pressure (25 °C, 15 mmHg) and water (20 mL) was added. After extraction with CH_2Cl_2 (3 × 20 mL), the organic phase was washed with brine (30 mL) and dried over magnesium sulfate. After filtration and removal of the solvent (25 °C, 15 mmHg), the crude mixture was chromatographed on a silica gel column (30 g SiO_2, EtOAc/petroleum ether: 20/80) leading to 3β-hydroxy-5,6β-epoxystigmastane (**11**) (0.542 g, 1.20 mmol, yield: 93%).

Spectral data: see Ref. [1].

3. Results and discussion

As mentioned above, we needed to have in our hands preparative amounts of highly pure oxyphytosterol derivatives especially 7keto-,7α-hydroxysitosterol, 7β-hydroxysitosterol and 5,6-α (and β)-epoxysitosterol. For that purpose, we started to study the regioselective hydrogenation of the 22–23 double bond of stigmasterol (**1**) which was already described by Kircher and Rosenstein in the early seventies [6]. These authors obtained several interesting results: when the hydrogenation of stigmasterol and derivatives was carried out in the presence of 5% Pd/C (entries 1–3), the starting material was still present (2–6%). More recently, Lichtfouse and Albrecht described the regioselective hydrogenation of the 22–23 double bond of 3b-acetoxystigmast-5-ene (obtained from 95% pure stigmasterol) over platinum dioxide: these authors claimed that sitosterol acetate was isolated in 86% yield with 91% purity as determined by GC [7].

According to these literature results, we first decided to check if we could reproduce them. For that purpose, commercially available stigmasterol was first purified on a silica gel column leading to the following mixture of products as determined by GC: 1.0% brassicasterol; 0.5% campesterol; 95.0% stigmasterol; 3.5% sitosterol. Thus, we assumed that stigmasterol has a purity ≥95%.

In the presence of Pd/C (entries 1–3) the hydrogenation of the starting material always delivered a mixture of three compounds, this being not interesting for our purpose. On the other hand, in the presence of PtO_2, we were not able to reproduce the Lichtfouse and Albrecht results [7], the starting material being the main product recovered (entries 4 [7]–4) (Table 1). This hydrogenation process was also utilized by Heupel and Nes but these authors worked only with analytical amounts of products and did not separate sitosterol from campesterol [8].

Table 1 – Hydrogenation of stigmasterol and stigmasterol derivatives over Pd/C and PtO_2

Entry	Starting product, g (mmol), AcOEt (mL)	Catalyst (g)	Stigmasterol, sitosterol, stigmastanol (%)
1 [6]	Stigmasterol, 0.825, (2.00) 100	5% Pd/C (0.40)	10, 33, 57
1	Stigmasterol, 0.825 (2.00), 100	10% Pd/C (0.40)	10, 36, 50
2 [6]	Stigmasterol acetate, 0.909 (2.00), 100	5% Pd/C (0.40)	2[a], 35[a], 63[a]
2	Stigmasterol acetate, 0.980 (2.15), 100	10% Pd/C, (0.042)	27[a], 43[a], 22[a]
3 [6]	Stigmasterol phenylacetate, 1.06 (2.00), 100	5% Pd/C, (0.40)	6[b], 66[b], 28[b]
3	Stigmasterol phenylacetate, 1.06 (2.00), 100	10% Pd/C (0.042)	28[b], 42[b], 30[b]
4 [7]	Stigmasterol acetate, 20.5 (45.08), 200	PtO_2 (Not indicated)	86%[c]
4	Stigmasterol acetate, 1.78 (3.91), 60	PtO_2 (0.042)	70[a], 23[a], 2[a]

[a] Acetate derivatives.
[b] Phenylacetate derivatives.
[c] Acetate derivative (purity: 91%).

Table 2 – Hydrogenation of stigmasterol derivatives over Raney-Nickel

Entry	Starting product, g (mmol), AcOEt (mL)	Raney-Nickel (g)	Starting material/ catalyst (w/w)	Stigmasterol, sitosterol, stigmastanol (%)
5 [6]	Stigmasterol acetate, 0.450 (1.00), Not indicated	1	0.45	12, 80, 8
5	Stigmasterol acetate, 0.450 (1.00), 50	1	0.45	34, 66, 0
6 [6]	Stigmasterol phenylacetate, 0.530 (1.00), Not indicated	1	0.53	0, 80, 20
6	Stigmasterol phenylacetate, 0.530 (1.00), 50	1	0.53	71, 29, 0

In the presence of Raney-Nickel, Kircher and Rosenstein used four different starting materials: stigmasterol acetate, stigmasterol trimethylacetate, stigmasterol phenylacetate and stigmasterol [6]. First of all, we tried to reproduce the hydrogenation reaction starting from stigmasterol acetate and stigmasterol phenylacetate (entries 5 [6]/5 and 6 [6]/6): despite several trials, we were unable to reproduce these results especially the total consumption of stigmasterol phenylacetate as indicated in entry 6 [6] (Table 2).

Starting from stigmasterol, it clearly appears, according to Kircher and Rosenstein, that when the ratio Raney-Nickel/stigmasterol decreases, the amount of sitosterol increases along with the complete consumption of stigmasterol (entries 7 [6] and 8 [6]).

Unfortunately, we were unable to reproduce these results. On the contrary, we noticed that when the ratio Raney-Nickel/stigmasterol increases, the complete consumption of stigmasterol was observed (entries 9–16, Table 3). It has also to be noted that these results show that the scale-up of the reaction does not allow the total consumption of stigmasterol (entry 17, Table 3) and that the respective amounts of "hydrogenated" compounds varies. Finally, we set up reaction conditions being easily reproducible leading only to the desired mixture sitosterol/stigmastanol. For that purpose, we

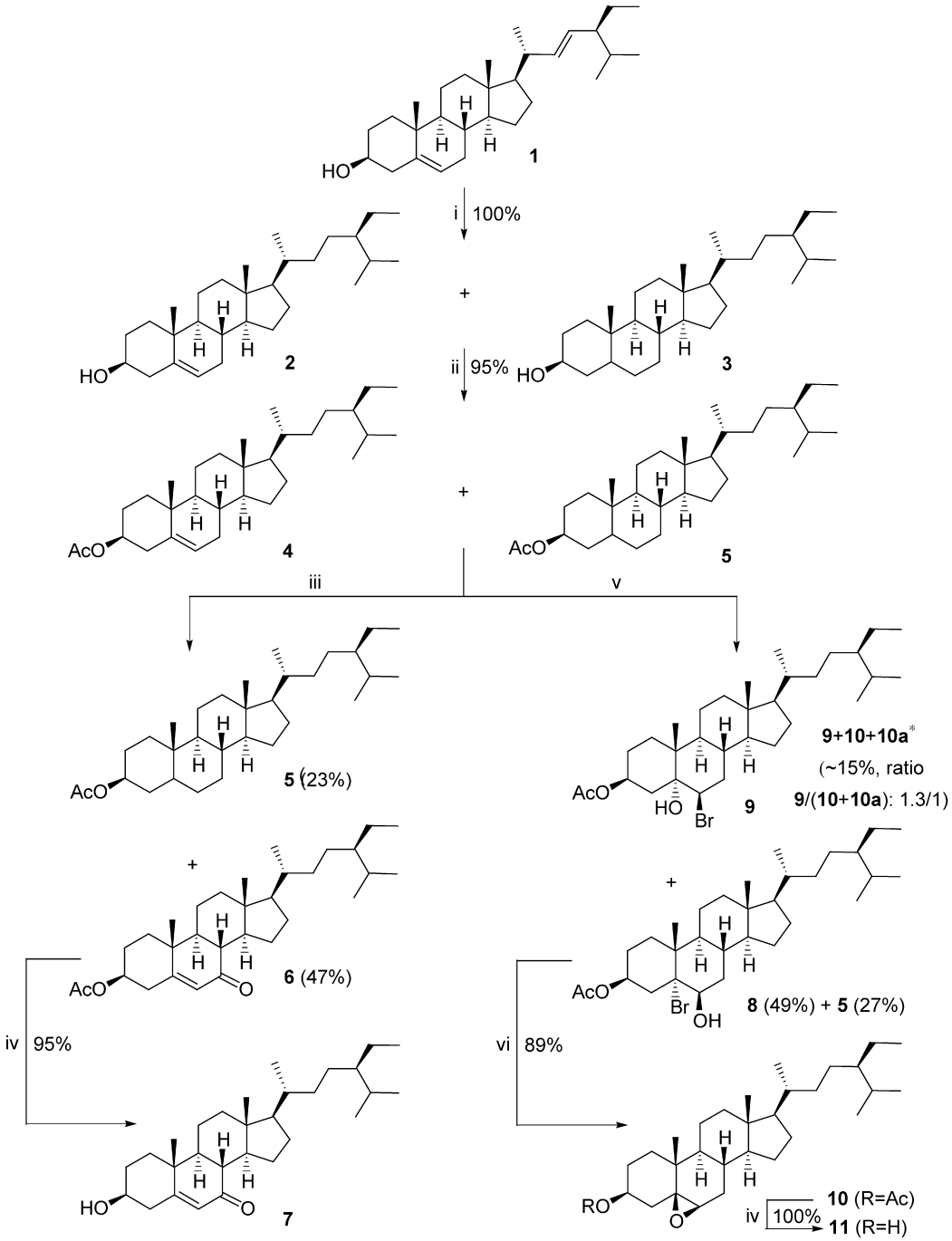
Table 3 – Hydrogenation of stigmasterol over Raney-Nickel[a]

Entry	Starting product, g (mmol), AcOEt (mL)	Catalyst (g)	Ra-Ni/stigmasterol (w/w)	Stigmasterol, sitosterol, stigmastanol (%)
7 [6]	Stigmasterol, 4.95 (12.00)	3	0.60	4–0[b], 77–71[b], 19–29[b]
8 [6]	Stigmasterol, 0.825 (2.00), 100	1	1.21	2, 64, 34
9	Stigmasterol, 1.50 (3.60), 100	1	0.67	28, 69, 1
10	Stigmasterol, 3.00 (7.28), 400	3	1.00	45, 48, 4
11	Stigmasterol, 1.50 (3.64), 200	1.5	1.00	26–59[c], 67–37[c], 5–2[c]
12	Stigmasterol, 1.00 (2.42), 140	1	1.00	41, 46, 11
13	Stigmasterol, 0.825 (2.00), 100	1	1.21	63, 30, 3
14	Stigmasterol, 2.47 (6.00), 350	3	1.21	27, 63, 7
15	Stigmasterol, 2.00 (4.85), 280	3	1.50	10, 76, 11
16	Stigmasterol, 1.50 (3.63), 200	3	2.00	0–0[d], 65–71[d], 32–21[d]
17	Stigmasterol, 3.00 (7.26), 400	6	2.00	20, 71, 7

[a] The ratio stigmasterol/sitosterol/stigmastanol does not reach 100%, this being due the presence of other sterols (see GC analaysis of our starting stigmasterol).
[b] 4 runs.
[c] 5 runs.
[d] 10 runs.

found out that some experimental requirements proved to be absolutely necessary: (1) commercially available stigmasterol has to be chromatographed over silica gel and analyzed by GC; (2) Raney-Nickel has to be prepared according to Ref. [2] and has to be used the same day; (3) the w/w ratio stigmasterol/Raney-Nickel has to be 1/2; (4) the reaction has to be carried out under hydrogen at atmospheric pressure; (5) the amount of stigmasterol has not to exceed 1.5 g (entry 15 *vs*. entry 16, Table 3).

Of course, at this stage, our hydrogenation procedure led to an unseparable mixture of sitosterol (**2**)/stigmastanol (**3**) [(**2**)/(**3**): 2.03 < ratio < 3.38], the presence of stigmasterol (**1**) being not detected. To obtain the desired 3b-hydroxystigmast-5-ene-7-one (**7**), the 3β-hydroxyl group present in sitosterol (**2**)/stigmastanol (**3**) was protected as an acetate leading to an unseparable mixture of the corresponding acetate derivatives. An allylic oxidation [9] was then carried out to deliver that time a mixture of two easily separable compounds, 3β-



i: H_2, Raney-Ni, AcOEt; ii: CH_3COCl, Py, CH_2Cl_2; iii: PCC, toluene, reflux; iv: Na_2CO_3, MeOH;
v: $CH_3CONHBr$, dioxan, $HClO_4$ cat.; vi: NaOAc, EtOH
*: compound **10a** = 3β-acetoxy-5,6α-epoxystigmastane.

Scheme 1 – Synthesis of oxyphytosterols starting from stigmasterol.

acetoxystigmast-5-ene-7-one (**6**) and 3β-acetoxystigmastane (**5**). Finally, the deprotection of the acetoxy group afforded readily the desired 3b-hydroxystigmast-5-ene-7-one (**7**) (overall yield starting from stigmasterol: 42%; purity ≥95% determined by GC) (Scheme 1). As we have shown earlier, the latter is the direct precusor of 7β- and 7α-hydroxysitosterol [1]. The 5,6β-epoxy derivative was also obtained starting from the sitosterol/stigmastanol mixture: after formation of the acetyl derivatives, a hydrobromination reaction was carried out leading to a complex mixture of compounds. A careful chromatography on a silica gel column was carried out affording 3β-acetoxystigmastane (**5**) along with an unseparable mixture of 3β-acetoxy-5α-hydroxy-6β-bromostigmastane (**9**), 3β-acetoxy-5,6α-epoxystigmastane (**10a**), 3β-acetoxy-5,6β-epoxystigmastane (**10**) (overall yield: ∼15%; ratio (**3**)/(**10** + **10a**): 1.3/1) and finally 3β-acetoxy-5α-bromo-6β-hydroxystigmastane (**8**) as a pure compound (49%). It has to be noted that the epoxy derivatives (**10**, **10a**) were already detected at this stage. Despite many trials, we were unable to obtain 3β-acetoxy-5α-hydroxy-6β-bromostigmastane (**9**) as a pure compound. On the other hand, when the mixture of compounds (**3**), (**9**), (**10**) and (**10a**) was treated with sodium acetate, a complex mixture of compounds was obtained and we were unable to isolate the 3β-acetoxy-5,6α-epoxystigmastane (**10a**) as a pure compound (Scheme 1). Nevertheless, the latter can be obtained according to our previously described method [2]. Finally, a deprotection reaction was carried out in the presence of sodium carbonate to afford quantitatively the desired 3α-hydroxy-5,6α-epoxystigmastane (**11**) (purity ≥95% determined by GC).

4. Conclusion

We have set up an easy to run and reproducible reaction sequence allowing a gram-scale access to 3b-hydroxystigmast-5-ene-7-one (**7**) (purity ≥95%), direct precursor of several oxyphytosterols and to 3β-hydroxy-5,6β-epoxystigmastane (**11**) (purity ≥95%), starting from commercially available stigmasterol.

Acknowledgements

DJD thanks the MRT for financial support. This work was supported by a grant from the Ministère de la Jeunesse, de l'Education Nationale et de la Recherche, France (RARE 015 No. 02 P0640). We thank ULP and CNRS for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2008.02.004.

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