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Characterisation of Novel 3-Carboxyalkyl-steranes Occurring in Geological Samples

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Abstract: Three novel series of 3-carboxyalkyl-steranes have been identified by synthesis in sediments from an evaporitic basin. These compounds may derive from precursor steroids bearing a polyfunctionalised side-chain at C-3, yet unknown in living organisms. Geochemical data suggest a microalgal or a bacterial origin.

INTRODUCTION

Steroids are produced by a wide range of organisms of marine as well as of continental origin. Due to the stability of their hydrocarbon skeleton, steroid derived compounds represent one of the most widespread classes of molecular fossils in the subsurface, where they undergo various biological and diagenetic transformations.[1,2]

The study of these sedimentary steroids has been shown to be useful for geochemical purposes such as the determination of the maturity level of the organic matter[3] or the establishment of correlations between petroleum and source-rocks.[4] Furthermore, detailed studies of sedimentary steroids bearing uncommon skeletons allowed, in some cases, to link these molecular fossils to particular biological precursors from specific environments,[5-7] providing useful indications on the organisms which contributed to the organic matter and/or on the palaeoenvironmental conditions of the organic matter deposition.

In addition, geochemical analysis of sediments and petroleum has led to the characterisation of some "orphan" steroids such as, for example, the 2α- or 3β-methylsteranes,[8,9] the 3β-ethylsteranes,[10] the 3β-alkylsteranes[11] or the 3β-carboxysteranes[12,13] which cannot yet be linked to any known biological precursor.

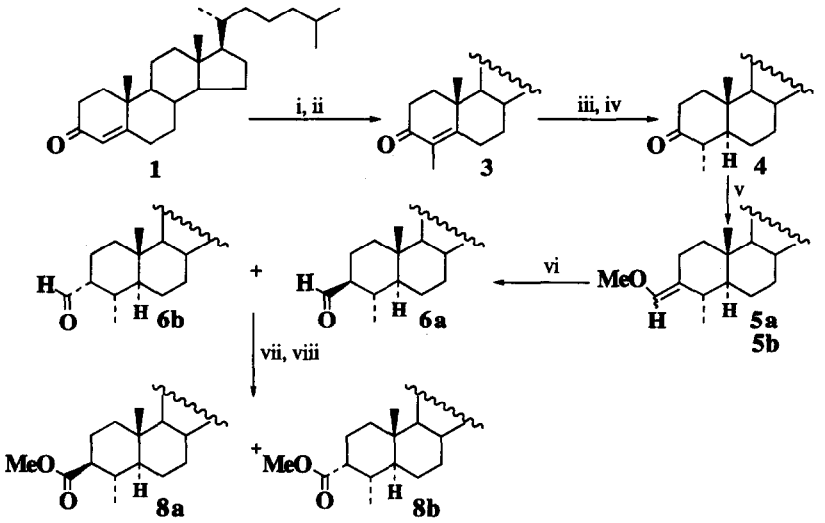
We report here the characterisation by synthesis of different homologues of novel 3-(carbomethoxyalkyl)-steroids (i.e. 3β-carbomethoxy-4α-methyl-5α-cholestane **8a**, 3β-(acetic acid)-5α-cholestane methyl ester **11a**, 3β-(acetic acid)-5α-stigmastane methyl ester **14a** and 3β-(propionic acid)-5α-cholestane methyl ester **18a** occurring (as carboxylic acids) in an Oligocene immature marl from the Mulhouse evaporitic basin (south of Alsace, France). Their identification in geological samples enabled us to consider novel hypotheses on the presence of unknown steroids bearing a functionalised C-3 alkylated side-chain in living organisms and on their diagenetic transformation pathways in the subsurface.

RESULTS AND DISCUSSION

The acid fraction was separated from the total organic extract of the sample and esterified as indicated in the experimental section. Coupled gas chromatography-mass spectrometry (GC-MS) analysis of the esters showed the occurrence, along with the previously identified 3β-carbomethoxy-steranes,[12] of three series of higher homologues. Two of them display a major fragment at *m/z* 289 and molecular ions at *m/z* 444, 458 and 472 which are compatible with either C_{29} - C_{31} 3-carbomethoxy-4-methyl-steranes or the methyl esters of 3-(acetic acid)-steranes. The third series shows a major fragment at *m/z* 303 and molecular ions at *m/z* 458, 472 and 486 which are in agreement with the methyl esters of C_{30} - C_{32} 3-(propionic acid)-steranes. The identification of these components was established by synthesis of steroids **8a**, **11**, **14** and **18a** and comparison of the mass spectra and GC elution times between the synthetic and the geological constituents. In the geological sample, the occurrence of 3-carboxy-4,23,24-trimethyl-5α-cholestanes was also tentatively established from the mass spectral data as indicated by the presence of an enhanced fragment at *m/z* 98.[5]

Synthesis of the 3-carbomethoxy-4α-methyl-5α-cholestanes 8a and 8b

Figure 1 shows the synthetic scheme used to obtain the 3-carbomethoxy-4α-methyl-5α-cholestanes **8a** and **8b.** Selective introduction of the methyl in position 4 was performed using the method described by Kirk and Petrow.[14] Reduction of 4-methyl-cholest-4-en-3-one **3** under thermodynamical equilibrium conditions with lithium in ammonia[15] gave a mixture of 4α-methyl-5α-cholestan-3-one **4** and of the corresponding 3α- and 3β-alcohols. The crude mixture was directly oxidised with Jones' reagent, yielding 4α-methyl-5α-cholestan-3-one **4**. A Wittig reaction with ketone **4** in presence of methoxymethylenetriphenylphosphonium chloride[16] furnished the two enol ethers **5** further hydrolysed into aldehydes **6a** and **6b**. The latter were separated by SiO_2 thin layer chromatography (TLC) and could be distinguished without ambiguity by their ^{1}H-NMR spectra. Oxidation of aldehyde **6a** (respectively **6b**), followed by esterification of carboxylic acid **7a** (**7b**) afforded 3-carbomethoxy-4α-methyl-5α-cholestane **8a** (**8b**).

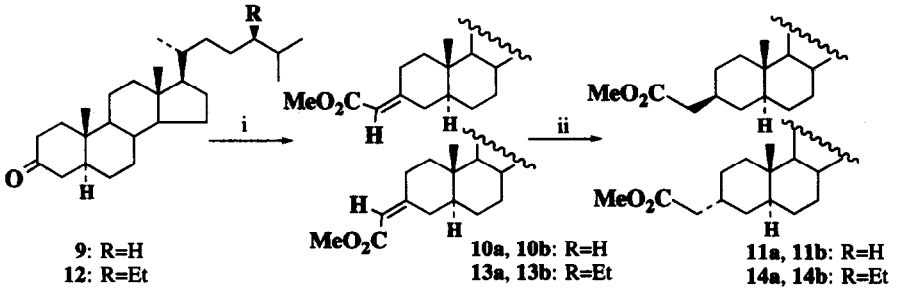


i: PhSH, $N(CH_2CH_2OH)_3$, HCHO; ii: Raney nickel, acetone; iii: Li, NH_3; iv: Jones' oxidation;
v: $MeOCH_2P(C_6H_5)_3Cl$, nBuLi, THF; vi: $HClO_4$; vii: Jones' oxidation; viii: CH_2N_2

Fig. 1. Synthesis of 3α- and 3β-carbomethoxy-4α-methyl-5α-cholestanes **8a** and **8b**

*Synthesis of methyl esters of 3-(acetic acid)-5α-cholestanes **11a, 11b** and 3-(acetic acid)-5α-stigmastanes **14a, 14b***

The synthetic scheme used for the preparation of compounds **11a, 11b, 14a** and **14b** is shown in figure 2. Intermediates **10a** and **10b** (respectively **13a** and **13b**) were obtained by a Wadsworth-Emmons reaction,[17] starting from 5α-cholestan-3-one **9** (5α-stigmastan-3-one **12**). Catalytic hydrogenation of **10a** and **10b** (**13a** and **13b**) with palladium on charcoal gave quantitatively a mixture of the two isomers **11a** and **11b** (**14a** and **14b**) which could not be separated on SiO_2 TLC or GC on non polar phase (DB5). They could, however, be distinguished by GC on moderately polar or polar phases (DB17, Supelcowax). The last eluting, major isomer corresponds to the naturally occurring compound, the 3α(H) configuration of which was assigned by analogy with relative elution times of compounds **18a** and **18b** (see below).



i: $(MeO)_2P(O)CH_2CO_2Me$, n-BuLi, THF; ii: H_2, Pd/C 10%, hexane

Fig. 2. Synthesis of methyl esters of 3-(acetic acid)-steranes **11a, 11b** and **14a, 14b**

*Synthesis of the methyl esters of 3-(propionic acid)-5α-cholestanes **18a, 18b***

Methyl esters of the 3-(propionic acid)-5α-cholestanes **18a** and **18b** were synthesised from cholestan-3-one **9** according to the scheme represented in figure 3. Contrary to compounds **11a** and **11b** (**14a** and **14b**), the compounds **18a** and **18b** could be separated by SiO_2 TLC. The major, more polar isomer **18a** was shown to have the indicated stereochemistry at C-3 from data of homonuclear and heteronuclear 2D NMR experiments. These experiments indeed allowed unambigous location of H-2_{ax} and the determination of its coupling constants (*dddd*, *J*=12.4, 12.4, 12.4, 3.5 Hz), the large coupling with H-3 implying that the latter is axial.

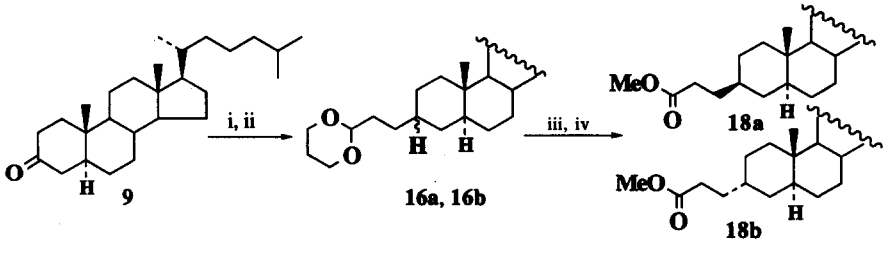


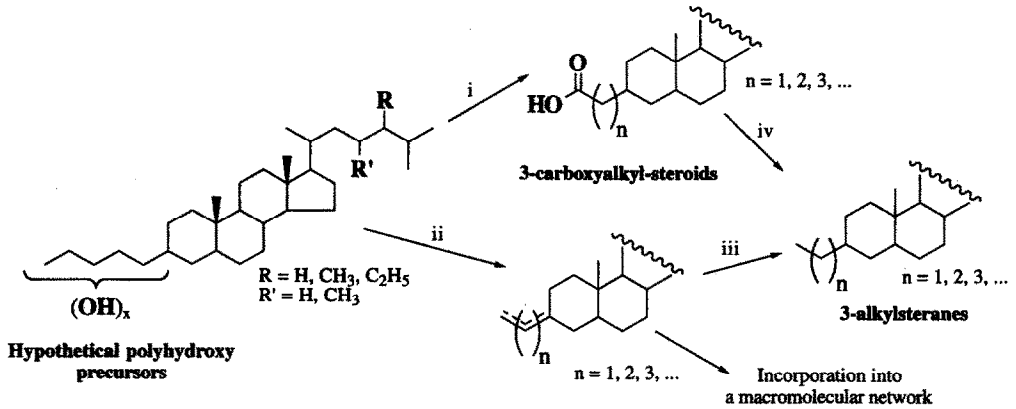
Fig. 3. Synthesis of the methyl esters of 3-(propionic acid)-5α-cholestanes **18a, 18b**

The occurrence of steroids in sediments and petroleum bearing one extra methyl group on ring A have been reported in the literature in several cases. Within these methylsteroids, the 4-methylsteroids[5,18-20] correspond to the most widespread group and are thought to derive mostly from dinoflagellates[5,21,22] although such compounds have also been found in other organisms such as the bacterium *Methylococcus capsulatus* [23,24] or some microalgae of the Prymnesiophyte group.[25]

Summons and Capon[9] have brought the first evidence for the occurrence of sedimentary steranes methylated on position 2 or 3. Until now, no known biological precursor could be attributed to these compounds, tentatively proposed to derive from microbiological methylation of Δ^2-sterenes[9] at an early stage of diagenesis. These authors have subsequently characterised in the same samples a series of 3β-ethylsteranes proposed to result from a two-step methylation mechanism starting from Δ^2-sterenes.[10]

Another alternative explanation for the origin of these unusual methylsteranes has been proposed more recently by Dahl *et al.*[11] These authors indeed detected 3-alkylsteranes with ring-A side chains ranging from C_1 to C_5 in a crude oil from the Monterey formation. Desulfurisation experiments using deuterated Raney nickel on sulfur-rich macromolecules[11] showed that 3-alkylsteroids were linked to the macromolecular matrix by the ring-A side chain, indicating that the biological precursors ("bacteriosteroids"[11]) probably possessed a polyfunctionalised ring-A side chain which could be derived from a C_5 sugar.

The occurrence of free 3-carboxysteroids in sediments from different origins have been reported by different authors.[12,13,26-28] These compounds, which have also been obtained by ruthenium tetroxide oxidation of petroleum asphaltenes[29] may derive from the same polyfunctionalised precursors ("bacteriosteroids") through well known geochemical transformations (figure 4) in a similar way as that observed within the sedimentary hopanoids.[30]



i: Oxidative cleavage; ii: Dehydration; iii: Reduction; iv: Decarboxylation / Reduction

Fig. 4. Hypothetical formation of 3-alkylsteroids and 3-carboxyalkyl-steroids in sediments

It should be noticed that 3-alkylsteranes with ring-A side chain ranging from C_1 up to C_5 have also been detected in the saturated hydrocarbon fractions of the Mulhouse basin sediments, strengthening the hypothesis of the existence of such polyfunctionalised precursors. The origin of highly functionalised 3-alkylated steroids is, up to now, not clear, but these compounds may occur as such in some particular organisms or derive from the (bacterial) transformation of steroids from algal origin,[11,13] as suggested by the occurrence of 3-carboxy dinosteroids in the Mulhouse basin sediments. Dinosteranes are indeed typical components of planktonic dinoflagellates.[5]

EXPERIMENTAL PART

Analytical aspects

Extraction and separation procedure: A crushed rock (100 g, 2.94 % total organic carbon) was extracted twice with a mixture of $CHCl_3$/MeOH 3:1 v/v and once with a mixture of toluene/MeOH 3:1 v/v at 50°C. After removing the solvent *in vacuo*, the total organic extract (1.08 g) was separated into neutral (0.59 g) and acidic (0.29 g) fractions on silica gel impregnated with potassium hydroxide.[31] The acid fraction was esterified with diazomethane and chromatographed on TLC (silica gel, CH_2Cl_2), furnishing the monoesters (26 mg), further separated on TLC (silica gel, CH_2Cl_2/hexane 1:1 v/v) in different subfractions, one of them being considerably enriched in 3-(carbomethoxyalkyl)-steranes. **Gas Chromatography (GC)**: Analyses were carried out on a Carlo Erba Fractovap 4160 chromatograph with "on-column" injector and flame ionisation detector, equipped with a DB5 capillary column (J&W, 30 m x 0.32 mm, film thickness: 0.25 µm), carrier gas: H_2, 0.7 kg/cm^2. **Gas Chromatography-Mass Spectrometry (GC-MS)**: EI analyses were mostly performed on a Varian 3400 gas chromatograph equipped with an "on-column" injector and with a DB5 fused silica column (30 m x 0.25 mm, film thickness : 0.1 µm) connected to a Finnigan MAT INCOS 50 mass spectrometer operating at 70 eV. Temperature was programmed from 35°C to 100°C at 10°C/min, 100°C-300°C at 4°C/min, then isothermal at 300°C. Co-elutions were performed on a Finnigan MAT TSQ 70 mass spectrometer, connected to a Varian 3400 gas chromatograph equipped with an "on-column" injector and with fused silica columns DB5 (apolar, 60 m x 0.25 mm, film thickness : 0.1 µm), DB17 (moderately polar, 25 m x 0.25 mm, film thickness : 0.1 µm) or Supelcowax (polar, 60 m x 0.25 mm, film thickness : 0.1 µm). Temperature was programmed from 35°C to 100°C at 10°C/min, 100°C-300°C at 3°C/min, then isothermal at 300°C. **Nuclear Magnetic Resonance**: ^{1}H NMR spectra were routinely realised on Bruker AM-400 and Bruker WP-200-SY spectrometers. The chemical shifts (δ) are reported in ppm downfield from TMS and are internally referenced to the residual protons in the deuterated solvent. Coupling constants (*J*) are given in Herz. For the compound **18a**, 2D-homonuclear (^{1}H-^{1}H COSY, NOESY) and heteronuclear (direct and long range ^{1}H-^{13}C correlations) experiments were perfomed on a Bruker ARX 500 spectrometer.

Synthesis

4-[(phenylthio)methyl]-cholest-4-en-3-one 2: Cholest-4-en-3-one **1** (3.85 g, 10.0 mmol) was refluxed under argon in presence of thiophenol (1.46 g, 13.3 mmol), of 35% aqueous formaldehyde (1.44 g, 16.6 mmol) and triethanolamine (4.96 g, 33.0 mmol) for 30 hours.[14] After cooling to room temperature and addition of 100 ml of distillate water, the crude was extracted with ether. The organic phase was washed with water, dried over $MgSO_4$ and the solvent removed under reduced pressure. Silica gel chromatography of the crude (hexane/ethyl acetate 6:1) afforded 1.52 g (3.0 mmol, 30% yield) of the thioether **2** (yellow oil). R_F=0.4 (SiO_2 TLC; hexane/ethyl acetate 6:1 v/v). EIMS (GC) 70eV, *m/z* (rel.int.): 506(M$^+$, 22%), 397(100), 283(28), 147(40), 123(60), 111(61). ^{1}H-NMR (200MHz, $CDCl_3$): δ (ppm) 0.69 (3H, *s*, H-18), 0.86 (6H, *d*, *J*=6.4 Hz, H-26 and H-27), 0.90 (3H, *d*, *J*=7.5 Hz, H-21), 1.15 (3H, *s*, H-19), 2.39 (2H, *m*), 2.69 (1H, *ddd*, *J*=14.4, 4.0, 4.0 Hz), 3.88 (2H, *s*, CH_2 in position 4), 7.15-7.41 (5H, *m*, H aromatic).

4-methylcholest-4-en-3-one 3: 5 g of Raney nickel, rinced with water and acetone, were refluxed in acetone for 30 minutes under argon. After cooling, 350 mg (0.7 mmol) of the thioether **2**, dissolved in a minimum volume of acetone, were added. The mixture was refluxed under argon for 3 hours. After decantation, the solvent was collected and the Raney nickel washed with methylene chloride. The organic phase was filtered over celite and the solvent removed *in vacuo*. The crude, after CC purification (silica gel, methylene chloride) furnished 221 mg (0.56 mmol, 80% overall yield) of compound **3**, which was recrystallised in methanol/methylene chloride. R_F=0.35 (CH_2Cl_2). Found: C=84.25, H=11.87, required C=84.36, H=11.63. Mp=91-92°C. EIMS (GC), 70eV, *m/z* (rel. int.): 398(M$^+$, 50%), 383(13), 289(9), 285(15), 275(27), 261(12),

163(30), 138(78), 123(100), 91(29). ^{1}H-NMR (200MHz, $CDCl_3$): δ (ppm) 0.71 (3H, *s*, H-18), 0.865 (3H, *d*, *J*=7.0 Hz, H-26 or H-27), 0.866 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.91 (3H, *d*, *J*=6.5 Hz, H-21), 1.16 (3H, *s*, H-19), 1.77 (3H, *d*, 1.2 Hz, methyl in position 4), 2.40 (2H, *m*), 2.75 (1H, *ddd*, *J*=14.7, 3.6, 2.9 Hz).

4α-methyl-5α-cholestan-3-one 4: The reaction was carried out under anhydrous conditions. 20 ml of ammonia, previously condensed at -78°C and dried with sodium were transferred in a two-necked flask. After addition of 250 mg of lithium, 390 mg (0.98 mmol) of 4-methylcholest-4-en-3-one **3** in 4 ml of anhydrous ether were added. The mixture was stirred at -78°C for 20 minutes, after which the excess of lithium was destroyed with methanol, and the crude, brought back to ambient temperature, was extracted with ether. The organic layer, washed with water and dried over $MgSO_4$ was concentrated *in vacuo*. The crude mixture was directly oxidised with Jones' reagent, extracted with ether, dried over $MgSO_4$ and subjected to CC (silica gel, hexane/ethyl acetate 20:1 v/v), yielding 192 mg (0.48 mmol, 49% overall yield) of 4α-methyl-5α-cholestan-3-one **4**. R_F=0.62 (CH_2Cl_2). Found: C=83.85, H=11.82; required C=83.93, H=12.07. Mp=111-112°C. EIMS (GC), 70eV, *m/z* (rel. int.): 400(M^+, 37%), 385(11), 260(9), 246(62), 245(100), 231(25), 177(20), 138(22), 123(22), 107(24), 95(40). ^{1}H-NMR (200MHz, $CDCl_3$): δ (ppm) 0.67 (3H, *s*, H-18), 0.861 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.865 (3H, *d*, *J*=6.7 Hz, H-26 or H-27), 0.90 (3H, *d*, *J*=7.3 Hz, H-21), 0.97 (3H, *d*, *J*=6.5 Hz, methyl at position 4), 1.07 (3H, *s*, H-19).

(Z) and (E) 3-methoxymethylene-4α-methyl-5α-cholestanes 5a and 5b: 1.1 ml of n-butyllithium in hexane (4 eq.) were added to 677 mg (6 eq.) of methoxymethylenetriphenylphosphonium chloride dissolved in 10 ml of anhydrous THF.[16] The mixture was stirred under argon for 10 minutes, after which 130 mg (0.32 mmol) of the ketone **4** in 3 ml of dry THF were added. The reaction was quenched with water after 1 hour and the crude mixture extracted with ether. The organic extract was washed with water, dried over $MgSO_4$ and the solvent removed under reduced pressure. TLC (silica gel, hexane/ethyl acetate 4:1 v/v) of the crude mixture afforded the two isomers **5a** and **5b** (32.5 mg, 0.076 mmol of one of the isomer; 48.5 mg, 0.110 mmol of the other isomer, 58% overall yield). Minor isomer: R_F=0.60 (hexane/ethyl acetate 4:1 v/v). Found: C=84.25, H=12.10, required C=84.04, H=12.22. Mp=129-130°C. EIMS (GC), 70eV, *m/z* (rel. int.): 428(M^+, 100%), 413(15), 381(15), 356(13), 351(12), 287(9), 243(11), 147(26), 121(36), 112(61), 109(36), 107(55), 105(33), 99(49), 95(91). ^{1}H-NMR (200MHz, CD_2Cl_2): δ (ppm) 0.65 (3H, *s*, H-18), 0.78 (3H, *s*, H-19), 0.86 (6H, *d*, *J*=6.6 Hz, H-26 and H-27), 0.90 (3H, *d*, *J*=7.7 Hz, H-21), 0.99 (3H, *d*, *J*=6.7 Hz, methyl in position 4), 3.48 (3H, *s*, H-methoxy), 5.67 (1H, *t*, *J*= 1.36 Hz, H-vinylic). Major isomer: R_F=0.53 (hexane/ethyl acetate 4:1 v/v). Mp=141-142°C. EIMS (GC), 70eV, *m/z* (rel. int.): 428(M^+, 100%), 413(18), 381(16), 356(12), 287(7), 243(9), 147(26), 121(36), 112(69), 109(37), 107(57), 105(36), 99(51), 95(86). ^{1}H-NMR (200MHz, CD_2Cl_2): δ (ppm) 0.66 (3H, *s*, H-18), 0.86 (6H, *d*, *J*=7.2 Hz, H-26 and H-27), 0.89 (3H, *s*, H-19), 0.904 (3H, *d*, *J*=6.6 Hz, H-21 or methyl in position 4), 0.906 (3H, *d*, *J*=7.3 Hz, H-21 or methyl in position 4), 2.66 (1H, *ddd*, *J*=10.5, 2.6, 2.6 Hz), 3.50 (3H, *s*, H-methoxy), 5.67 (1H, *t*, *J*=1.5 Hz, H-vinylic).

3-formyl-4α-methyl-5α-cholestanes 6a and 6b: 2 ml of 70% perchloric acid were added to 57 mg (0.13 mmol) of a mixture of the two isomers **5a** and **5b** solubilised in 10 ml ether.[16] After 10 minutes, the solution was extracted with ether, the organic phase washed with water and dried over $MgSO_4$. The solvent was removed *in vacuo*. TLC (silica gel, hexane/ethyl acetate 1:1 v/v) afforded the two aldehydes **6a** (14 mg, 0.03 mmol, 23%yield) and **6b** (30 mg, 0.07 mmol, 54% yield). 3β-formyl-4α-methyl-5α-cholestane **6a**: R_F=0.58 (hexane/ethyl acetate 1:1 v/v). Mp=112-113°C. EIMS (GC), 70eV, *m/z* (rel. int.): 414(M^+, 52%), 399(19), 262(17), 260(67), 259(100), 245(13), 231(30), 191(50), 123(41), 121(41), 109(42), 108(45), 107(46), 95(65), 81(65). ^{1}H-NMR (400MHz, CD_2Cl_2): δ (ppm) 0.66 (3H, *s*, H-18), 0.82 (3H, *d*, *J*=6.4 Hz, methyl in position 4), 0.84 (3H, *s*, H-19), 0.859 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.863 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.90 (3H, *d*, *J*=6.6 Hz, H-21), 1.98 (1H, *ddd*, *J*=12.4, 3.6, 3.6 Hz), 9.50 (1H, *d*, *J*=4.1 Hz, H-aldehyde). 3α-formyl-4α-methyl-5α-cholestane **6b**: R_F=0.61 (hexane/ethyl acetate 1:1 v/v). Mp=69-70°C. EIMS (GC), 70eV, m/z (rel. int.): 414(M^+, 49%), 399(26), 356(27), 344(27), 315(7), 273(13), 260(56), 259(100), 245(17), 231(27), 191(40), 121(47), 109(48), 107(52), 95(77), 81(72). ^{1}H-NMR (400MHz,

CD_2Cl_2): δ (ppm) 0.65 (3H, *s*, H-18), 0.84 (3H, *s*, H-19), 0.857 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.862 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.90 (3H, *d*, *J*=6.6 Hz, H-21), 1.10 (3H, *d*, *J*=7.2 Hz, methyl in position 4), 2.35 - 2.38 (1H, *m*, H-3), 9.91 (1H, *m*, *Js* < 1.5 Hz, H-aldehyde).

3-carboxy-4α-methyl-5α-cholestanes 7a and 7b: 1 ml of Jones' reagent was added to 13.5 mg (0.033 mmol) of the aldehyde **6a**, solubilised in a mixture of acetone/ether. After 20 minutes under stirring, extraction with ether, drying over $MgSO_4$ and removal *in vacuo* of the solvent, TLC of the crude (silica gel, methylene chloride) furnished 12 mg (0.028 mmol, 85% yield) of the acid **7a**. The same procedure was applied for the aldehyde **6b** (13.0 mg, 0.031 mmol), yielding 9.5 mg of the acid **7b** (0.022 mmol, 71% overall yield). 3β-carboxy-4α-methyl-5α-cholestane **7a**: Found: C=80.87, H=11.67, required C=80.87, H=11.70. Mp=203-204°C. EIMS (GC), 70eV, *m/z* (rel. int.): 430(M^+, 46%), 415(33), 370(2), 290(9), 276(65), 275(100), 261(14), 231(6), 208(14), 207(32), 121(23), 107(30), 95(22). ^{1}H-NMR (400MHz, $CDCl_3$): δ (ppm) 0.65 (3H, *s*, H-18), 0.85 (3H, *s*, H-19), 0.862 (6H, *d*, *J*=6.3 Hz, H-26 and H-27), 0.865 (3H, *d*, *J*=6.8 Hz, methyl in position 4), 0.90 (3H, *d*, *J*=6.5 Hz, H-21). 3α-carboxy-4α-methyl-5α-cholestane **7b**: R_F=0.05 (CH_2Cl_2). Found: C=81.35, H=11.74, required C=80.87, H=11.70. Mp=199-200°C. EIMS (GC), 70eV, *m/z* (rel. int.): 430(M^+, 40%), 415(59), 369(3), 290(11), 276(65), 275(100), 261(22), 231(8), 208(24), 207(25), 121(21), 95(28). ^{1}H-NMR (400MHz, $CDCl_3$): δ (ppm) 0.65 (3H, *s*, H-18), 0.82 (3H, *s*, H-19), 0.863 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.868 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.90 (3H, *d*, *J*=6.5 Hz, H-21), 0.97 (3H, *d*, *J*=6.9 Hz, methyl in position 4), 2.63-2.66 (1H, *m*, H-3).

3-carbomethoxy-4α-methyl-5α-cholestanes 8a and 8b: 6 mg (0.014 mmol) of acid **7a**, solubilised in 2 ml of methylene chloride were esterified with freshly distillated diazomethane,[32] affording quantitatively the methyl ester **8a**. The same conditions were used for the acid **8b** (4.0 mg, 0.009 mmol). 3β-carbomethoxy-4α-methyl-5α-cholestane **8a**: R_F=0.55 (CH_2Cl_2/hexane 1:1 v/v). Mp=103-104°C. EIMS (GC), 70eV, *m/z* (rel. int.): 444(M^+, 55%), 429(25), 385(3), 331(3), 290(78), 289(100), 275(18), 262(23), 231(15), 222(21), 221(53), 181(16), 180(29), 121(81), 109(39), 108(52), 107(52), 95(61), 81(54). ^{1}H-NMR (400MHz, $CDCl_3$): δ (ppm) 0.65 (3H, *s*, H-18), 0.79 (3H, *d*, *J*=6.4 Hz, methyl in position 4), 0.85 (3H, *s*, H-19), 0.860 (3H, *d*, *J*=6.2 Hz, H-26 or H-27), 0.865 (3H, *d*, *J*=6.2 Hz, H-26 or H-27), 0.90 (3H, *d*, *J*=6.5 Hz, H-21), 3.66 (3H, *s*, H-methoxy). 3α-carbomethoxy-4α-methyl-5α-cholestane **8b**: R_F=0.75 (CH_2Cl_2/hexane 1/1 v:v). Mp=136-137°C. EIMS (GC), 70eV, *m/z* (rel. int.): 444(M^+, 37%), 429(31), 369(5), 290(70), 289(100), 275(17), 262(24), 231(17), 222(37), 221(52), 181(20), 180(21), 121(81), 109(40), 108(44), 107(54), 95(62), 81(56). ^{1}H-NMR (400MHz, $CDCl_3$): δ (ppm) 0.64 (3H, *s*, H-18), 0.81 (3H, *s*, H-19), 0.862 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.867 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.892 (3H, *d*, *J*=6.8 Hz, H-21 or methyl in position 4), 0.896 (3H, *d*, *J*=6.5 Hz, H-21 or methyl in position 4), 2.612 (1H, *ddd*, *J* =5.0, 5.0, 2.5 Hz, H-3), 3.63 (3H, *s*, H-methoxy).

(Z) and (E) 3-(acrylic acid)-5α-cholestane, methyl esters 10a and 10b: 3.96 mmol (3 eq.) of n-butyllithium in hexane were added to 3.96 g (5.28 mmol, 4 eq.) of trimethylphosphonoacetate dissolved in 2 ml of dry THF, and the reaction stirred under argon.[17] After 30 minutes, 5α-cholestan-3-one **9** (500 mg, 1.32 mmol) in 2 ml of THF was added dropwise. The reaction was quenched after 1 hour with 2 ml of water and extracted with ether. After washing the organic layer and drying it over $MgSO_4$ the solvent was removed under reduced pressure. CC (silica gel, methylene chloride) of the crude afforded 567 mg (1.28 mmol, 97 % overall yield) of a mixture of the two isomers **10a** and **10b**, which were not separable by CC, but could be distinguished by gas chromatography, in a ratio of 45/55. R_F=0.83 (CH_2Cl_2). Found: C=81.53, H=11.53, required C=81.39, H=11.38. Minor isomer: EIMS (GC), 70 eV, *m/z* (rel. int.): 442(M^+, 100%), 427(19), 368(7), 329(17), 288(22), 287(52), 255(21), 219(13), 180(34), 161(29), 147(45), 121(51), 119(45), 107(59), 93(60), 81(74). Major isomer: EIMS (GC), 70eV, *m/z* (rel. int.): 442(M^+, 100%), 427(11), 368(4), 329(6), 302(20), 288(24), 287(61), 255(8), 219(14), 180(28), 147(27), 126(31), 121(36), 119(34), 107(43), 105(41), 95(61), 81(58).^{1}H-NMR of mixture (400 MHz, $CDCl_3$): δ (ppm) 0.65 (3H, *s*, H-18), 0.856 (3H, *d*, *J*=6.6

Hz, H-26 or H-27), 0.861 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.896 (3H, *d*, *J*=7.1 Hz, H-21), 0.905 (3H, *s*, H-19), 3.67 (3H, *s*, H-methoxy), 5.58 (m, H-vinylic).

3-(acetic acid)-5α-cholestane, methyl esters 11a and 11b: 105 mg (20% weight) of Pd/C 10% were added to 528 mg (1.19 mmol) of a mixture of **10a** and **10b**, dissolved in 20 ml of hexane. The reaction was performed under hydrogen and was stirred for 8 hours. After purging with argon, the crude was filtered through celite and the solvent removed *in vacuo*, yielding quantitatively the two esters **11**, epimeric at position 3, which could not be separated by CC (silica gel, methylene chloride/hexane 1:1 v/v), but could be distinguished by gas chromatography with a moderately polar (DB17) or polar (Supelcowax) capillary column in a ratio of approximately 3/1. R_F=0.38 (CH_2Cl_2/hexane 1:1 v/v). Found: C=81.78, H=12.07, required C=81.02, H=11.78. EIMS of the mixture, 70eV, *m/z* (rel. int.): 444(M^+, 32%), 429(20), 331(20), 303(11), 290(67), 289(100), 275(18), 262(18), 221(45), 216(17), 181(8), 121(40), 107(67), 95(61), 93(52), 81(64). ^{1}H-NMR of the mixture (400MHz, $CDCl_3$): δ (ppm) 0.64 (3H, *s*, H-18), 0.75 and 0.78 (3H, 2*s*, H-19), 0.859 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.863 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.90 (3H, *d*, *J*=6.5 Hz, H-21), 3.656 and 3.661 (3H, 2*s*, H-methoxy).

(Z) and (E) 3-(acrylic acid)-5α-stigmastane, methyl esters 13a and 13b: 1.62 mmol (6 eq.) of n-butyllithium in hexane were added to a stirred solution of 400 mg (2.16 mmol, 8 eq.) of trimethylphosphonoacetate in 2 ml of dry THF.[17] After 30 minutes, 115 mg (0.27 mmol) of 5α-stigmastan-3-one in 2 ml of dry THF were added dropwise. After 1 hour, the reaction was quenched with water. CC (silica gel, methylene chloride/hexane 1:1 v/v) of the crude obtained after extraction with ether afforded 110 mg (0.23 mmol, 87% overall yield) of the two isomers **13a** and **13b**, which were not separable by CC (45/55 ratio by GC). R_F=0.83 (CH_2Cl_2). Found: C=81.70, H=11.65, required C=81.64, H=11.56. Minor isomer: EIMS (GC), 70eV, *m/z* (rel. int.): 470(M^+, 100%), 455(13), 396(6), 329(14), 303(13), 288(27), 287(52), 255(22), 180(38), 161(32), 147(50), 133(41), 121(54), 107(63), 105(59), 95 (83), 81(80), 57(100). Major isomer: EIMS (GC), 70eV, *m/z* (rel. int.): 470(M^+, 100%), 455(10), 396(6), 396(4), 357(4), 329(8), 302(27), 288(31), 287(68), 255(11), 219(17), 180(38), 147(36), 126(37), 121(45), 119(41), 109(37), 107(56), 105(52), 95(77), 81(73). ^{1}H-NMR of the mixture (400 MHz, $CDCl_3$): δ (ppm) 0.66 (3H, *s*, H-18), 0.81 (3H, *d*, *J*=6.9 Hz, H-26 or H-27), 0.83 (3H, *d*, *J*=7.2 Hz, H-26 or H-27), 0.84 (3H, *t*, *J*=7.5 Hz, H-29), 0.90 (3H, *d*, *J*=6.9 Hz, H-21), 0.91 (3H, *s*, H-19), 3.47 (1H, *m*), 3.67 (3H, *s*, H-methoxy), 5.58 (m, H-vinylic).

3-(acetic acid)-5α-stigmastane, methyl esters 14a and 14b: Catalytic hydrogenation of compounds **13a** and **13b** was performed with the same procedure of that used for the hydrogenation of compounds **11**. The reaction was quantitative, affording a mixture of methyl esters **14a** and **14b** in a ratio of approximately 3/1, which could not be separated by CC (silica gel, methylene chloride/hexane 1:1 v/v), but could be resolved by GC with a moderately polar capillary column (DB17). R_F=0.38 (CH_2Cl_2/hexane 1:1 v/v). Found: C=81.00, H=12.10, required C=81.29, H=11.94. EIMS of the mixture, 70eV, *m/z* (rel. int.): 472(M^+, 24%), 457(13), 359(1), 303(11), 290(75), 289(100), 275(18), 221(44), 216(16), 181(8), 121(41), 107(67), 95(62), 81(64). ^{1}H-NMR of the mixture (400 MHz, $CDCl_3$): δ (ppm) 0.64 (3H, *s*, H-18), 0.75 and 0.78 (3H, 2*s*, H-19), 0.81 (3H, *d*, *J*=7.0 Hz, H-26 or H-27), 0.83 (3H, *d*, *J*=7.4 Hz, H-26 or H-27), 0.84 (3H, *t*, *J*=7.4 Hz, H-29), 0.90 (3H, *d*, *J*=6.5 Hz, H-21), 1.95 (1H, *ddd*, *J*=12.4, 3.8, 3.8 Hz), 2.19 (2H, *d*, *J*=7.1 Hz, H-30), 3.656 and 3.659 (3H, 2*s*, H-methoxy).

Unsaturated dioxanes 15a, 15b: 0.3 ml of n-butyllithium in hexane (1.5 eq.) were added to a suspension of 360 mg of the phosphonium salt of 2-(2-bromoethyl)-1,3-dioxane (0.78 mmol, 3eq.) in anhydrous toluene. The red ylide was stirred under argon for 30 minutes, and 100 mg of 5α-cholestan-3-one (0.26 mmol, 1eq.) in anhydrous THF were added. After 90 minutes the reaction was quenched with water and the crude mixture extracted with ether. The organic extract was dried over $MgSO_4$, the solvent removed *in vacuo*. TLC (silica gel, CH_2Cl_2/hexane 80:20 v/v) of the crude mixture yielded 127 mg of a colorless oil composed of the two dioxanes

15a and **15b** (0.26 mmol) in a ratio of 2/1 (estimation by GC). R_F=0.4 (CH_2Cl_2/hexane 80:20 v/v). EIMS (GC), 70eV, *m/z* (rel. int.) of the minor isomer: 483 (0.25%), 408(0.85), 87(100), 59(6.3). EIMS (GC), 70eV, *m/z* (rel. int.) of the major isomer: 483(0.25), 408(0.6), 88(100), 59(5.6). ^{1}H-NMR of the mixture (400 MHz, $CDCl_3$): δ (ppm) 0.65 (3H, *s*, H-18), 0.85 (3H, *s*, H-19), 0.857 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.862 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.89 (3H, *d*, *J*=6.6 Hz, H-21), 1.95 (2H, *ddd*, *J*=12.4, 3.3, 3.3 Hz), 2.32 (2H, *m*), 3.76 (2H, *m*)

Dioxanes 16a, 16b: The hydrogenation of the dioxanes **15a** and **15b** was performed according to the same procedure used for the reduction of compounds **11**, yielding a mixture of the two isomers **16a** and **16b** in a ratio of 3/1 (GC; 87% overall yield). R_F=0.71 (hexane/ethyl acetate 5:1 v/v). Found: C=81.61, H=12.05, required C=81.41, H=12.01. EIMS (GC), 70eV, *m/z* (rel. int.) of the minor isomer: 486(M^+, 3%), 458(2), 412(23), 410(100), 382(16), 255(12), 229(15), 147(8), 87(42). EIMS (GC), 70eV, *m/z* (rel. int.) of the major isomer: 486(M^+, 4%), 410(38), 395(7), 331(4), 257(7), 255(16), 229(4), 187(5), 147(6), 87(100). ^{1}H-NMR of the mixture (400MHz, $CDCl_3$): δ (ppm) 0.64 (3H, *s*, H-18), 0.73 (major isomer) and 0.77 (3H, 2*s*, H-19), 0.857 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.862 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.89 (3H, *d*, *J*=6.5 Hz, H-21), 1.95 (2H, *ddd*, *J*=12.4, 3.3, 3.3 Hz), 3.75 (2H, *m*), 4.09 (2H, *m*), 4.47 (major isomer) and 4.50 (1H, 2*t*, *J*=5.2 Hz).

3α- and 3β-(propionic acid)-5α-cholestanes 17a, 17b: An excess of Jones' reagent was added to a mixture of the two isomers **16** (0.095 mmol) dissolved in ether/acetone (1:1 v/v). After 45 minutes under stirring at 0°C, isopropanol and water were added and the crude mixture extracted with ether and chloroform. The organic extract was washed with water, the solvent removed in vacuo and the crude separated by TLC to furnish 25 mg (59% overall yield) of the two acids **17a, 17b** in a ratio of 7/3 (estimation from ^{1}H-NMR spectrum). R_F=0.05 (CH_2Cl_2). Found: C=80.91, H=11.84, required C=81.02, H=11.79. EIMS, 70eV, *m/z* (rel.int.): 444(M^+, 46%), 429(36), 290(89), 289(100), 275(25), 262(21), 221(32), 121(39), 95(40), 85(37). ^{1}H-NMR (400MHz, $CDCl_3$): δ (ppm) 0.64 (3H, *s*, H-18), 0.74 (major isomer) and 0.78 (3H, 2*s*, H-19), 0.86 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.90 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 1.96 (1H, *ddd*, *J*=12.4, 3.1, 3.1 Hz), 2.4-2.3 (2H, *m*, H α of the carboxylic group).

3α- and 3β-(propionic acid)-5α-cholestane methyl esters 18a, 18b: 14mg of a mixture of the compounds **17** in CH_2Cl_2 were quantitatively esterified with diazomethane.[32] The two isomers **18** were separated by TLC (silica gel, hexane/CH_2Cl_2 1:1 v/v) to furnish 10 mg (71% overall yield) of the 3β-(propionic acid)-5α-cholestane methyl ester **18a** and 4 mg (29% overall yield) of the 3α-(propionic acid)-5α-cholestane methyl ester **18b**, the major and more polar isomer corresponding to the naturally-occurring compound. The stereochemistry at C-3 for the compound **18a** was established by homonuclear (^{1}H-^{1}H NOESY, COSY) and heteronuclear (direct and long range ^{1}H-^{13}C) experiments. R_F=0.4 (hexane/CH_2Cl_2 1:1 v/v). 3β-(propionic acid)-5α-cholestane methyl ester **18a**: Found: C=81.06, H=12.05, required C=81.16, H=11.86. EIMS (GC), 70eV, *m/z* (rel. int.): 458(M^+, 40%), 443(27), 318(12), 304(76), 303(100), 289(21), 262(19), 236(16), 235(57), 194(13), 121(15), 107(12), 95(17). ^{1}H-NMR (400MHz, $CDCl_3$): δ (ppm) 0.64 (3H, *s*, H-18), 0.74 (3H, *s*, H-19), 0.856 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.861 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.89 (3H, *d*, *J*=6.6 Hz, H-21), 1.96 (1H, *ddd*, *J*=12.4, 3.4, 3.4 Hz), 2.31 (2H, *t*, *J*=7.7 Hz, H α to ester), 3.66 (3H, *s*, H-methoxy). 3α-(propionic acid)-5α-cholestane methyl ester **18b**: Found: C=81.17, H=11.99, required C=81.16, H=11.86. EIMS (GC), 70eV, *m/z* (rel. int.): 458(M^+, 52%), 443(37), 318(14), 304(78), 303(100), 289(20), 262(21), 235(61), 194(16), 121(17), 107(15), 95(19). ^{1}H-NMR (400MHz, $CDCl_3$): δ (ppm) 0.64 (3H, *s*, H-18), 0.78 (3H, *s*, H-19), 0.862 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.867 (3H, *d*, *J*=6.6 Hz, H-26 or H-27), 0.90 (3H, *d*, *J*=6.6 Hz, H-21), 1.94 (1H, *ddd*, *J*=12.3, 3.4, 3.4 Hz), 2.26-2.32 (2H, *m*, H α to ester), 3.67 (3H, *s*, H-methoxy).

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