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RESULTS (Product analyses)

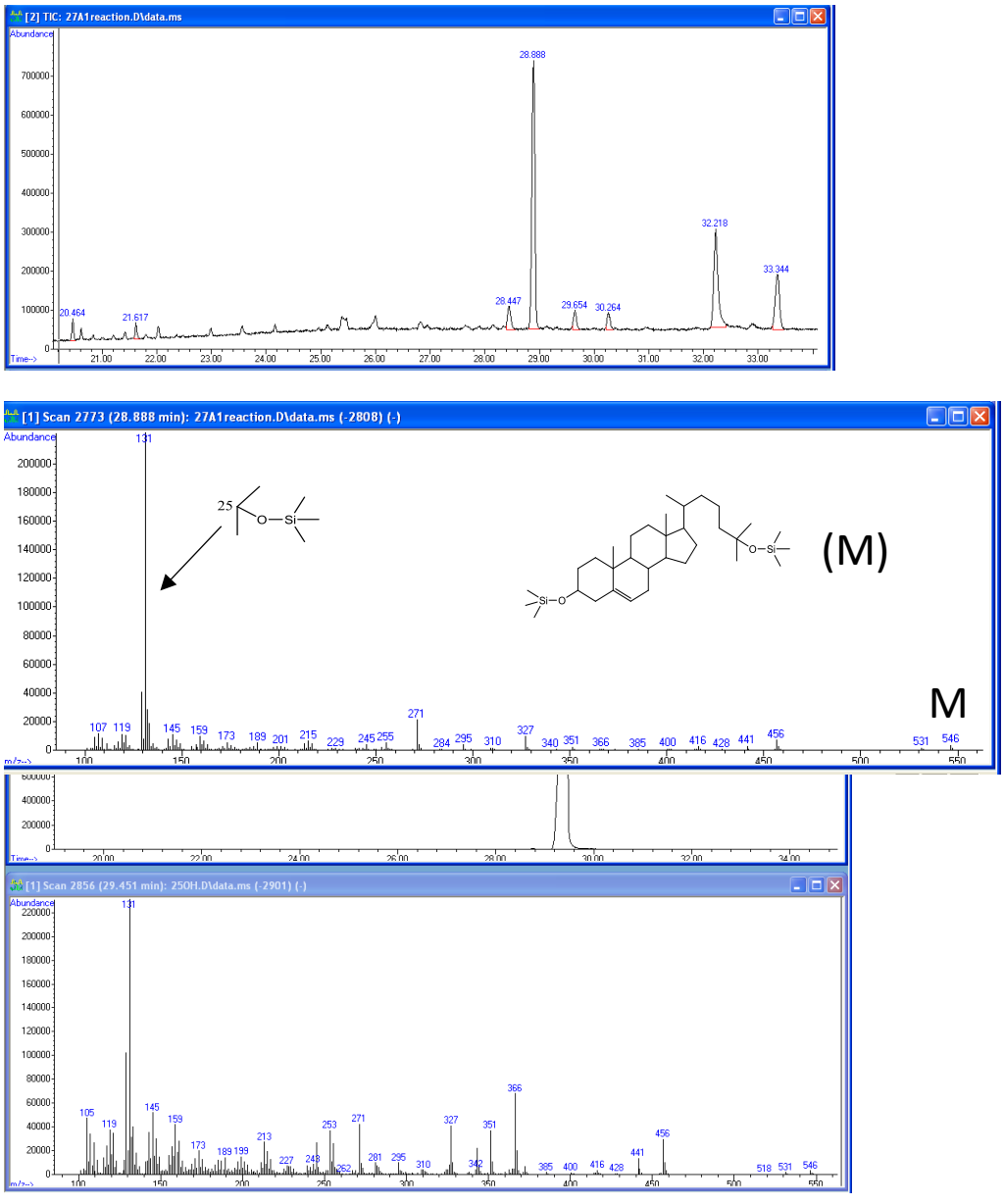
*Spectral Changes in CYP27A1 and Product Analysis upon addition of 25-Hydroperoxycholesterol (**1**)*

Addition of the 25-hydroperoxide **1** to CYP27A1 gave a difference spectrum with a through shifting within 10 min from 422 to 416 nm (Figure 1).

*25-Hydroxycholesterol (**4**).* Thin layer chromatography (TLC) of the $MeCl_2$ extract showed that most of **1** had been reduced to the corresponding 25-hydroxycholesterol **4** (R_f = 0.74 rel. to **1**, magenta colour response to acid/heat, identical to authentic **4**) (Scheme 1). GC-MS data of the TMS derivative were identical to authentic **4** showing a

major peak at t_R = 28.85 min, and a characteristic MS fragmentation pattern: *m/z* = 546 (M), *m/z* = 456 (M – 25-OTMS), *m/z* = 271 (M – [C24-27, 25OTMS + C3-5, 3OTMS]), *m/z* = 131 (base peak) (C25-27, 25OTMS fragment).

Figure S1. *Top panel*. GC of extract from CYP27A1 incubation with 25-hydroperoxide **1**. Peak at 28.888 is the 25-hydroxycholesterol (**4**) and peak at 33.344 min was characterized as **5**. *Lower panel*. MS fragmentation pattern of peak at 28.888 min (25-hydroxycholesterol **4**).



*25,27-dihydroxycholesterol (**5**)*. A small polar spot on the TLC (R_f = 0.17 rel. to **1**; brown colour to acid/heat) suggested the presence of a triol product, that correlated to a new

peak in the GC-MS profile of the TMS derivative at t_R = 33.35 min (>30% yield rel. to **4**), which we assign to 25,27-dihydroxycholesterol (**5**) (Scheme 1) based on its GC-MS fragmentation pattern: *m/z* = 619 (M-15); *m/z* = 531 (M-27OTMS); *m/z* = 219 (C25-27 + 25,27diOTMS side chain fragment); *m/z* = 147 (base peak) (*m/z* = 219 fragment, minus one TMS group).

Scheme S1. Structure of the TMS derivative of 25,26(27)-dihydroxycholesterol (**5**).

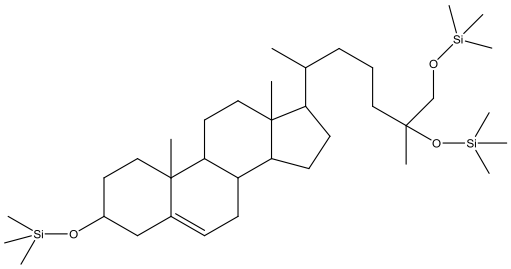
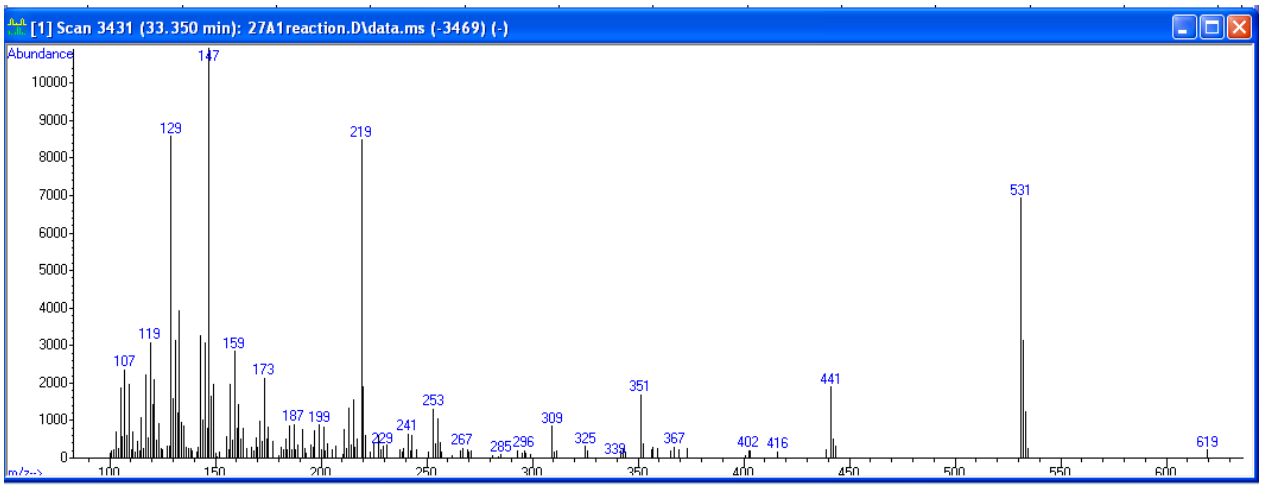


Figure S3. MS of peak at t_R = 33.35 min characterized as 25,26(27)-dihydroxycholesterol



*Spectral Changes in CYP27A1 and Product Analysis upon addition of the epimeric 20ξ-Hydroperoxycholesterols (**2** and **3**)*

Addition of the 20α-hydroperoxycholesterol (**2**) to CYP27A1 induced difference spectrum (within 10 min) with a maximum at 404 nm and a minimum at 425 nm (Figure 1), a spectral response that does not seem to correspond to any of the reported types of P450 responses. TLC analysis showed a small brown spot in the triol region (R_f < 0.2 rel. to **2**), and a small peak at t_R = 27.436 min (<10%) in the GC-MS analysis. Excess persilylated **2** and **3** decompose at the GC injection site to give pregnelone 3-TMS (t_R = 20.72 min).

Addition of the 20β-hydroperoxy-20-*iso*-cholesterol (**3**) to CYP27A1 also induced a difference spectrum similar to that elicited by the 20α-hydroperoxide **2** with a maximum at 408 nm and a minimum at 426 nm (Figure 1). However, no metabolites were detected in the $MeCl_2$ extracts as indicated by TLC and GC-MS analyses.

20α,27-dihydroxycholesterol. TLC analysis of the $MeCl_2$ extract of the CYP27A1 incubation with the 20α-hydroperoxide **2** showed a small brown spot in the triol region (R_f < 0.2 rel. to **2**), and a small peak at t_R = 27.436 min (<10%) in the GC-MS analysis. Although the MS fragmentation pattern of this compound is consistent with 20α,27-dihydroxycholesterol, the 27-position of the second side chain hydroxyl is difficult to ascertain from the fragmentation pattern: *m/z* 461 (loss of C22-27 + OTMS side chain fragment); 201 (base peak) (C20-27 + OTMS side chain fragment).

Scheme S2. Proposed MS fragments of GC peak at t_R = 27.436 min, assigned to 20α,27-dihydroxycholesterol.

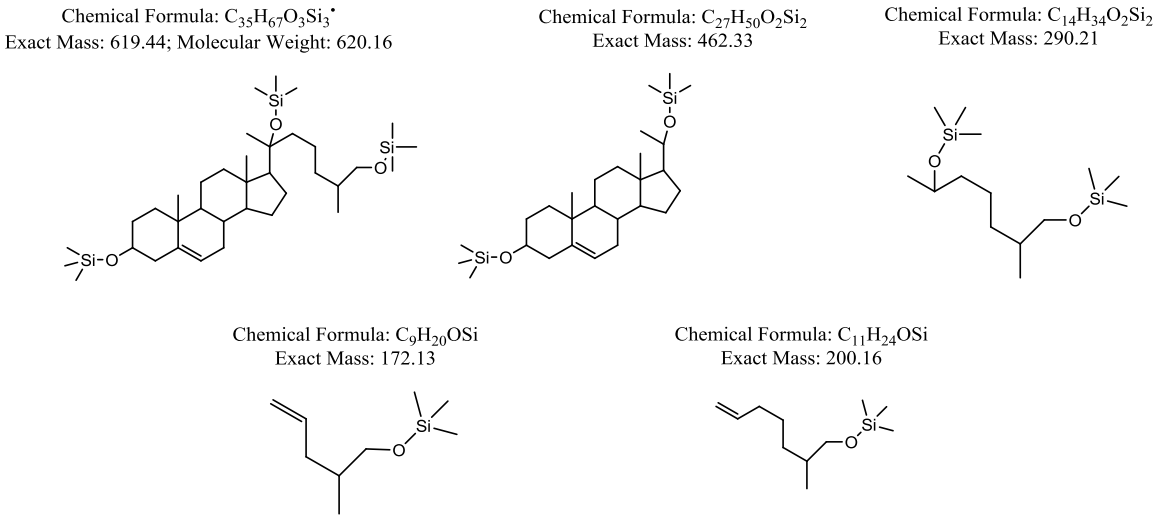
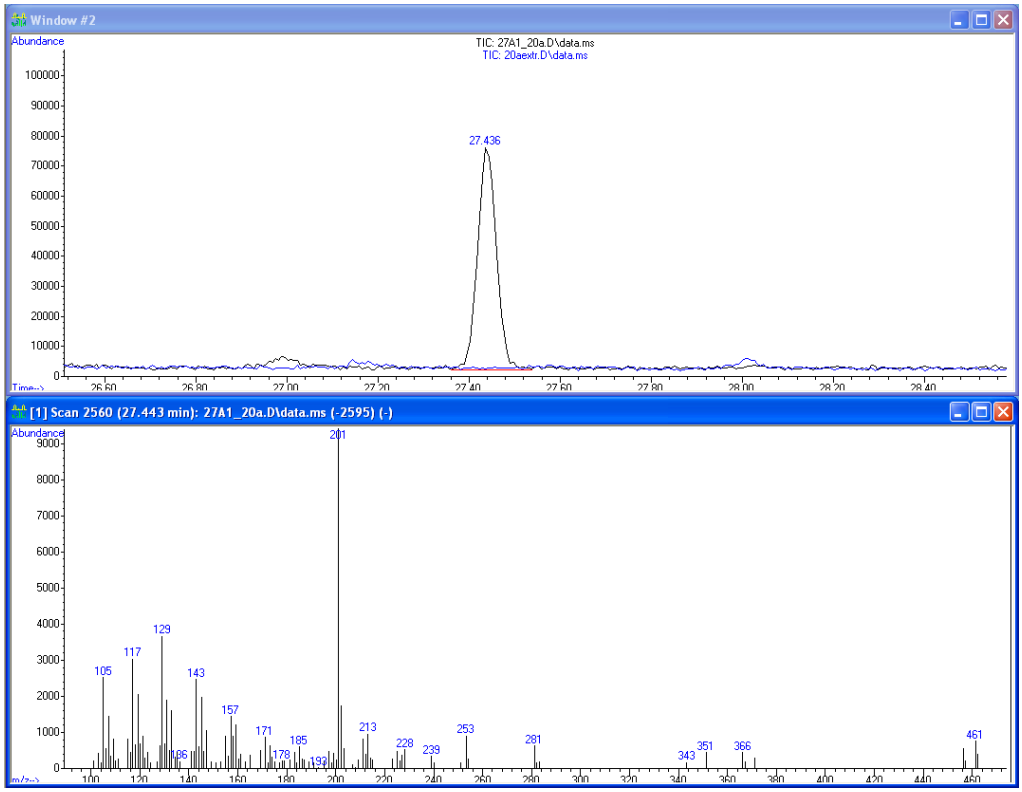


Figure S4. MS fragmentation of peak t_R = 27.436 min assigned as 20α,27-dihydroxycholesterol.



*Spectral Changes in CYP11A1 and Product Analysis upon addition of 25-Hydroperoxycholesterol (**1**)*

Addition of the 25-hydroperoxide **1** to CYP11A1 gave a difference spectrum that developed within 10 min in a stable spectrum with a maximum at 386 nm and a minimum at 419 nm (Figure 1). This type of a response is indicative of water displacement from the coordination sphere of the haem iron and is usually elicited by P450 substrates.

*25-hydroxycholesterol (**4**).* Analysis of the $MeCl_2$ extract as described above showed that most of **1** had been reduced to 25-hydroxycholesterol (**4**) (Scheme 1), which was in all respects identical to authentic **4** (TLC, R_f = 0.74 rel. to **1**, magenta colour response to acid/heat; GC-MS of TMS derivative, t_R = 28.85 min, and identical MS fractionation as compared to authentic **2**, see above).

*22,25-dihydroxycholesterol (**5**).* TLC of the $MeCl_2$ extract revealed a small polar spot (R_f = 0.15 rel. to **1**; brown colour to acid/heat) while GC-MS analysis of the TMS derivatives confirmed the presence of a new product at t_R = 29.688 (yield <30% rel. to **2**), to which we assign the 22,25-dihydroxycholesterol (**5**) structure (Scheme 1) based on the following characteristic MS ion fragments: *m/z* = 619 (M-15); *m/z* = 475 (M - 159, cleavage of the C22-23 bond); *m/z* = 261 (side chain fragment upon cleavage of the C20-22 bond), *m/z* = 171 (base peak) (261 minus 22OTMS); *m/z* = 159 (C23-27, 25OTMS fragment formed upon cleavage of the C22-23 bond); *m/z* = 131 (C25-27, 25OTMS fragment formed upon cleavage of the C24-25 bond).

Scheme S3. Proposed fragments observed in the MS fragmentation pattern of peak at t_R = 29.688 min characterized as *22,25-dihydroxycholesterol (**5**).*

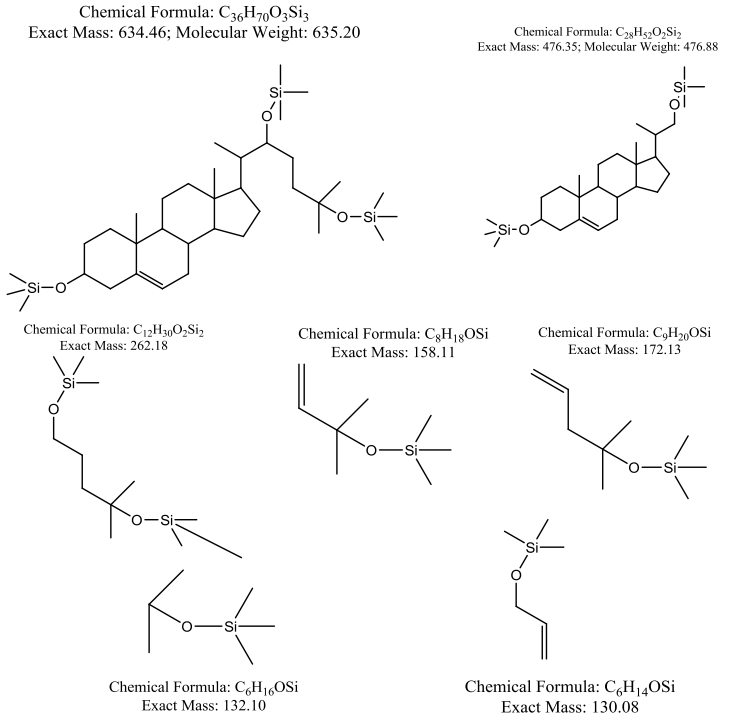
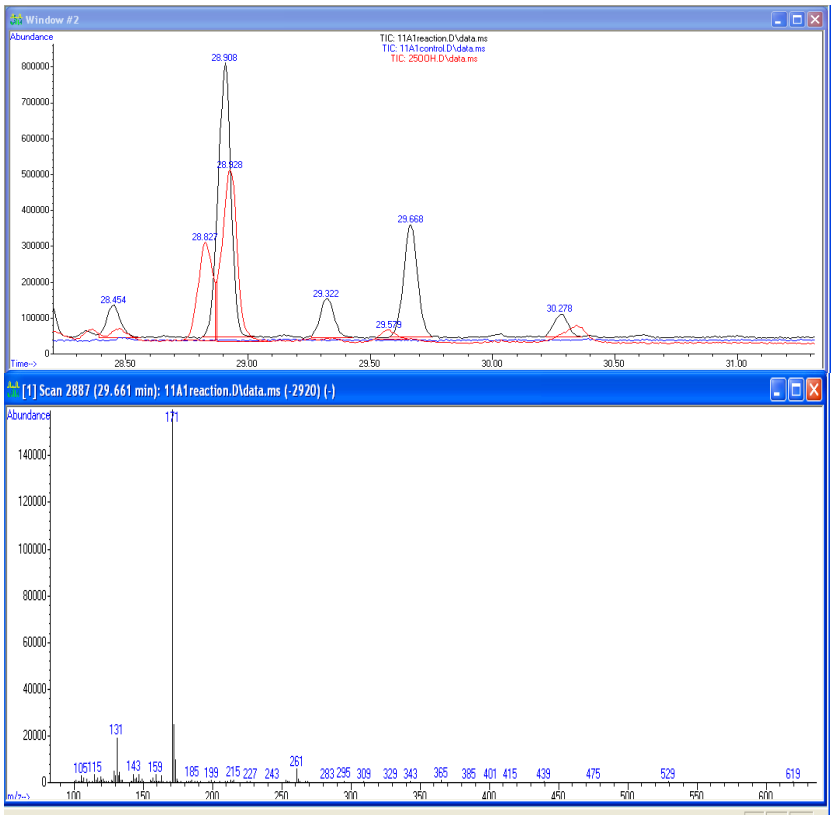


Figure S5. GC-MS analysis of the TMS-derivatives from the incubation of CYP11A1 with **1**. *Top panel*: Red trace: Incubation of **1** without CYP11A1; blue trace: Incubation of CYP11A1 alone; black trace: incubation of CYP11A1 with **1**. *Lower panel*: MS of peak at 29.668 min (*22,25-dihydroxycholesterol* ***5****).*

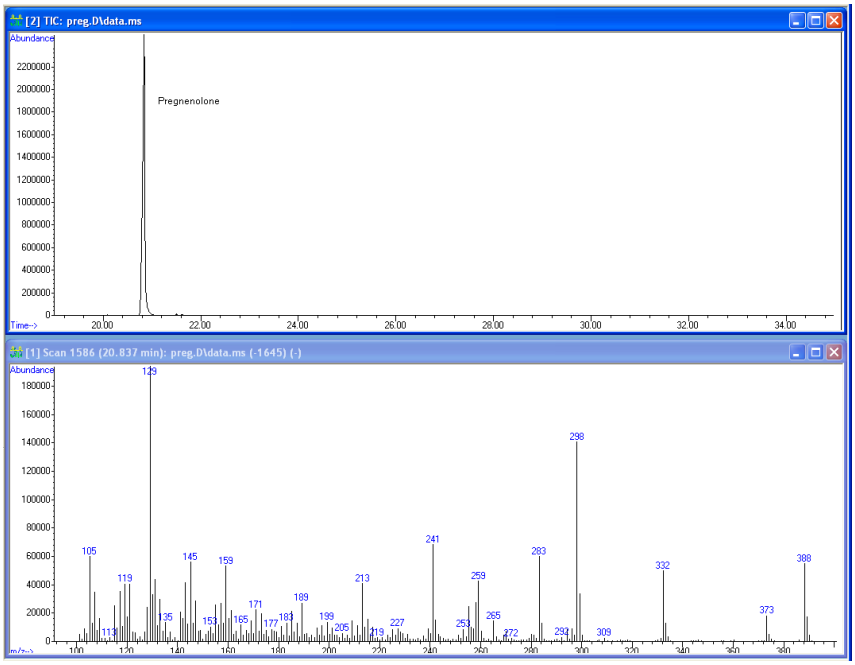


*Spectral changes in CYP11A1 and Product Analysis upon addition of the 20α-Hydroperoxycholesterol (**2**)*

Addition of the 20α-hydroperoxycholesterol (**2**) to CYP11A1 induced within 1 min a classical type 1 difference spectrum with a maximum at 387 nm and a minimum at 419 nm. The isomeric 20β-hydroperoxy-20-*iso*-cholesterol (**3**) gave a similar type of response; except the maximum was shifted to 403 nm and minimum to 423 nm and it took a longer time (~10 min) for the spectrum to develop (Figure 1).

Pregnenolone. Persilylated 20ξ-hydroperoxycholesterols **2** and **3** both decompose upon injection in the flash heater zone of the GC to give the TMS derivative of pregnenolone as a single product (t_R = 20.8 min; MS = *m/z* 388 (M), 373, 332, 298 (M minus 3-OTMS), 283, 259 and 129 (base peak, fragment C2-4 + 3-OTMS).

Figure S7. MS of authentic 3-TMS pregnenolone.



*CYP11A1 and 20α-Hydroperoxycholesterol (**2**).* Extraction of the incubation mixture with $MeCl_2$ followed by TLC analysis showed that the 20α-hydroperoxide **2** was transformed to a single glycol product. The metabolite was identified as 20α,22R-dihydroxycholesterol (**7**) (R_f = 0.3 rel. to cholesterol, purple colour response to acid/heat). TMS derivatization and comparison of GC-MS data with those of a synthetic reference sample showed identical GC mobility and MS fragmentation pattern, confirming the assigned structure.

*20α,22R-dihydroxycholesterol (**7**)*

Figure S6. GC-MS analysis of CYP11A1 and 20α-hydroperoxycholesterol (**2**) incubation mixture showing the TMS derivative of metabolite 20α,22R-dihydoxycholesterol (**7**) as single product at t_R = 29.261 min. Excess **2** decomposes to the 3-TMS pregnenolone at t_R = 20.722 min with MS fragmentation pattern identical to authentic prenenolone.

Top panel: GC of TMS derivatives of persilylated metabolite mixture showing 20α,22R-dihydoxycholesterol (**7**) at t_R = 29.261 min and pregnenolone at t_R = 20.722 min (decomposition product of excess **2**). *Lower panel*: MS of 3-TMS-pregenolone at t_R = 20.722 min (decomposition product of excess **2**), identical to authentic 3-TMS-pregenolone.

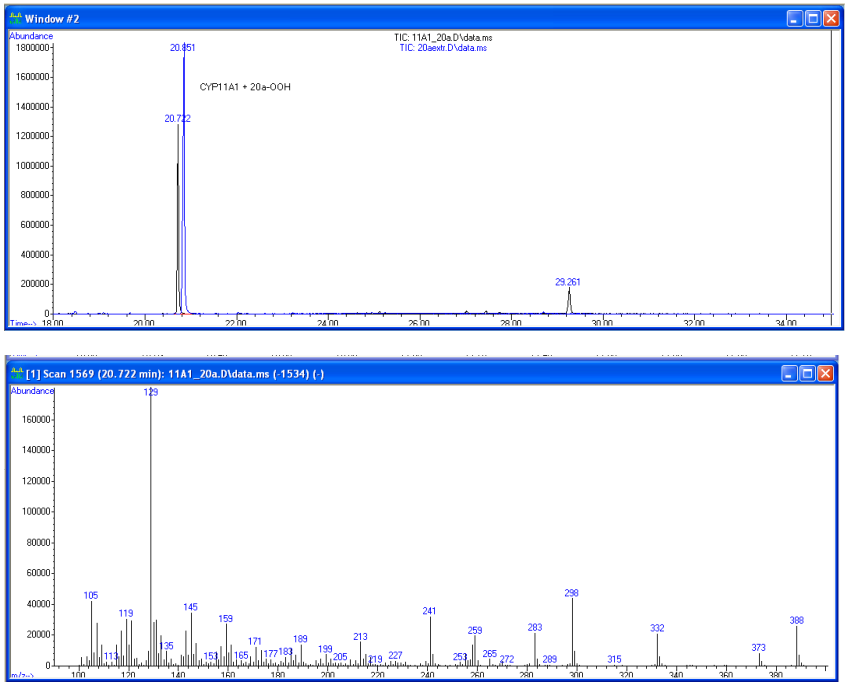


Figure S8. *Top panel*: GC of TMS derivative of metabolite at t_R = 29.26 min, identified as 20α,22R-dihydoxycholesterol (**7**). *Lower panel*: MS fragmentation pattern identical to that of authentic **7** (Figure S9): MS = *m/z* 389 (loss of C22-27 and 20-TMS), *m/z* 299 (loss of C22-27 and 22-OTMS and 3-TMS), *m/z* 173 (base peak, fragment C22-27 + 22-OTMS produced upon cleavage of the C20-22 bond).

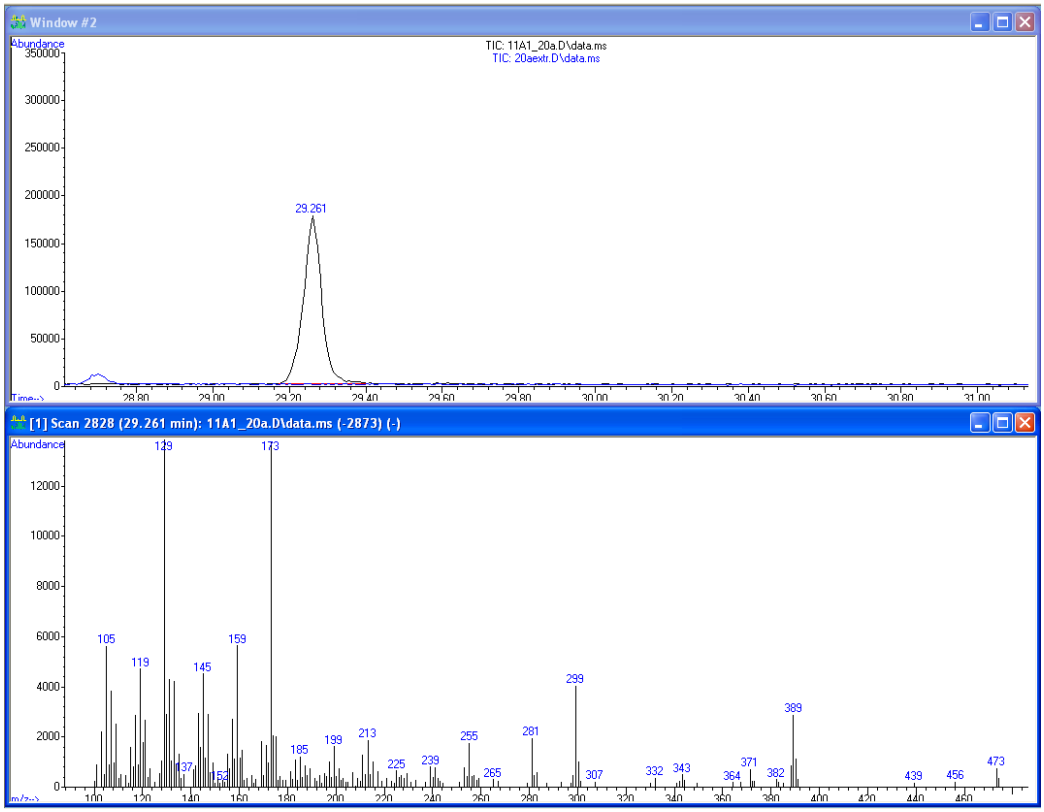
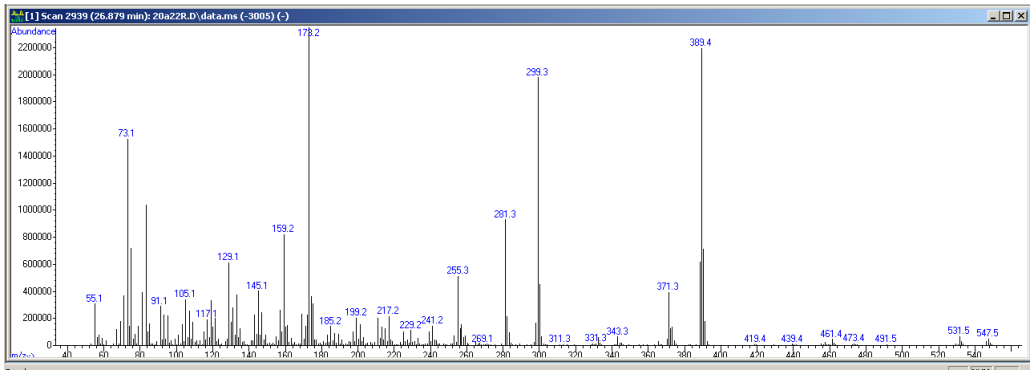
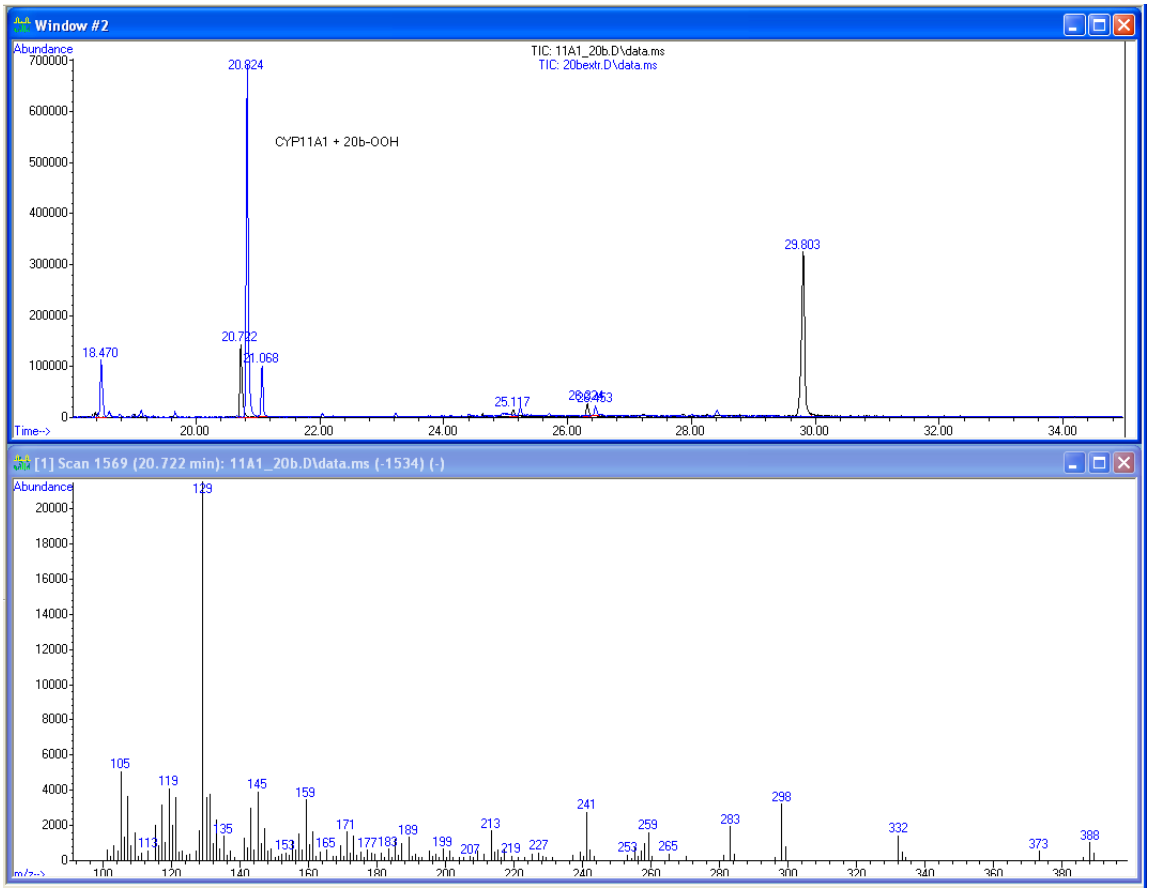


Figure S9. MS of authentic 20α,22R-dihydoxycholesterol (**7**).



*CYP11A1 and 20β-hydroperoxy-20-iso-cholesterol (**3**).* Extraction of the incubation mixture with $MeCl_2$ followed by TLC analysis showed that **3** was transformed to a single glycol product. The metabolite was identified as the 20β,21-dihydroxy-20-*iso*-cholesterol (**8**) (R_f = 0.2 rel. to cholesterol, purple colour response to acid/heat) (Scheme 1). TMS derivatization and comparison of GC-MS data with those of a synthetic reference sample showed identical GC mobilities and MS fragmentation pattern, confirming the assigned structures (Figure S9).

Figure S9. GC-MS analysis of CYP11A1 and 20β-hydroperoxy-20-iso-cholesterol (**3**) incubation mixture showing the TMS derivative of metabolite 20β,21-dihydroxy-20-*iso*-cholesterol (**8**) as single product at t_R = 29.80 min. Excess **3** decomposes to 3-TMS pregnenolone at t_R = 20.722 min with MS fragmentation pattern identical to authentic pregnenolone. *Top panel*: GC of TMS derivatives of CYP11A1 and **3** metabolites. Peak at t_R = 20.722 min is pregnenolone (decomposition product of excess **3**), and the peak at t_R = 29.803 min is identified as 20β,21-dihydroxy-20-*iso*-cholesterol (**8**). *Middle panel*: MS of peak at t_R = 20.722 min, identical to pregnenolone. *Lower panel*: MS of peak at t_R = 29.803 min, identical to authentic 20β,21-dihydroxy-20-*iso*-cholesterol (**8**) (Figure S10). Characteristic ions at , *m/z* 544 (M - 20-OTMS), 459 (M - C22-27 and 20-OTMS), 255, 129 (base peak).



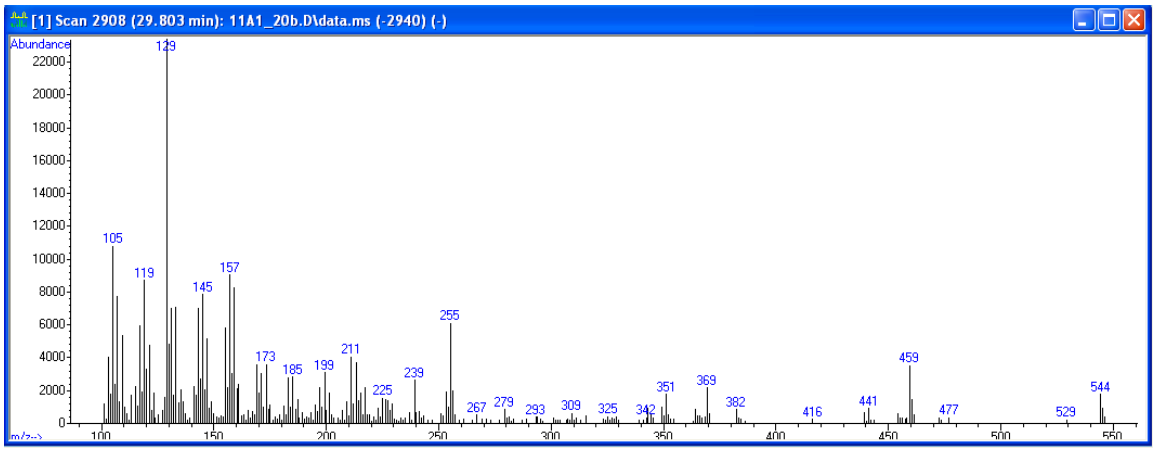
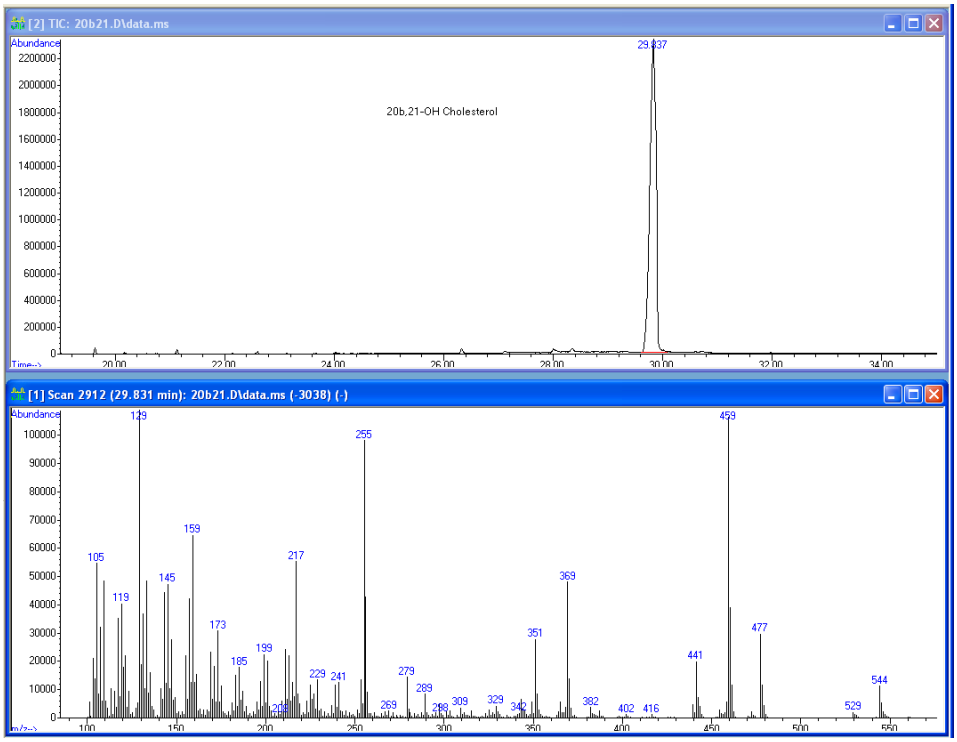


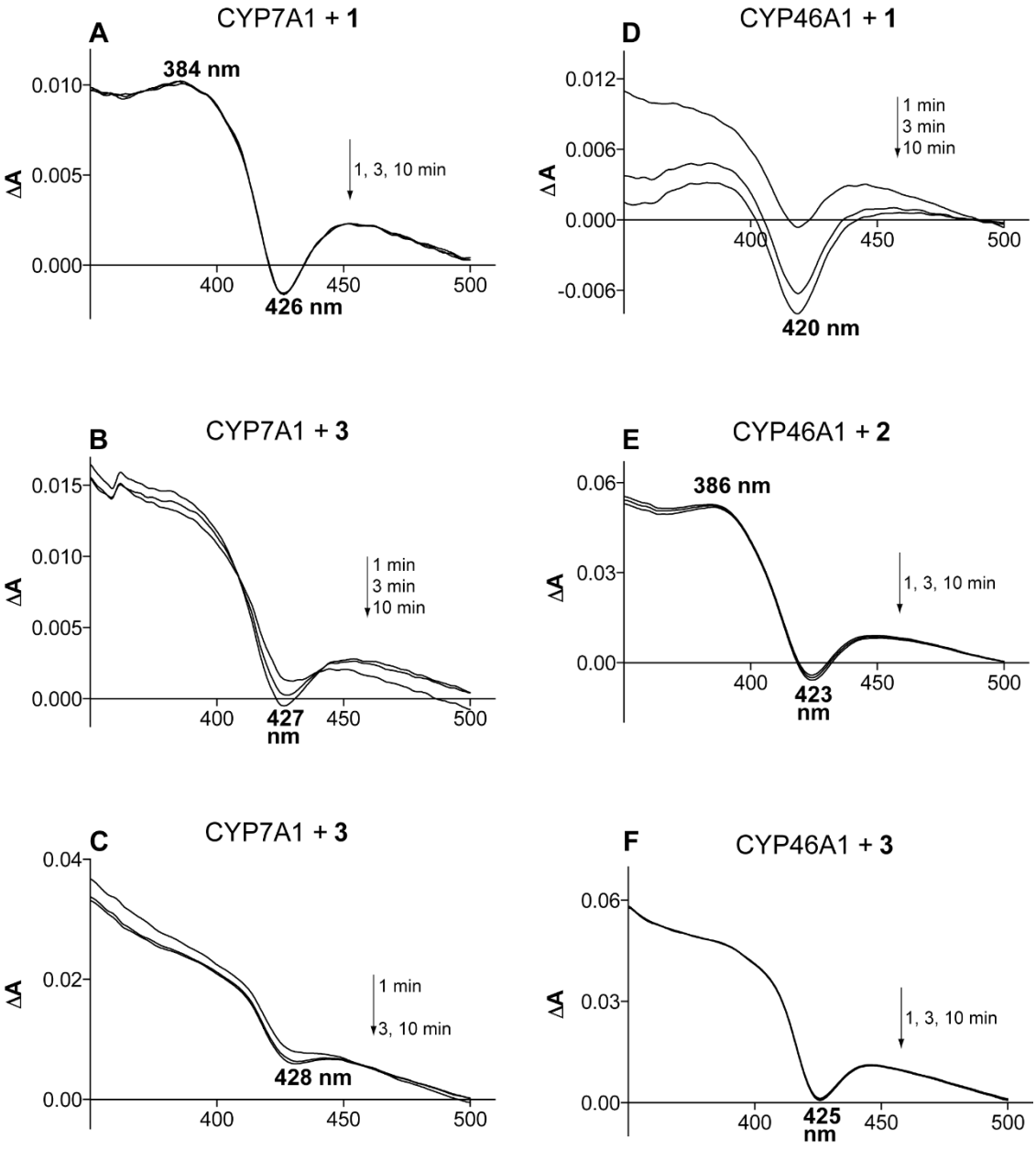
Figure S10. GC-MS of authentic 20β,21-dihydroxy-20-*iso*-cholesterol (**8**).



Spectral changes in CYP7A1 and CYP46A1 and product analysis

Addition of the 25-hydroperoxide **1** and 20ξ-hydroperoxides **2** and **3** to CYP7A1 introduced significant light scattering in the P450 difference spectra as indicated by a lack of well-defined peaks or troughs. Yet there was no visible protein precipitation in the cuvette, which could be a reason for increased solution light scattering. Light scattering was the smallest upon the addition of 25-hydroperoxide **1** and produced difference spectrum with a maximum at 384 nm and a minimum at 426 nm, parameters close to those of type 1 spectral response (maximum at 380-390 nm and minimum at 415-420 nm). Light scattering was also observed in the incubation of 20ξ-hydydroperoxides **2** and **3** with CYP46A1 but was not visible upon the addition of 25-hydroperoxide **1** (Figure S11). In all cases no major reaction products were observed by TLC and GC-MS analyses.

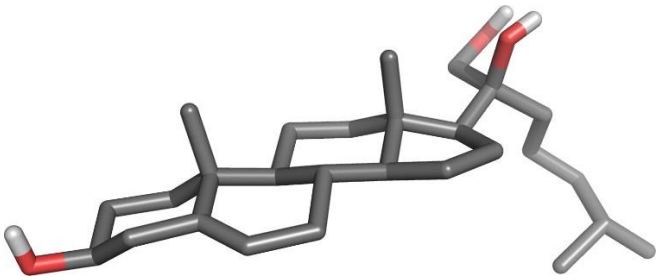
Figure S11. Difference spectra developed within 1-10 min after addition of 25-hydroperoxycholesterol (**1**), 20α-hydroperoxycholesterol (**2**) or 20β-hydroperoxy-20-*iso*-cholesterol (**3**) to CYP7A1 and CYP46A1. A) CYP7A1 and **1**, B) CYP7A1 and **2**, C) CYP7A1 and **3**, D) CYP46A1 and **1**, E) CYP46A1 and **2**, F) CYP46A1 and **3**.



*The 20α-hydroperoxycholesterol (**2**) and its 20β-hydroperoxy-20-iso-cholesterol (**3**)* were isolated from air-aged cholesterol [12a] and characterized upon borohydride reduction to the known 20β-hydroxy-20-*iso*-cholesterol and 20α-hydroxycholesterol, which were in all respects identical to the authentic 20-hydroxysterols [15d]. Incubation of CYP11A1 with the 20α-hydroperoxide **2** gave the known 20α,22R-dihydroxycholesterol (**7**) while the metabolite of the 20β-hydroperoxide **3** was identified as the 20β,21-dihydroxy-20-*iso*-cholesterol (**8**) by comparison of its chromatographis and mass spectral properties with those of a synthetic sample [15d].

*The epimeric 20α,21-dihydroxycholesterol and 20β,21-dihydroxy-20-iso-cholesterol (**8**)* were prepared from 21-hydroxypregnenolone and addition of the side chain via a Grignard reaction. The assigned configurations were previously established by converting the epimeric glycols to the 3,21-dibenzoates and transformation to the known 20α- and 20β-hydroxysterols [15a]. X-ray crystallography of the 20α,21-dihydroxycholesterol isomer confirmed the assigned configuration.

Figure S12



X-ray crystallography of 20α,21-dihydroxycholesterol