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Substrate analog studies of the ω-regiospecificity of *Mycobacterium tuberculosis* cholesterol metabolizing cytochrome P450 enzymes CYP124A1, CYP125A1 and CYP142A1

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ABSTRACT

We report the synthesis and evaluation of a series of cholesterol side-chain analogs as mechanistic probes of three important *Mycobacterium tuberculosis* cytochrome P450 enzymes that selectively oxidize the ω-position of the methyl-branched cholesterol side-chain. To probe the structural requirements for the thermodynamically disfavored ω-regiospecificity we compared the binding of these substrate analogs to each P450, determined the turnover rates, and characterized the enzymatic products. The results are discussed in the context of the structure-activity relationships of the enzymes and how their active sites enforce ω-oxidation.

1. Introduction

Mycobacterium tuberculosis (Mtb) is the pathogenic bacterium that causes pulmonary tubercular infection (TB). Deaths due to active TB infection, or arising from complications from HIV/AIDS in conjunction with TB, continue to claim millions of lives per year.[1] In addition, 10% of individuals harboring latent infections (approximately 2 billion people) experience activation to infectious TB each year.[1] The emergence of multi-drug resistant (MDR-TB) and extremely drug resistant (XDR-TB) Mtb strains has rendered the frontline antibiotics less effective and complicates efforts to halt the spread of TB infection, particularly in developing countries. New drugs and new drug targeting strategies are urgently needed to slow the spread of tuberculosis infection.

Like nearly all bacteria studied to date, *M. tuberculosis* is not able to synthesize steroids de novo.[2–4] Nevertheless, Mtb has evolved a remarkable ability to thrive and persist within the human macrophage, and cholesterol (**1**) is essential for its entry into the macrophage.[5] Bacterial survival in the macrophage relies on the uptake of lipids, including cholesterol (**1**), from the human host cells during infection and their subsequent breakdown to derive energy and carbon equivalents for building biomolecules such as lipids.[2,6,7] Cholesterol (**1**) is an important source of carbon and energy during both chronic and latent phases of infection.

The steps necessary to utilize cholesterol (**1**) can be divided into roughly four phases: (a) uptake of cholesterol (**1**) into the cell, (b) its oxidation to cholest-4-en-3-one (**1k**), (c) side-chain degradation, and (d) steroid ring degradation.[2] Cholesterol (**1**) (cholest-5-en-3β-ol) is transported into the cell by the MCE (mammalian cell entry) family of proteins,[8] and is then converted to cholest-4-en-3-one (**1k**) by a cholesterol oxidase.[9] An essential P450 activity is required to activate the side-chain for entry into β-oxidation, and finally, the steroid ring system is degraded. The structures of cholesterol (**1**) and cholest-4-en-3-one (**1k**) are shown in Fig. 1. Three Mtb P450 isoforms, CYP125A1, CYP142A1 and CYP124A1, catalyze steroid side-chain oxidation.[10–15] CYP125A1 is the primary enzyme responsible for this in vivo activity though

Abbreviations: CYP, cytochrome P450; BSTFA, *N,O*-bis(trimethylsilyl)trifluoro acetamide; DIBAL-H, diisobutylaluminum hydride; MβCD, methyl β-cyclodextrin; MCE, mammalian cell entry; MDR-TB, multi-drug resistant tuberculosis; Mtb, *Mycobacterium tuberculosis*; P450, cytochrome P450; PCC, pyridinium chlorochromate; TB, tuberculosis; TBS, *tert*-butyldimethylsilyl; XDR-TB, extremely drug resistant tuberculosis.

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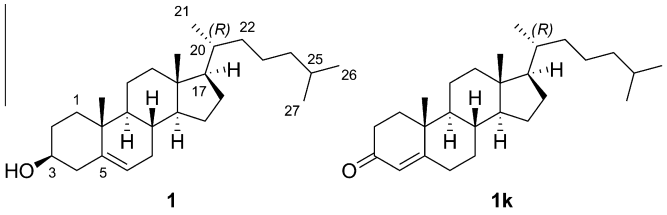


Figure 1. Cholesterol (**1**) and cholest-4-en-3-one (**1k**).

CYP142A1 provides functional redundancy in some Mtb strains.[2,11,16] In vivo, CYP142A1 but not CYP124A1 fully restores the growth phenotype for the △CYP125A1 strain.[9] The cholest-4-en-3-one (**1k**) side-chain is oxidized at the ω-position in three consecutive steps, first to the alcohol, then to the aldehyde and finally to the carboxylic acid.[2,10,11,17] The newly formed carboxylic acid undergoes transesterification with coenzyme-A (CoA) in an ATP-dependent step that primes the acyl-CoA chain for β-oxidation. Three cycles of β-oxidation produces one acetyl-CoA and two propionyl-CoA equivalents.

The critical function of these P450 enzymes for Mtb infection and survival makes them potential drug targets.[2,18] These particular bacterial P450 isoforms show low sequence identity with human cholesterol (**1**) side-chain oxidizing P450 enzymes. CYP125A1 is distributed widely in various actinomycete genomes and parallels in gene organization and functional assignment led to our initial understanding of the *M. tuberculosis* CYP125A1 isoform.[19] All three P450 enzymes catalyze the ω-oxidation of the steroid side-chain.[17] Although CYP124A1 and CYP125A1 exhibit high sequence identity and are closely related, CYP124A1 also oxidizes fatty acids and isoprenoids but CYP125A1 does not.[11,20] CYP142A1 is more distantly related to CYP125A1 than CYP124A1. Thermodynamic considerations suggest that the terminal portion of the cholesterol (**1**) side-chain would be most easily oxidized at the tertiary 25-position to give 25-hydroxycholesterol, a known potent modulator of cellular processes. However, this product is not detectably formed. Instead, only products resulting from the thermodynamically disfavored ω-oxidation of the primary methyl group are observed.[17]

We report here the synthesis and evaluation of a series of cholesterol (**1**) analogs bearing side-chain and ring modifications as substrates of CYP125A1, CYP142A1 and CYP124A1. The results provide a better understanding of the strategies used by each enzyme to oxidize its substrates with high regiospecificity. They also provide much-needed insight for the design of substrate-analogs as mechanism-based inhibitors of a critical energy-harvesting pathway in *M. tuberculosis.*

2. Results

2.1. Synthesis of cholesterol side-chain analogs 2–10

The structures of cholesterol (**1**), the chemically synthesized cholesterol side-chain analogs (**2**–**10**) and the other, commercially available steroids (**11**–**13**) used in this study are shown in Fig. 2. Our initial strategy for the synthesis of the desired analogs **2**–**10** began with the TBS ether of commercially available 5-pregnen-3β-ol-20-one (**14**).[21–23] Despite reports of similar steroids undergoing Wittig condensation,[24–28] all reaction conditions tested were unsuccessful. An approach involving vinyl Grignard addition to C-20 followed by oxidative rearrangement of the resulting tertiary alcohol was thus adopted (Supplementary data).[23,29] It was envisioned that aldehyde (20*E*)-**15** would provide easy access to our desired sterols **2**–**10** via Wittig condensation and subsequent side-chain functionalization.

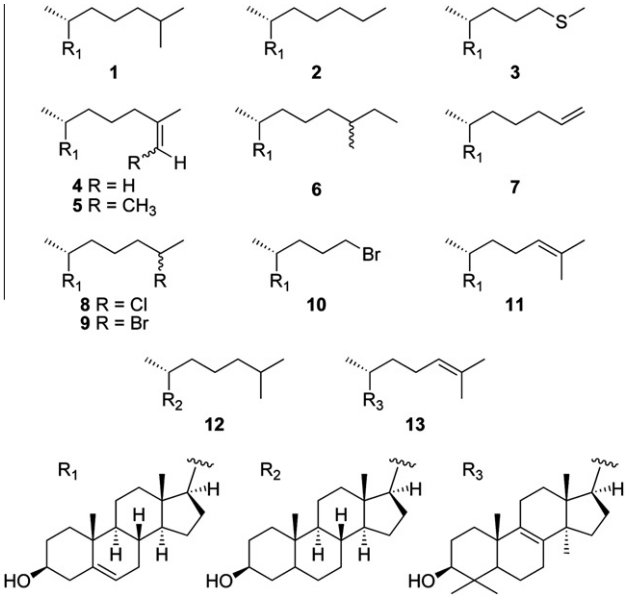
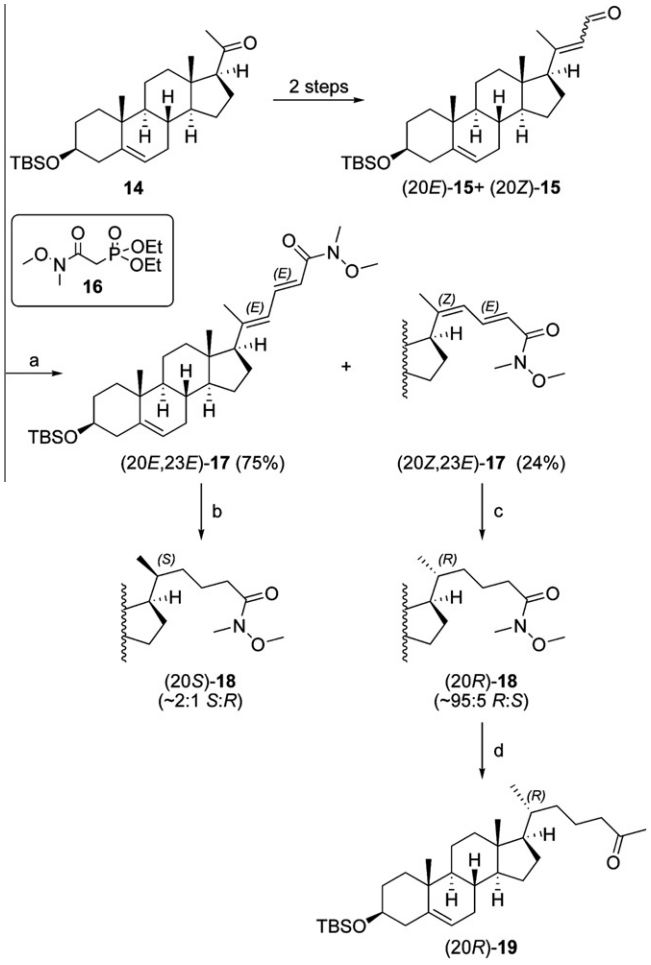


Figure 2. The sterols used in this study.

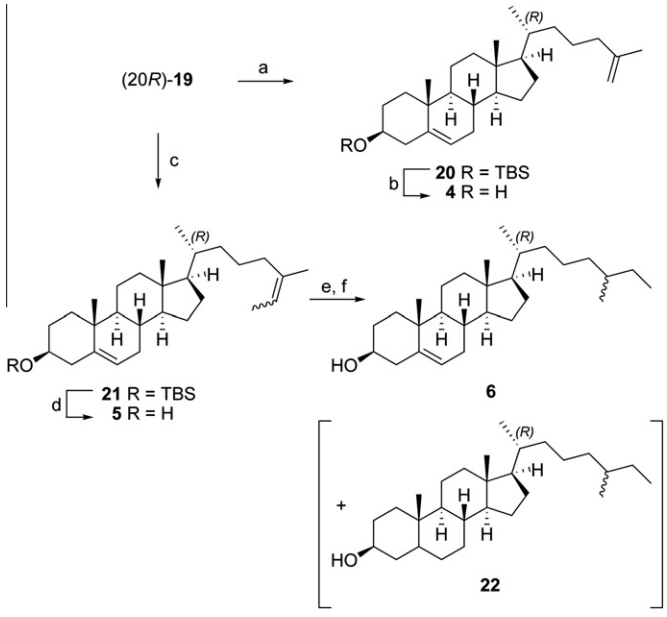


Scheme 1. Synthesis of ketone (20*R*)-**19** from TBS-protected 5-pregnen-3-ol-20-one (**14**) via a mixture of isomeric aldehydes (20*E*)-**15** and (20*Z*)-**15** (~3:1). Reagents and conditions: (a) Phosphonate ester **16**, NaH, THF, 0 °C, 99%, ~3:1 (20*E*,23*E*)-**17**: (20*Z*,23*E*)-**17**. (b) H_2, PtO_2, 1,4-dioxane/AcOH (50:1), 88%, ~2:1 (20*S*)-**18**:(20*R*)-**18**. (c) H_2, PtO_2, 1,4-dioxane/AcOH (50:1), 81%, ~95:5 (20*R*)-**18**:(20*S*)-**18**. (d) Methylmagnesium bromide, THF, −78 °C, 83%.

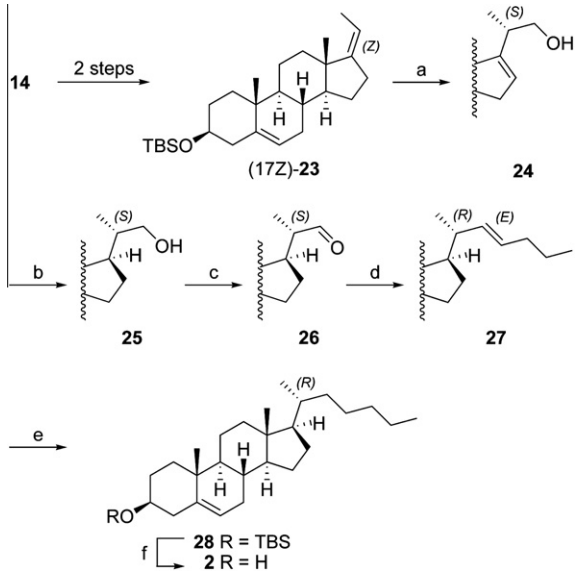
Horner-Wittig addition of diethyl 2-(methoxy(methyl)amino)-2-oxoethylphosphonate[30] (**16**) to a mixture of (20*E*)-**15** and (20*Z*)-**15** proceeded in near quantitative yield (Scheme 1), with exclusive formation of the 23(24)*E* alkene[30] affording only two chromatographically separable, isomeric α,β,γ,δ-unsaturated Weinreb amides, (20*E*,23*E*)-**17** and (20*Z*,23*E*)-**17**. Catalytic hydrogenation of various 20(22)*E* alkenes[26–28,31] and conjugated 20(22),23(24) dienes[32–34] generally, but not always,[35] occurs stereoselectively to provide the natural 20*R* stereochemistry of cholesterol (**1**). In this case, hydrogenation of both (20*E*,23*E*)-**17** and (20*Z*,23*E*)-**17** separately over PtO_2[28,36] proceeded in good yield (Scheme 1), with no observed reduction of the 5(6) double bond. Unfortunately, reduction of the major isomer (20*E*,23*E*)-**17** afforded an inseparable mixture of (20*S*)-**18** and (20*R*)-**18**, with the undesired 20*S* stereochemistry predominating (20*S*:*R* ~2:1). Hydrogenation of (20*Z*,23*E*)-**17** afforded the desired (20*R*)-**18** almost exclusively (20*S*:*R* ~95:5), with the stereochemistry at C-20 assigned based on comparison of the ^1H NMR spectra of **18** with data reported for similar compounds.[28]

Reaction of methylmagnesium bromide with (20*R*)-**18** afforded the methyl ketone (20*R*)-**19** (Scheme 1), and subsequent condensation with either methyl or ethyltriphenylphosphonium halides provided alkenes **20** (89% yield) and **21** (48%, 25*E*:*Z* ~1:1), respectively (Scheme 2). Acid-catalyzed hydrolysis of the silyl moiety of **20** and **21** afforded the desired sterols **4** and **5**, respectively, in excellent yields. Alkene **21** was also subjected to catalytic hydrogenation prior to TBS-deprotection, which afforded a diastereomeric mixture of (25*S*)- and (25*R*)-**5** contaminated with a small amount of saturated **22** (~20%). While this strategy (Schemes 1 and 2) provided the desired cholesterol analogs **4**–**6**, the production and challenging chromatographic separation of the diastereomeric mixtures obtained was unattractive. A more efficient, alternative route for the synthesis of sterols **2**, **3** and **7**–**10** was thus developed (**Scheme 3**).

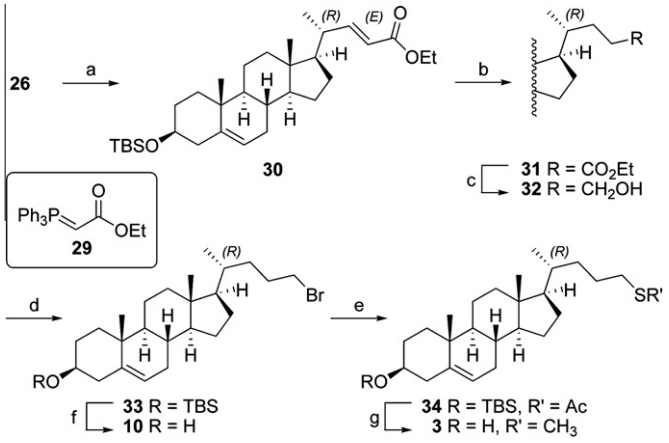
Again starting with **14**, (17*Z*)-**23** was available in two steps (Supplementary data)[22] and a $BF_3{\cdot}Et_2O$-catalyzed ene condensation



Scheme 2. Synthesis of sterols **4**–**6** from ketone (20*R*)-**19**. Reagents and conditions: (a) Methyltriphenylphosphonium iodide, *n*-BuLi, THF, −78 °C, 89%. (b) Dowex 50W-X8(H) resin, CH_2Cl_2/MeOH (1:1), quantitative. (c) Ethyltriphenylphosphonium bromide, *n*-BuLi, THF, −78 °C, 48%. (d) Dowex 50W-X8(H) resin, CH_2Cl_2/MeOH (1:1), 96%. (e) H_2, PtO_2, 1,4-dioxane/AcOH (50:1). (f) HCl, CH_2Cl_2/MeOH (1:1), quantitative over 2 steps, ~4:1 **6**:**22**.



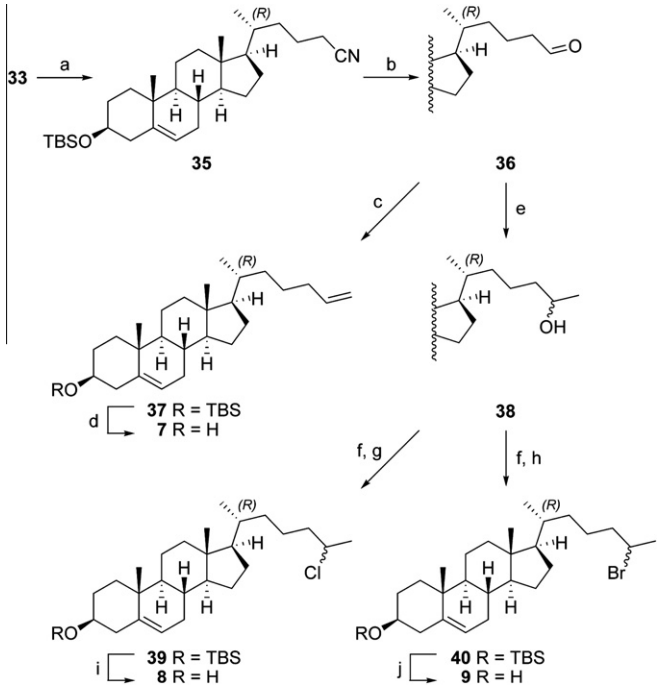
Scheme 3. Synthesis of 27-norcholesterol (**2**) from compound **14**, via aldehyde **26**. Reagents and conditions: (a) $(CHO)_n$, $BF_3{\cdot}Et_2O$, CH_2Cl_2, 74%. (b) H_2, PtO_2, EtOAc, 86%. (c) PCC, NaOAc, CH_2Cl_2, 87%. (d) *n*-Butyltriphenylphosphonium bromide, *n*-BuLi, THF, −78 °C, 87%. (e) H_2, PtO_2, 1,4-dioxane/AcOH (50:1), 96%. (f) Dowex 50W-X8(H) resin, CH_2Cl_2/MeOH (1:1), 97%.



Scheme 4. Synthesis of sterols **3** and **10** from aldehyde **26**. Reagents and conditions: (a) Stabilized ylide **29**, CH_2Cl_2, reflux, 93%. (b) H_2, PtO_2, 1,4-dioxane/AcOH (50:1), 97%. (c) $LiAlH_4$, Et_2O, 98%. (d) CBr_4, PPh_3, imidazole, CH_3CN/Et_2O (1:3), 95%. (e) KSAc, acetone, 95%. (f) Dowex 50W-X8(H) resin, CH_2Cl_2/MeOH (1:1), 92%. (g) (i) NaOMe, THF/MeOH (1:1), 0 °C. (ii) MeI. (iii) HCl (2:1), 68% over 3 steps.

with paraformaldehyde cleanly provided the homoallylic alcohol (20*S*)-**24** in 74% yield (Scheme 3).[37,38] Catalytic hydrogenation of (20*S*)-**24** (PtO_2, EtOAc) afforded alcohol **25** stereoselectively, with the angular C-18 methyl group directing hydrogen addition from the less hindered α-face of the molecule.[39] Oxidation (PCC, CH_2Cl_2) of **25** provided aldehyde **26** as a key intermediate, which permitted simple construction of **2**, **3**, and **7**–**10**.

Wittig condensation of **26** with *n*-butyltriphenylphosphonium bromide gave **27** (87% yield; Scheme 3) as predominantly one isomer, tentatively assigned as (22*E*)-**27** based on comparison of ^{13}C NMR spectral data with that reported for similar compounds, and in accord with literature precedent.[40–42] Catalytic hydrogenation of **27** (PtO_2, 1,4-dioxane/AcOH) then provided **28** and acid-catalyzed removal of the TBS group yielded 27-norcholesterol (**2**) in near-quantitative yield.



Scheme 5. Synthesis of sterols **7**–**9** from bromide **33**, via aldehyde **36**. Reagents and conditions: (a) KCN, DMF, 80 °C, 98%. (b) DIBAL-H, Et_2O, −78 °C, 76%. (c) Methyltriphenylphosphonium bromide, *n*-BuLi, THF, −78 °C, quantitative. (d) Dowex 50W-X8(H) resin, CH_2Cl_2/MeOH (1:1), 93%. (e) Methylmagnesium bromide, THF, −78 °C, 90%. (f) MsCl, Et_3N, CH_2Cl_2. (g) LiCl, THF, reflux, quantitative over 2 steps. (h) LiBr, THF, reflux, quantitative over 2 steps. (i) Dowex 50W-X8(H) resin, CH_2Cl_2/MeOH (1:1), 89%. (j) Dowex 50W-X8(H) resin, CH_2Cl_2/MeOH (1:1), 88%.

Wittig addition of stabilized ylide ethyl 2-(triphenylphosphoranylidene)acetate[43] (**29**) to aldehyde **26** gave the α,β-unsaturated ester (22*E*)-**30** in 93% yield (Scheme 4), with the coupling constants for H-22 (δ_H 6.81 ppm, dd) and H-23 (5.71 ppm, d) revealing the 22*E* stereochemistry (J = 15.6 Hz) of the major isomer (>85%). Catalytic hydrogenation of **30**, $LiAlH_4$-mediated reduction of ester **31**, and subsequent bromination of alcohol **32** afforded the primary bromide **33** in excellent yield. Bromide **33** was an important intermediate in the synthesis of sterols **3** and **7**–**9** (Schemes 4 and 5) and also yielded **10** via acid-catalyzed cleavage of the TBS ether (Scheme 4).

Reaction of bromide **33** with potassium thioacetate provided **34** (95% yield; Scheme 4) and sodium methoxide-mediated thioester hydrolysis, followed by alkylation of the thiolate generated in situ with methyl iodide, and TBS-deprotection afforded the desired 25-thia-27-norcholesterol (**3**) in 68% yield (over the three steps). Alternatively, reaction of **33** with potassium cyanide afforded nitrile **35** in 98% yield (Scheme 5), which then underwent diisobutylaluminum hydride (DIBAL-H)-mediated reduction to aldehyde **36** (76% yield). Subsequent Wittig condensation with methyltriphenylphosphonium bromide afforded the terminal alkene **37**, with cleavage of the TBS moiety providing sterol **7** (93% yield).

Addition of methylmagnesium bromide to aldehyde **36** afforded alcohol **38** in 90% yield (Scheme 5). Direct conversion of **38** to either **39** or **40** (PPh_3 and CCl_4 or CBr_4) was unsuccessful. Thus, **38** was converted to the corresponding mesylate, which was reacted without purification with LiCl or LiBr to afford chloride **39** and bromide **40**, respectively, in quantitative yields. Finally, acid-catalyzed removal of the TBS ether afforded the desired halogenated norcholesterols **8** and **9** in high yields.

2.2. Spectroscopic characterization of P450-substrate analog complexes

We probed the specific interaction of substrate analogs **2**–**13** with the active site heme of each cytochrome P450 enzyme using UV–vis spectrophotometry. Specific ligand binding in the P450 active site usually perturbs the spin-state of the heme iron from the low-spin, 6-coordinate water-bound form to the high-spin, 5-coordinate form (a Type-I spectrum).[44] A broad peak centered between 385–390 nm and a trough at 418–422 nm characterizes a Type-I difference binding spectrum.[45] A Type-Ia ligand produces a mirror image of the Type-I spectrum and indicates a shift of equilibrium from the high-spin, 5-coordinate form to the low-spin, 6-coordinate form of the heme iron. Type-II difference spectra, on the other hand, typically have a peak between 422–435 nm and an absorbance minimum at 390–405 nm that indicates replacement of the coordinated water molecule by a heteroatom of the ligand, often the lone pair of electrons on a nitrogen atom of an imidazole or triazole ring.

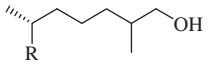
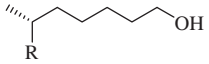
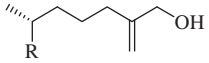
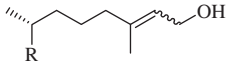
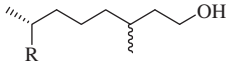
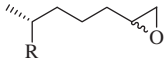
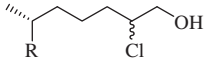
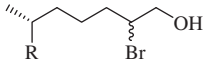
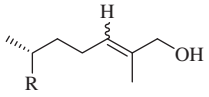
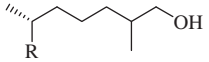
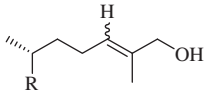
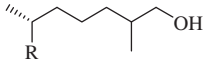
Table 1 lists the observed characteristic spin shifts for each cytochrome P450-substrate complex. Most of the compounds tested (**1–13** and **1k**), with some exceptions, bound in the active sites as Type-I ligands. Interestingly 25-thia-27-norcholesterol (**3**), cholesta-5,25-dienol (**4**), and 24-bromochol-5-enol (**10**) bind in the CYP125A1 active site as reverse-Type I ligands. A significant fraction of the resting ferric CYP125A1 enzyme is in the high-spin state,[10,14,15,46] thus, the observed Type-Ia binding spectra for compounds **3**, **4**, and **10** with CYP125A1 is consistent with stabilization of the low-spin, water-bound form of the enzyme upon ligand binding. The observation of Type-I/Type-Ia binding spectra for nearly all of the compounds indicated that the main structural determinant of binding resides in the steroid nucleus rather than the side chain and suggested that they may also be substrates for the P450 enzymes.

2.3. Substrate analog affinities for each P450 enzyme

Steroid stock solutions typically require solubilization by cyclodextrins, such as methyl β-cyclodextrin (MβCD), or other compatible detergents in order to measure binding and observe catalytic activity.[11] We attempted to determine the dissociation constants (K_d) across the panel of compounds with each P450 isoform by monitoring the concentration-dependence of the low-spin/high-spin equilibrium. In most cases we were unable to accurately determine K_d values because the signals exhibited complex titration behavior (Supplementary data). The reversible and erratic signals likely result from partitioning of the steroid between three phases: the enzyme active site, the medium, and bound with the detergent (MβCD).[47] In many cases the high-spin absorbance rapidly increased in amplitude in a concentration dependent manner but then quickly decayed over time. The absorbance at ~650 nm increased in amplitude and then reversed with time, paralleling the reversible Soret absorbance and providing further support for rapid and reversible binding in the P450 active site. We observed in several other cases that a substrate analog produced different binding spectra at different concentration ranges: for example, the titration of CYP124A1 with 26-methylcholesta-5,25(26)-dienol (**5**) showed a distinct Type-I binding spectrum at low concentrations (0.05–0.2 μM), but a reverse-Type-I binding spectrum at higher concentrations. Generally, each of the enzymes exhibited a large amplitude Type-I binding spectrum at sub-stoichiometric ligand:enzyme ratios.

We also examined the absolute spectra of the P450-steroid complexes and this data supports our conclusion that each compound binds as a Type I ligand within the active site of each enzyme. For several of the more stable P450-steroid complexes we

Table 1
The characteristic binding shifts detected for each substrate with each P450 enzyme, and the side-chain of the primary oxidations products

#	Compound name	Characteristic binding type			Side-chain of primary oxidation product
		CYP124A1	CYP125A1	CYP142A1	
1	Cholesterol	I	I	I	
2	27-Norcholesterol	I	I	I	
3	25-Thia-27-norcholesterol	I	IA	I	ND[a]
4	Cholesta-5,25-dienol	I	IA	I	
5	26-Methylcholesta-5,25(26)-dienol	I/IA[b]	I	I	
6	26-Methylcholesterol	I	I	I	
7	27-Norcholesta-5,25-dienol	I	I	I	
8	25-Chloro-27-norcholesterol	I	I	I	
9	25-Bromo-27-norcholesterol	I	I	I	
10	24-Bromochol-5-enol	I	IA	I	ND[a]
11	Desmosterol	I	I	I	
12	Coprostanol	I	I	I	
13	Lanosterol	I	I	I	
1k	Cholest-4-en-3-one	I	I	I	

[a] ND = not detected.
[b] Two distinct binding modes were obsderved: Type I at low substrate concentrations (0.05–0.20 M) and Type Ia at higher concentrations.

Table 2
Apparent turnover numbers (k_{cat}^{app}) for CYP124A1, CYP125A1 and CYP142A1 with cholesterol side-chain and ring analogs are listed in the first three columns. The second set of three columns lists the relative turnover numbers (k_{rel}), normalized to cholesterol (**1**)

#	Compound name	Apparent turnover number k_{cat}^{app} (min^{-1})			Relative turnover number (k_{rel})		
		CYP124A1	CYP125A1	CYP142A1	CYP124A1	CYP125A1	CYP142A1
1	Cholesterol	1.5 ± 0.2[a]	28 ± 3[a]	16.7 ± 1.3[a]	100	100	100
2	27-Norcholesterol	0.7 ± 0.1	5.1 ± 1.6	1.9 ± 0.4	48.6	18.0	11.7
3	25-Thia-27-norcholesterol	ND[b]	ND[b]	ND[b]	NA[c]	NA[c]	NA[c]
3	Cholesta-5,25-dienol	0.4 ± 0.03	10.7 ± 1.4	1.1 ± 0.2	26.2	37.0	6.7
5	26-Methylcholesta-5,25(26)-dienol	0.3 ± 0.05	8.1 ± 2.1	ND[b]	22.4	29.9	NA[c]
6	26-Methylcholesterol	ND[b]	19.0 ± 2.0	9.8 ± 1.6	NA[c]	68.1	58.7
7	27-Norcholesta-5,25-dienol	0.03 ± 0.005	2.2 ± 0.7	1.1 ± 0.3	1.9	8.0	6.7
8	25-Chloro-27-norcholesterol	0.45 ± 0.033	7.29 ± 1.5	8.97 ± 1.4	29.8	26.0	53.7
9	25-Bromo-27-norcholesterol	1.01 ± 0.5	18.22 ± 2.3	17.94 ± 1.7	67.3	65.1	107.4
10	24-Bromochol-5-enol	ND[b]	ND[b]	ND[b]	NA[c]	NA[c]	NA[c]
11	Desmosterol	1.37 ± 0.3	13.29 ± 2.2	6.92 ± 1.1	91.2	47.5	41.4
12	Coprostanol	1.03 ± 0.3	14.24 ± 2.1	7.2 ± 1.8	68.4	50.9	43.1
13	Lanosterol	ND[b]	10.37 ± 1.6	22.09 ± 3.1	NA[c]	37.0	132.3

[a] Values from Johnston et al. *J. Biol. Chem.* **2010**, *285*, 36352.
[b] ND = not detected.
[c] NA = not applicable.

were able to measure equilibrium dissociation constants (Supplementary data) and they were generally high-affinity interactions that were on the order of the binding parameters for the substrate, *i.e.* the K_d^{app} values of the analogs were similar to the apparent values of the natural substrates cholesterol (**1**) and cholest-4-en-3-one (**1k**).[9–13]

2.4. Conversion of sterols to their respective ketone forms

Previous studies have established that cholest-4-en-3-one (**1k**) is the better, and likely physiological, substrate for all three enzymes.[2,10,11,15] We tested the compounds with sterol (**1–13)** and ketosteroid (**2k–13k)** ring cores. The alcohols were converted enzymatically to the corresponding ketones using cholesterol oxidase and the conversion was confirmed by GC–MS analysis. Having demonstrated specific binding of the substrate analogs within the active sites of all three enzymes, we next tested whether each would be turned over by the different enzymes.

2.5. Monooxygenation products formed by Mtb CYP124A1, CYP125A1, and CYP142A1

Sterols **1**–**13** and ketones **1k–13k** were evaluated as substrates for the P450 enzymes. Nearly all of the compounds were substrates for the three enzymes, with the exception of 25-thia-27-norcholesterol (**3**), 24-bromochol-5-enol (**10**), and ketones **3k** and **10k**. Nevertheless, compounds **3** and **10** were both found to bind specifically in the active site of each enzyme (Table 1). Table 2 lists the apparent turnover number (k_{cat}^{app}) for compounds **1–13** (data for ketones **1k–13k** not shown). We were unable to detect enzymatic oxidation of **3** and **3k** beyond the non-enzymatic sulfoxide formation. Although, both CYP125A1 and CYP142A1 catalyzed the oxidation of lanosterol (**13**) and its ketone form **13k**, CYP124A1 did not, suggesting differences in the ability of the active sites to accommodate the extra methyl groups on the A and D rings of lanosterol (**13**) and **13k**. We were also unable to detect the oxidation of 26-methylcholesterol (**6**) and ketone **6k** by CYP124A1 or the oxidation of 26-methylcholesta-5,25(26)-dienol (**5**) and **5k** by CYP142A1.

2.6. Catalytic activity comparison

Table 2 lists the apparent turnover numbers (k_{cat}^{app}) as well as the relative turnover numbers (k_{rel}) for each compound **1–13** with each enzyme (the oxidation of ketones **1k–13k** parallaled these results, data not shown). As already noted, we were unable to detect oxidation of 25-thia-27-norcholesterol (**3**) and 24-bromochol-5-enol (**10**) by the three enzymes. The rest of the tested compounds were substrates. Substrates with methyl branching (**2**, **3**, **7**, **10**) exhibited lower turnover numbers than substrates without methyl branching at the 25-position (**4–6**, **8**, **9**), in contrast to what we found previously with CYP124A1.[20] Our data with all these substrate analogs parallels our earlier finding that CYP125A1 is generally the best catalyst. However, and interestingly, CYP142A1 exhibits the highest turnover numbers with lanosterol (**13**), suggesting that its active site is more tolerant of structural modifications to the A and D rings.

We observed exclusively the formation of ω-oxidation products, and found evidence for secondary oxidation at the same site to produce aldehyde and carboxylic acid products. Two examples of this are cholesta-5,25-dienol (**4**) and 26-methylcholesta-5,25(26)-dienol (**5**). We have previously found that the three enzymes oxidized the side-chain of cholesterol (**1**) and cholest-4-en-3-one (**1k**) in a similar manner to form alcohol, aldehyde and carboxylic acid products.[11] CYP124A1 also catalyzes such sequential oxidation at the same site in non-steroidal substrates such as geranylgeraniol.[20]

2.7. 27-Norcholesta-5,25-diene (7) is not a mechanism-based inhibitor

Like most of the steroids examined, the unsaturated **7** binds specifically and with high relative affinity in the active sites of the P450 enzymes. Catalytic assays demonstrated that compound **7** is a substrate for all three P450 isoforms, in each case forming the terminal epoxide. However, in a competition between epoxidation of the terminal double bond in analog **4** and allylic, terminal hydroxylation, only terminal hydroxylation was observed. A similar selectivity preference was observed in the oxidation of desmosterol (**11**) and lanosterol (**13**). We were not able to detect inhibition by these olefinic substrates beyond the competitive inhibition that occurs during the pre-incubation experimental setup, which is corrected for in the calculations.

3. Discussion

Cytochrome P450 enzymes oxidize a wide variety of substrates, often with very precise regio- and stereoselectivity.[44] Enzymatic oxidation of unactivated hydrocarbons is the most energetically demanding cytochrome P450-catalyzed reaction.[48] The primary intermediate in the catalytic cycle is the fleeting oxoferryl species ($Fe{=}O^{+\cdot}$, Compound **I**)[49] that abstracts a hydrogen atom from the substrate to produce a substrate-centered radical and Fe^{IV}–OH. The reaction products are formed by recombination of the substrate radical with the hydroxy radical equivalent. Based on thermodynamic principles the oxidized product distribution should reflect the ease with which C–H bonds undergo homolytic bond cleavage, that is, the primary C–H bonds are stronger than those at secondary and tertiary positions and are disfavored sites of reaction.[17,48] Many cytochrome P450 enzymes, however, have evolved to catalyze oxidation at these difficult primary positions, including the ω-oxidation of various steroid side-chains by CYP124A1, CYP125A1, and CYP142A1. It appears in these cases that ω-oxidation is facilitated by preventing more reactive positions of the substrate from encountering the reactive oxoferryl species.[17]

CYP124A1 binds and oxidizes a wider variety of substrates than CYP125A1 and CYP142A1, including fatty acids and alcohols, isoprenoid alcohols and diphosphates, and steroids.[11,20] However, CYP124A1 is a less efficient catalyst than CYP142A1 and CYP125, at least for cholesterol (**1**).[11] CYP124A1 catalyzes ω-oxidation of methyl branched substrates and a co-crystal structure of CYP124A1 with phytanic acid suggests a mechanism by which the active site enforces unfavorable regioselectivity.[11,20] A small hydrophobic cavity located near the heme group binds one of the terminal methyl groups such that the other is positioned near the heme iron for oxidation. Substrates lacking a terminal methyl branch bind in the active site with the end of the lipid chain bound in the small hydrophobic pocket above the heme, preventing oxidation of the methyl group. Oxidation occurs non-selectively at the ω-1 and ω-2 positions due to minor conformations in which the methyl terminus is not sequestered in the cavity.[11,17] CYP124A1 catalyzes the ω-oxidation of lipids of varying lengths, which suggests that the observed strict ω-regiospecificity is controlled by constraints near the heme, whereas the rest of the active site has some tolerance for structural variation.[11,20] Indeed, the co-crystal structure with phytanic acid revealed several large cavities (200 and 300 $Å^3$) along the length of the substrate, while the terminal portion is tightly bound by a hydrophobic complementary surface.

In contrast, CYP125A1 for which only the ω-hydroxylation of steroid side-chains has been observed, has a 'letterbox shaped' active site.[8,12,14] The crystal structures of sterols in complex with CYP125A1 suggest that many lipophilic interactions with the steroid ring nucleus clamp the substrate and position the side-chain at the correct distance for ω-oxidation.

The CYP142A1 crystal structure[12] reveals a hybrid of the CYP124A1 and CYP125A1 active sites, with a 'letterbox' portion of the active site, consistent with our observation that CYP142A1

does not oxidize fatty acids or methyl branched lipids. However, the CYP142A1 co-crystal structure uncovered a small pocket near the heme iron that may function similarly to that in CYP124A1 by binding one methyl group and thereby placing the other methyl terminus near the heme iron for oxidation.

3.1. Nor series of steroid side-chain analogs

Exclusive ω-oxidation of steroid side-chains is observed for the three P450 isoforms. All three P450 isoforms tolerate a variety of substrate structural modifications while retaining their precise ω-regiospecificity. Modifications to either the steroid rings (vide infra, Section 3.3) or the steroid side-chain were tolerated, as judged from the k_{rel} values (Table 2). It appears that additions to the side-chain are more readily tolerated than deletions: for example, the relative turnover numbers of 27-norcholesterol (**2**) and 27-norcholesta-5,25-dienol (**7**) show that they are poorer substrates than cholesterol (**1**). All three enzymes produced only products resulting from ω-oxidation with none of the ω-1 or ω-2 oxidation products observed previously in the oxidation of unbranched fatty acids by CYP124A1.[20] CYP124A1 thus appears to bear some resemblance to the 'letterbox' active site of CYP125A1,[10,14] in that tight binding of the sterol nucleus defines the distance the sterol side-chain can extend towards the heme. Indeed, strong support for this model is provided by the inability of these enzymes to oxidize 24-bromochol-5-enol (**10**) despite its specific binding in the active site. Based on previous studies, it is likely that the truncated side-chain is too short for the terminal atom to be accessible to the iron-based oxidizing species, presumably because the steroid nucleus prevents the side-chain from moving closer to the heme iron, whereas the phytanic acid CYP124A1 co-crystal structure[20] shows how lipids of varying chain length are able to bypass this steric block and reach the heme for ω-oxidation. Thus, the steroid nucleus appears to influence the binding interactions with the substrate of CYP124A1 much like the CYP125A1 'letterbox' active site and it appears that the binding interactions of the steroid rings also control substrate mobility in CYP124A1. Indeed, the turnover numbers of CYP124A1 with methyl branched fatty acids and isoprenoid alcohols (15-methylpalmitic acid, phytanic acid, farnesol, geranylgeraniol: k_{cat} = 7.6 ± 1.5, 9.9 ± 2.7, 15.5 ± 2.8, 9.6 ± 3.1 min^{-1}, respectively)[20] are similar to those for methyl branched steroidal substrates [cholesterol (**1**), cholest-4-en-3-one (**1k**): k_{cat} = 1.5 ± 0.2, 11.7 ± 1.3 min^{-1}].[11] The turnover numbers of the unbranched analogs [27-norcholesterol (**2**), cholesta-5,25-dienol (**4**), 25-thia-27-norcholesterol (**3**), and 24-bromochol-5-enol (**10**): k_{cat} = 0.7 ± 0.1, 0.03 ± 0.005 min^{-1}, ND, and ND, respectively) and the lack of detectable ω-1 and ω-2 oxidation products from these substrates supports this model. The turnover of unbranched palmitic acid (k_{cat} = 0.07 ± 0.003 min^{-1}) was greatly diminished relative to branched substrates, importantly with the thermodynamically favored ω/ω-1/ ω-2 product ratios.[20]

3.2. Methyl branching side-chain analogs

Structural modifications in the methyl branched side-chain were generally well tolerated among the enzymes, much better so than the removal of carbon atoms as observed in the *nor* series. Similar to earlier findings with CYP124A1, methyl branching here led to an increase in turnover number. However, the effects of the additional branch are less clear when comparing the turnover numbers of steroids. 27-Norcholesterol (**2**) shows slightly higher activity than most of the branched analogs, indicating that the effects of methyl branching in steroids are much more subtle than those in simple fatty acids with CYP124A1. In all of the steroid products we only detected ω-oxidation of the side-chain. Interestingly the terminal olefin of 27-norcholesta-5,25-dienol (**7**) was oxidized to the oxirane ring, as confirmed by the mass spectrum and relative retention times, as well as the appearance of the diol following acid treatment (Supplementary data). The epoxide was detected with each enzyme, a prerequisite in some cases for mechanism-based inhibition by olefins of cytochrome P450 enzymes (vide infra, Section 3.4). [44]

3.3. Steroid ring analogs

CYP125A1 and CYP142A1 accommodate modifications of the steroid ring structure, as indicated by their apparent k_{cat} values with lanosterol (**13**). Lanosterol (**13**) has three additional methyl groups, two at the 4′ position and one at the 14′ position at the juncture of rings C and D (Fig. 2). CYP125A1 and CYP142A1 show k_{rel} values for lanosterol (**13**) of 37 and 132, respectively, when compared to cholesterol (**1**) (Table 2). These high activities indicate that the two enzymes readily adjust to the presence of the additional methyl groups. As expected, the only product observed was the terminal alcohol resulting from ω-hydroxylation. In contrast, CYP124A1 did not detectably oxidize lanosterol (**13**) and thus, despite its ability to bind and oxidize linear hydrocarbon substrates, cannot cope with expansion of the sterol core. Coprostanol (**12**), which has a saturated A/B ring system, was turned over efficiently by all three enzymes, as shown by the k_{rel} values. Comparison of the turnover numbers of coprostanol (**12**), cholesterol (**1**) and cholest-4-en-3-one (**1k**) suggests that three features of the steroid A/B ring system contribute to the catalytic efficiency: (a) the level of oxidation at the 3 position, and both (b) the presence and (c) position of a double bond in the A/B ring system. The absolute k_{cat} values parallel our earlier finding that CYP125A1 is the best steroid oxidant, followed by CYP142A1 and finally CYP124A1 (Table 2). Under our incubation conditions, two of the substrates, cholesta-5,25-dienol (**4**) and 26-methylcholesta-5,25(26)-dienol (**5**), with unsaturated, branched side-chains, were found to be oxidized to the respective carboxylic acids (Table 1). However, it is likely that the other analogs would undergo similar secondary oxidation in extended incubations.

3.4. Mechanism-based inhibition: 27-norcholesta-5,25-dienol (7)

27-Norcholesta-5,25-dienol (**7**) undergoes olefin epoxidation. Olefin epoxidation is known to cause inactivation of some P450 enzymes,[44] either through heme or protein alkylation, but time-dependent inhibition was not observed in extended incubations of up to 3 hours. The epoxidation of compound **7** therefore does not appear to be associated with inactivation of any of the three enzymes.

3.5. Conclusions and outlook

In summary, we have investigated a series of synthetic cholesterol (**1**) side-chain analogs (**2–10**) and commercially available ring analogs (**11–13**) as substrates for CYP124A1, CYP125A1, and CYP142A1. Our results indicate that there are differences in the tolerance of the three enzyme active sites to modifications of the side-chain and steroid core, but, most importantly, the enzymes do tolerate substantial changes. Thus each P450 active site tolerates a range of structural modifications to the steroid side chain and ring nucleus. The minimal sidechain must extend to at least C25, but the sidechain can accept additional carbon units at various positions extending from C25 to the methyl branched terminus. Furthermore, CYP125A1 and CYP142A1 tolerate modifications of the A and B ring system better than CYP124A1. This information

provides a starting point for the development of specific inhibitors of these enzymes.

4. Materials and methods

4.1. General

All materials and reagents were of the highest commercial grade available. All chemicals, unless noted otherwise, were obtained from Sigma–Aldrich (St. Louis, MO or Castle Hill, NSW, Australia). DNA restriction enzymes were obtained from New England Biolabs. Cholesterol (**1**), cholest-4-en-3-one (**1k**), desmosterol (**11**), coprostanol (**12**), and lanosterol (**13**) were obtained from Sigma–Aldrich. All sterol stocks were solubilized in 9% (w/v) methyl-β-cyclodextrin (MβCD) at between 4–10 mM, and diluted further as necessary. 26-Hydroxycholesterol and 26-cholestanoic acid were obtained from Avanti Polar Lipids (Alabaster, AL) and Research Plus, Inc (Barnegat, NJ). The cloning, over-expression, purification and general characterization of CYP124A1, CYP125A1, and CYP142A1 have been reported previously.[10–15,20,46] Ferredoxin (*Spinacia oleracea*), ferredoxin-NADP$^+$ reductase (*S. oleracea*), catalase (bovine), glucose-6-phosphate dehydrogense, and cholesterol oxidase (*Streptomyces* sp.) were obtained from Sigma–Aldrich.

Anhydrous solvents were dried according to established procedures and were distilled under vacuum or N_2 immediately prior to use. THF and Et_2O were distilled off sodium-benzophenone ketyl. CH_2Cl_2, pyridine, and $BF_3{\cdot}Et_2O$ were dried over and distilled from CaH_2. Moisture or air sensitive experiments were conducted in oven-dried glassware under a N_2 or Ar atmosphere. Flash column chromatography was performed on Silica gel 60 (0.04–0.06 mM, 230–400 mesh, Scharlau or Merck). TLC was performed on Kieselgel 60 F254 plates (Merck, aluminum backed). Compounds were visualized by treatment with PMA (5% phosphomolybdic acid in EtOH), $KMnO_4$ (1.0 g $KMnO_4$, 5.0 g Na_2CO_3 and 0.5 g NaOH dissolved in 100 mL H_2O), or vanillin (6.0 g vanillin and 2.5 mL concentrated H_2SO_4 dissolved in 250 mL EtOH) dips. ^{1}H and ^{13}C NMR spectra were recorded on either a Varian Inova 400, Bruker AV400 or Bruker AV500 spectrometer using $CDCl_3$ (99.8 atom% D) or C_6D_6 (99.96 atom% D) as solvent. ^{1}H and ^{13}C signals are recorded in part per million (ppm) on the δ scale, with the residual solvent peaks ($CDCl_3$: δ_H 7.24 and δ_C 77.0; C_6D_6:δ_H 7.18 and δ_C 128.0) as internal references. GC–MS analyses (positive ion EI mode) were performed on a Shimadzu GC–MS QP5000 or QP2010-plus GC–MS, operating at 70 eV, connected to a Shimadzu GC-17A or GC-2010 gas chromatograph fitted with a DB-5 column (30 m, internal diameter 0.25 mM, J&W Scientific), or on an Agilent 6850 gas chromatograph fitted with an HP-5MS column (30 m, internal diameter 0.25 mM, Agilent), coupled to an Agilent 5973 network mass selective detector operating at 70 eV. Standard GC–MS programs: Shimadzu GC–MS, DB-5 column: split mode; column flow 1.0 mL min^{-1}; total flow 102.2 mL min^{-1}; injector 250 °C; detector 250 °C; oven 200 °C (1.0 min equilibration) held for 1.0 min, ramp 20 °C min^{-1} to 300 °C and held for 44.0 min (total program time 50.0 min). Agilent GC–MS, HP-5MS column: split mode; column flow 1.0 mL min^{-1}; total flow 10.0 mL min^{-1}; injector 250 °C; detector 230 °C; oven 70 °C (1.0 min equilibration) held for 1.0 min, ramp 10 °C min^{-1} to 300 °C and held for 20.0 min (total program time 44.0 min). Melting points were measured on a Buchi Dr Tottoli apparatus and are uncorrected. High resolution mass spectra were recorded on a Bruker micrOTOFQ spectrometer (positive ion ESI mode) or at the UC Berkeley Mass Spectrometry Facility using EI-MS. Elemental microanalyzes were performed by the Microanalytical Service, School of Chemistry and Molecular Biosciences, at the University of Queensland. Optical rotations were measured at the sodium D line (589 nm) at ambient temperature using a 1 mL quartz cell with a 10 cm path length, using a Jasco P-2000 polarimeter.

4.2. Sterol synthesis

4.2.1. (20*E*,23*E*)-*N*-Methoxy-*N*-methyl-24-(3β-(*tert*-butyldimethylsilyloxy)-chola-5,20(22),23-triene)carboxamide and [(20*E*,23*E*)-17] and (20*Z*,23*E*)-*N*-methoxy-*N*-methyl-24-(3β-(*tert*-butyldimethylsilyloxy)-chola-5,20(22),23-triene)carboxamide [(20*Z*,23*E*)-17]

NaH (60% dispersion in mineral oil, 190 mg, 4.75 mmol) was added portion-wise to a solution of diethyl 2-(methoxy(methyl)amino)-2-oxoethylphosphonate[30] (**16**) (1.030 g, 4.31 mmol) in anhydrous THF (15 mL) stirring under a N_2 atmosphere at 0 °C. The mixture was stirred for a further 10 min following the completion of effervescence of H_2 then a solution of isomeric aldehydes (20*E*)-**15** and (20*Z*)-**15** (~75%:25%, 912 mg, 2.00 mmol) in anhydrous THF (10 mL) was added dropwise. The reaction mixture was allowed to warm to room temperature and was stirred for 16 h. Saturated aqueous NH_4Cl solution (10 mL) was added to quench the reaction and the aqueous and organic layers were separated. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic extract was washed with brine (20 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, 100% CH_2Cl_2 to 2% EtOAc in CH_2Cl_2) to afford the major unsaturated Weinreb amide (20*E*,23*E*)-**17** (810 mg, 1.49 mmol, 75%) and the minor isomer (20*Z*,23*E*)-**17** (261 mg, 0.48 mmol, 24%; total 99% yield) as white solids.

4.2.1.1. (20*E*,23*E*)-*N*-Methoxy-*N*-methyl-24-(3β-(*tert*-butyldimethylsilyloxy)-chola-5,20(22),23-triene)carboxamide.

[(20*E*,23*E*)-**17**]: mp 175–177°C. $[\alpha]_D^{24}$ −7.2 (*c* 0.77, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.55 (s, 3H, H_3-18), 0.82–1.30 (m, 16H, incl. 0.86 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.97 [s, 3H, H_3-19]), 1.33–1.61 (m, 6H), 1.62–2.04 (m, 10H, incl. 1.90 (d, 3H, *J* = 0.8 Hz, H_3-21), 2.10–2.30 (m, 3H), 3.23 (s, 3H, RCON(CH_3)(OCH_3)), 3.46 (m, 1H, H-3), 3.69 (s, 3H, RCON(CH_3)(OCH_3)), 5.29 (m, 1H, H-6), 6.09 (d, 1H, *J* = 11.6 Hz, H-22), 6.36 (d, 1H, *J* = 14.9 Hz, H-24), 7.68 (dd, 1H, *J* = 14.9, 11.6 Hz, H-23). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 13.0 (C-18), 18.2 (ROSi(CH_3)$_2$C(CH_3)$_3$), 19.2, 19.4 (C-19), 21.0, 24.4, 24.8, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 31.8, 32.0, 32.2, 32.5, 36.6 (C-10), 37.4, 38.6, 42.8, 44.8 (C-13), 50.3, 56.5, 59.7, 61.7, 72.6 (C-3), 116.6, 120.9 (C-6), 124.2, 139.8, 141.6 (C-5), 149.0 (C-20), 168.0 (C-25). Anal. Calcd for $C_{33}H_{55}NO_3Si$: C, 73.14; H, 10.23; N, 2.58. Found: C, 73.16; H, 10.19; N, 2.48. HRMS (ESI): Calcd for $C_{33}H_{56}NO_3Si$ ([M+H]$^+$): 542.4029. Obsd: 542.4029.

4.2.1.2. (20*Z*,23*E*)-*N*-methoxy-*N*-methyl-24-(3β-(*tert*-butyldimethylsilyloxy)-chola-5,20(22),23-triene)carboxamide.

[(20*Z*,23*E*)-**17**]: Mp 161–163 °C. $[\alpha]_D^{23}$ −20.6 (*c* 0.28, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.04 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.63 (s, 3H, H_3-18), 0.82–1.08 (m, 16H, incl. 0.87 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.96 [s, 3H, H_3-19]), 1.10–1.81 (m, 12H), 1.82–2.05 (m, 5H, incl. 1.87 (s, 3H, H_3-21), 2.10–2.30 (m, 2H), 3.23 (s, 3H, RCON(CH_3)(OCH_3)), 3.47 (m, 1H, H-3), 3.68 (s, 3H, RCON(CH_3)(OCH_3)), 5.30 (m, 1H, H-6), 6.22 (d, 1H, *J* = 11.8 Hz, H-22), 6.32 (d, 1H, *J* = 14.8 Hz, H-24), 7.69 (dd, 1H, *J* = 14.8, 11.8 Hz, H-23). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 13.9 (C-18), 18.2 (ROSi(CH_3)$_2$C(CH_3)$_3$), 19.4 (C-19), 20.7, 24.1, 24.7, 24.9, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 31.97, 32.03 (2C), 32.4, 36.7 (C-10), 37.3, 37.6, 42.8, 46.4 (C-13), 50.2, 51.5, 55.8, 61.7, 72.5 (C-3), 116.2, 121.0 (C-6), 127.5, 140.2, 141.6 (C-5), 149.4 (C-20), 168.0 (C-25). Anal. Calcd for $C_{33}H_{55}NO_3Si$: C,

73.14; H, 10.23; N, 2.58. Found: C, 73.18; H, 10.28; N, 2.48. HRMS (ESI): Calcd for $C_{33}H_{56}NO_3Si$ ([M+H]$^+$): 542.4029. Obsd: 542.4033.

4.2.2. (20*S*)-*N*-Methoxy-*N*-methyl-24-(3β-(*tert*-butyldimethylsilyloxy)-chol-5-ene)carboxamide [(20*S*)-18]

A suspension of PtO_2 (40 mg, 0.18 mmol) in a mixture of 1,4-dioxane and AcOH (50:1, 20 mL) was degassed-purged twice with N_2 (g), then twice with H_2 (g). Triene (20*E*,22*E*)-**17** (800 mg, 1.48 mmol) was added as a solution in a mixture of 1,4-dioxane and AcOH (50:1, 5 mL) and the reaction mixture was stirred under a H_2 atmosphere at room temperature for 5 h. The mixture was then filtered through a pad of Celite™ and silica gel, which was washed thoroughly with CH_2Cl_2, and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, 15–20% EtOAc in petroleum spirit 40–60) to afford (20*S*)-**18** (264 mg, 0.48 mmol, 33%), and a mixture comprised of approximately 45% (20*S*)-**18** and 55% (20*R*)-**18** (444 mg, 0.81 mmol, 55%; total 88% yield, ~2:1 *S*:*R*) as white solids.

4.2.2.1. (20*S*)-*N*-Methoxy-*N*-methyl-24-(3β-(*tert*-butyldimethylsilyloxy)-chol-5-ene)carboxamide [(20*S*)-18]. Mp 82–84 °C. $[\alpha]_D^{24}$ −26.2 (*c* 0.14, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 6H, ROSi(C*H*$_3$)$_2$C(CH$_3$)$_3$), 0.65 (s, 3H, H$_3$-18), 0.78–1.32 (m, 24H, incl. 0.82 [d, 3H, *J* = 6.6 Hz, H$_3$-21], 0.87 [s, 9H, ROSi(CH$_3$)$_2$C(C*H*$_3$)$_3$], 0.97 [s, 3H, H$_3$-19]), 1.35–1.62 (m, 8H), 1.62–1.85 (m, 4H), 1.88–2.00 (m, 2H), 2.08–2.42 (m, 4H), 3.16 (s, 3H, RCON(C*H*$_3$)(OCH$_3$)), 3.45 (m, 1H, H-3), 3.66 (s, 3H, RCON(CH$_3$)(OC*H*$_3$)), 5.29 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(*C*H$_3$)$_2$C(CH$_3$)$_3$), 12.1 (C-18), 18.3 (ROSi(CH$_3$)$_2$*C*(CH$_3$)$_3$), 18.7, 19.4 (C-19), 21.1, 21.4, 24.2, 25.9 (3C, ROSi(CH$_3$)$_2$C(*C*H$_3$)$_3$), 28.0, 31.9 (2C), 32.1, 32.4, 35.0, 35.1, 36.6 (C-10), 37.4, 39.7, 42.4 (C-13), 42.8, 50.2, 55.5, 56.8, 61.2, 72.6 (C-3), 121.2 (C-6), 141.5 (C-5), 178.3 (C-25). GC–MS (EI): *m/z* (%) 545 (0.1, M$^+$), 530 (2, M$^+$ –CH$_3$), 490 (10), 489 (37), 488 (100, M$^+$ -*t*Bu), 470 (2), 458 (3), 253 (1), 207 (5), 159 (4), 158 (3), 147 (3), 133 (3), 131 (3), 130 (1), 119 (3), 116 (1), 115 (1), 105 (4), 103 (1), 95 (4), 88 (1), 81 (7), 75 (19), 60 (1), 57 (2), 55 (5). Anal. Calcd for $C_{33}H_{59}NO_3Si$: C, 72.60; H, 10.89; N, 2.57. Found: C, 72.92; H, 10.83; N, 2.53. HRMS (ESI): Calcd for $C_{33}H_{59}NNaO_3Si$ ([M+Na]$^+$): 568.4162. Obsd: 568.4143.

4.2.3. (20*R*)-*N*-Methoxy-*N*-methyl-24-(3β-(*tert*-butyldimethylsilyloxy)-chol-5-ene)carboxamide [(20*R*)-18].

A mixture of triene (20*Z*,22*E*)-**17** (240 mg, 0.44 mmol) and PtO_2 (12 mg, 0.05 mmol) in 1,4-dioxane and AcOH (50:1, 5 mL) was degassed-purged twice with N_2 (g), then twice with H_2 (g). The reaction mixture was stirred under a H_2 atmosphere at room temperature for 20 h and then was filtered through a pad of Celite™ and silica gel, which was washed thoroughly with CH_2Cl_2. The filtrate was concentrated in vacuo and the residue was purified by flash column chromatography (silica gel, 80% CH_2Cl_2 in petroleum spirit 40–60) to afford (20*R*)-**18** (195 mg, 0.36 mmol, 81%; ~95% 20*R*, 5% 20*S*) as a white solid. Mp 122–124 °C. $[\alpha]_D^{24}$ −13.4 (*c* 0.37, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 6H, ROSi(C*H*$_3$)$_2$C(CH$_3$)$_3$), 0.65 (s, 3H, H$_3$-18), 0.80–1.30 (m, 24H, incl. 0.82 [d, ~0.14H, *J* = 6.6 Hz, H$_3$-21 of 20*S*-isomer], 0.86 [s, 9H, ROSi(CH$_3$)$_2$C(C*H*$_3$)$_3$], 0.92 [d, ~2.86H, *J* = 6.6 Hz, H$_3$-21 of 20*R*-isomer], 0.97 [s, 3H, H$_3$-19]), 1.32–1.60 (m, 8H), 1.62–1.87 (m, 4H), 1.88–2.02 (m, 2H), 2.10–2.42 (m, 4H), 3.15 (s, 3H, RCON(C*H*$_3$)(OCH$_3$)), 3.45 (m, 1H, H-3), 3.65 (s, 3H, RCON(CH$_3$)(OC*H*$_3$)), 5.28 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(*C*H$_3$)$_2$C(CH$_3$)$_3$), 11.8 (C-18), 18.3 (ROSi(CH$_3$)$_2$*C*(CH$_3$)$_3$), 18.6, 19.4 (C-19), 21.0, 21.2, 24.3, 25.9 (3C, ROSi(CH$_3$)$_2$C(*C*H$_3$)$_3$), 28.2, 29.7, 31.90, 31.92, 32.1, 32.4, 35.6, 36.7, 36.6 (C-10), 37.4, 39.8, 42.3 (C-13), 42.8, 50.2, 55.8, 56.8, 61.2, 72.6 (C-3), 121.1 (C-6), 141.6 (C-5), 178.3 (C-25). GC–MS (EI): *m/z* (%) 545 (0.1, M$^+$), 530 (2, M$^+$ –CH$_3$), 490 (11), 489 (37), 488 (100, M$^+$ -*t*Bu), 470 (2), 458 (4), 253 (1), 207 (5), 159 (4), 158 (3), 147 (3), 133 (3), 131 (3), 130 (1), 119 (3), 116 (1), 115 (1), 105 (4), 103 (1), 95 (4), 88 (1), 81 (7), 75 (19), 60 (1), 57 (2), 55 (5). Anal. Calcd for $C_{33}H_{59}NO_3Si$: C, 72.60; H, 10.89; N, 2.57. Found: C, 72.67; H, 10.78; N, 2.55. HRMS (ESI): Calcd for $C_{33}H_{59}NNaO_3Si$ ([M+Na]$^+$): 568.4162. Obsd: 568.4147.

4.2.4. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-25-oxo-27-norcholest-5-ene [(20*R*)-19]

Methylmagnesium bromide (3.0 M in Et_2O, 0.4 mL, 0.60 mmol) was added dropwise to a solution of Weinreb amide (20*R*)-**18** (180 mg, 0.33 mmol) in anhydrous THF (4 mL) stirring under a N_2 atmosphere at −78 °C. The reaction mixture was stirred at −78 °C for 1 h, then allowed to warm to room temperature and stirred for a further 24 h. Saturated aqueous NH_4Cl solution (10 mL) was added to quench the reaction and the mixture was extracted with Et_2O (20 mL) and CH_2Cl_2 (3 × 20 mL). The combined organic extract was washed with brine (20 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, 50–60% CH_2Cl_2 in petroleum spirit 40–60) to afford methyl ketone (20*R*)-**19** (137 mg, 0.27 mmol, 83%; ~95% 20R, 5% 20*S*) as a white solid. Mp 135–137 °C, lit.[50] mp 120–122 °C. $[\alpha]_D^{24}$ −12.9 (*c* 0.38, $CHCl_3$). Synthesis of (20*R*)-**19** via a different synthetic route has been reported,[50,51] and the ^{1}H and ^{13}C NMR spectral data obtained here are consistent with the limited data available in the literature.[50] ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 6H, ROSi(C*H*$_3$)$_2$C(CH$_3$)$_3$), 0.65 (s, 3H, H$_3$-18), 0.80–1.27 (m, 24H, incl. 0.81 [d, ~0.16H, *J* = 6.6 Hz, H$_3$-21 of 20*S*-isomer], 0.87 [s, 9H, ROSi(CH$_3$)$_2$C(C*H*$_3$)$_3$], 0.91 [d, ~2.84H, *J* = 6.6 Hz, H$_3$-21 of 20*R*-isomer], 0.97 [s, 3H, H$_3$-19]), 1.27–1.85 (m, 12H), 1.88–2.02 (m, 2H), 2.08–2.18 (m, 4H, incl. 2.11 [s, 3H, H$_3$-26]), 2.20–2.44 (m, 3H), 3.46 (m, 1H, H-3), 5.29 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(*C*H$_3$)$_2$C(CH$_3$)$_3$), 11.8 (C-18), 18.3 (ROSi(CH$_3$)$_2$*C*(CH$_3$)$_3$), 18.6, 19.4 (C-19), 20.4, 21.0, 24.3, 25.9 (3C, ROSi(CH$_3$)$_2$C(*C*H$_3$)$_3$), 28.2, 29.9, 31.90, 31.92, 32.1, 35.4, 35.6, 36.6 (C-10), 37.4, 39.7, 42.3 (C-13), 42.8, 44.3, 50.2, 55.8, 56.8, 72.6 (C-3), 121.1 (C-6), 141.6 (C-5), 209.4 (C-25). GC–MS (EI): *m/z* (%) 500 (0.3, M$^+$), 485 (3, M$^+$ –CH$_3$), 444 (34), 443 (94, M$^+$ -*t*Bu and/or M$^+$ -C$_3$H$_5$O), 429 (1), 425 (1), 415 (1), 387 (1), 385 (1 M$^+$ -TBS), 369 (1, M$^+$ -OTBS), 367 (3), 351 (21), 341 (1), 329 (2), 309 (6), 295 (3), 253 (11), 241 (8), 227 (6), 213 (11), 199 (9), 185 (10), 173 (13), 161 (18), 159 (30), 145 (48), 133 (20), 131 (14), 119 (24), 115 (7), 113 (9), 105 (25), 95 (40), 85 (4), 81 (37), 75 (100), 71 (6), 67 (16), 58 (9), 57 (8), 55 (22), 43 (40). Anal. Calcd for $C_{32}H_{56}O_2Si$: C, 76.73; H, 11.27. Found: C, 76.58; H, 11.28. HRMS (ESI): Calcd for $C_{32}H_{56}NaO_2Si$ ([M+Na]$^+$): 523.3947. Obsd: 523.3947.

4.2.5. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)cholesta-5,25-diene (20)

A solution of *n*-BuLi (1.1 M in hexanes, 0.27 mL, 297 μMol) was added dropwise to a solution of methyltriphenylphosphonium iodide (180 mg, 450 μMol) in anhydrous THF (3 mL) stirring under a N_2 atmosphere at −78 °C. The resulting orange solution was stirred at −78 °C for 40 min, then allowed to warm to room temperature and stirred for a further 10 min. The reaction mixture was recooled to −78 °C and a solution of ketone (20*R*)-**19** (34 mg, 68 μMol) in anhydrous THF (1 mL) was added dropwise. The orange reaction mixture was allowed to warm to room temperature and stirred for a further 20 h. Saturated aqueous NH_4Cl solution (10 mL) was added to quench the reaction and the mixture was extracted with Et_2O (20 mL) and CH_2Cl_2 (3 × 20 mL). The combined organic extract was washed with brine (20 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, 25% CH_2Cl_2 in petroleum spirit 40–60) to afford diene **20** (30 mg,

60 μMol, 89%; ~95% 20*R*, 5% 20*S*) as a white solid. Mp 125–127 °C. $[\alpha]_D^{23}$ −15.6 (*c* 0.09, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.04 (s, 6H, $ROSi(CH_3)_2C(CH_3)_3$), 0.66 (s, 3H, H_3-18), 0.80–1.61 (m, 33H, incl. 0.81 [d, ~0.15H, *J* = 6.6 Hz, H_3-21 of 20*S*-isomer], 0.87 [s, 9H, $ROSi(CH_3)_2C(CH_3)_3$], 0.91 [d, ~2.85H, *J* = 6.6 Hz, H_3-21 of 20*R*-isomer], 0.97 [s, 3H, H_3-19]), 1.65–1.74 (m, 4H, incl. 1.69 [s, 3H, H_3-27]), 1.75–1.85 (m, 2H), 1.87–2.03 (m, 4H), 2.10–2.18 (m, 1H), 2.20–2.30 (m, 1H), 3.46 (m, 1H, H-3), 4.62–4.68 (m, 2H, H_2-26), 5.29 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$) δ −4.6 (2C, $ROSi(CH_3)_2C(CH_3)_3$), 11.8 (C-18), 18.3 ($ROSi(CH_3)_2C(CH_3)_3$), 18.7 (C-21), 19.4 (C-19), 21.1, 22.4 (C-27), 24.1, 24.3, 25.9 (3C, $ROSi(CH_3)_2C(CH_3)_3$), 28.2, 31.91, 31.94, 32.1, 35.6, 35.7, 36.6 (C-10), 37.4, 38.3, 42.3 (C-13), 42.8, 50.2, 56.1, 56.8, 72.6 (C-3), 109.5 (C-26), 121.2 (C-6), 141.6 (C-5), 146.4 (C-25). GC–MS (EI): *m/z* (%) 498 (0.3, M^+), 483 (2, $M^+ - CH_3$), 443 (11, $M^{+\cdot} - C_4H_7$), 442 (36), 441 (100, M^+ -*t*Bu), 429 (1), 423 (3), 415 (1), 387 (1), 385 (2), 371 (1), 367 (2, M^+ -OTBS), 365 (11), 341 (1), 329 (2), 281 (2), 255 (4), 245 (3), 235 (4), 207 (4), 159 (10), 147 (8), 133 (6), 131 (5), 121 (7), 115 (3), 111 (9), 109 (12), 95 (16), 83 (3), 81 (16), 75 (77), 69 (15), 57 (4), 55 (13), 41 (8). Anal. Calcd for $C_{33}H_{58}OSi$: C, 79.45; H, 11.72. Found: C, 79.13; H, 11.71.

4.2.6. (20*R*)-Cholesta-5,25-dien-3β-ol (4)

Dowex 50W-X8(H) standard grade resin (cat) was added to a solution of TBS ether **20** (22 mg, 44.1 μMol) in a mixture of CH_2Cl_2 (1 mL) and MeOH (1 mL). The mixture was stirred at room temperature for 66 h, and then the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, 5–17% EtOAc in petroleum spirit 40–60) to afford sterol **4** (17 mg, 44.1 μMol, quantitative) as a white solid. mp 118–120 °C, lit.[52] mp 120.5–121.5 °C. $[\alpha]_D^{24}$ −17.4 (*c* 0.09, $CHCl_3$), lit.[52] $[\alpha]_D^{25}$ −40.2 (*c* unspecified, $CHCl_3$). Synthesis of **4** has reportedly been accomplished via different synthetic routes,[52,53] however no spectral data has been reported previously. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.66 (s, 3H, H_3-18), 0.79–1.66 (m, 26H, incl. 0.91 [d, 3H, *J* = 6.5 Hz, H_3-21], 0.99 [s, 3H, H_3-19]), 1.69 (s, 3H, H_3-27), 1.74–1.88 (m, 3H), 1.88–2.07 (m, 3H), 2.25 (m, 2H), 3.50 (m, 1H, H-3), 4.65 (dd, 2H, *J* = 8.9, 0.8 Hz, H_2-26), 5.33 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.8 (C-18), 18.7 (C-21), 19.4 (C-19), 21.1, 22.4 (C-27), 24.1, 24.3, 28.2, 31.7, 31.9 (2C), 35.6, 35.7 (C-20), 36.5 (C-10), 37.2, 38.3, 39.8, 42.3, 42.3 (C-13), 50.1, 56.1, 56.8, 71.8 (C-3), 109.5 (C-26), 121.7 (C-6), 140.8 (C-5), 146.4 (C-25). GC–MS (EI): *m/z* (%) Free alcohol: 384 (64, M^+), 369 (16, $M^+ - CH_3$), 367 (5, $M^{+\cdot}$-OH), 366 (15, $M^{+\cdot}$-H_2O), 351 (24), 327 (5), 299 (30), 271 (46), 255 (21), 243 (10), 229 (16), 213 (34), 199 (20), 187 (14), 173 (20), 159 (41), 145 (52), 131 (35), 119 (43), 111 (29), 105 (77), 95 (69), 83 (16), 81 (78), 69 (63), 55 (100), 41 (54). TMS ether: 456 (66, M^+), 441 (13, $M^+ - CH_3$), 367 (20, $M^{+\cdot}$-OTMS), 366 (67), 351 (33), 343 (8), 327 (44), 310 (2), 299 (2), 281 (4), 271 (3), 255 (9), 245 (10), 227 (3), 213 (10), 199 (5), 187 (4), 173 (13), 159 (17), 145 (24), 129 (93), 121 (26), 111 (14), 105 (28), 95 (32), 91 (30), 89 (1), 83 (7), 81 (31), 75 (68), 73 (100, TMS^+), 69 (63), 55 (60), 41 (36). HRMS (ESI): Calcd for $C_{27}H_{44}NaO$ ($[M+Na]^+$): 407.3290. Obsd: 407.3275. HRMS (EI): TMS ether: Calcd for $C_{30}H_{52}OSi$ (M^+): 456.3787. Obsd: 456.3792.

4.2.7. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-26-methylcholesta-5,25(26)-diene (21)

A solution of *n*-BuLi (0.9 M in hexanes, 0.44 mL, 396 μMol) was added dropwise to a solution of ethyltriphenylphosphonium bromide (99%, 368 mg, 981 μMol) in anhydrous THF (4 mL) stirring under a N_2 atmosphere at −78 °C. The resulting orange solution was stirred at −78 °C for 30 min, then allowed to warm to room temperature and stirred for a further 30 min. The reaction mixture was re-cooled to −78 °C and a solution of ketone (20*R*)-**19** (41 mg, 82 μMol) in anhydrous THF (1 mL) was added dropwise. The orange reaction mixture was allowed to warm to room temperature and stirred for a further 40 h. The solvent was then evaporated and the residue was purified by flash column chromatography (silica gel, 100% petroleum spirit 40–60 to 9% EtOAc in petroleum spirit 40–60) to afford diene **21** (20 mg, 39 μMol, 48%; ~50% 25*E*, 50% 25*Z*) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of *E* and *Z* isomers): δ 0.04 (s, 6H, $ROSi(CH_3)_2C(CH_3)_3$), 0.65 & 0.66 (2 overlapping s, 3H, H_3-18), 0.78–2.06 (m, 46H, incl. 0.87 [s, 9H, $ROSi(CH_3)_2C(CH_3)_3$], 0.98 [s, 3H, H_3-19]), 2.15 (m, 1H), 2.25 (m, 1H), 3.46 (m, 1H, H-3), 5.17 (m, 1H, H-26), 5.30 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$) (mixture of *E* and *Z* isomers): δ −4.6 (2C, $ROSi(CH_3)_2C(CH_3)_3$), 11.8 (C-18), 13.2, 13.3, 15.6, 18.3 ($ROSi(CH_3)_2C(CH_3)_3$), 18.69, 18.73, 19.4 (C-19), 21.1, 23.4, 24.2, 24.3, 24.4, 25.9 (3C, $ROSi(CH_3)_2C(CH_3)_3$), 28.2, 31.8, 31.91, 31.94, 32.1, 35.63 (C-20), 35.64, 35.7 (C-20), 35.8, 36.6 (C-10), 37.4, 39.8, 40.1, 42.3 (C-13), 42.8, 50.2, 56.07, 56.10, 56.8, 72.6 (C-3), 117.9 (C-26), 118.6 (C-26), 121.2 (C-6), 136.2 (C-25), 136.4 (C-25), 141.6 (C-5). GC–MS (EI) *m/z* (%) *More volatile isomer:* 512 (0.2, M^+), 497 (1, $M^+ - CH_3$), 457 (7, $M^{+\cdot} - C_4H_7$), 456 (25), 455 (64, M^+ -*t*Bu), 437 (2), 381 (1, M^+ -OTBS), 379 (9), 359 (1), 331 (1), 309 (1), 281 (1), 255 (2), 235 (3), 207 (5), 159 (10), 131 (5), 125 (4), 115 (3), 105 (12), 97 (4), 95 (19), 83 (10), 81 (20), 75 (100), 69 (26), 57 (9), 55 (25), 41 (20). *Less volatile isomer:* 512 (0.1, M^+), 497 (1, $M^+ - CH_3$), 457 (7, $M^{+\cdot} - C_4H_7$), 456 (24), 455 (62, M^+ -*t*Bu), 437 (2), 381 (1, M^+ -OTBS), 379 (8), 359 (1), 331 (2), 309 (1), 281 (2), 255 (3), 235 (3), 207 (6), 159 (10), 131 (5), 125 (4), 115 (3), 105 (12), 97 (5), 95 (20), 83 (11), 81 (19), 75 (100), 69 (27), 57 (9), 55 (25), 41 (20). HRMS (ESI): Calcd for $C_{34}H_{60}NaOSi$ ($[M+Na]^+$): 535.4311. Obsd: 535.4294.

4.2.8. (20*R*)-26-Methylcholesta-5,25(26)-dien-3β-ol (5)

Dowex 50W-X8(H) standard grade resin (cat) was added to a solution of TBS ether **21** (8 mg, 15.6 μMol) in a mixture of CH_2Cl_2 (1 mL) and MeOH (1 mL). The mixture was stirred at room temperature for 44 h, and then the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, 5–9% EtOAc in petroleum spirit 40–60) to afford sterol **5** (6 mg, 15.1 μMol, 96%; ~50% 25*E*, 50% 25*Z*) as a white solid. $[\alpha]_D^{24}$ −13.2 (*c* 0.10, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of *E* and *Z* isomers): δ 0.66 (2 overlapping s, 3H, H_3-18), 0.78–1.70 (m, 32H, incl. 0.89 & 0.91 [2 overlapping d, 3H, *J* = 2.9 Hz, H_3-21], 0.99 [s, 3H, H_3-19]), 1.74–2.05 (m, 6H), 2.24 (m, 2H), 3.50 (m, 1H, H-3), 5.17 (m, 1H, H-26), 5.33 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$) (mixture of *E* and *Z* isomers): δ 11.8 (C-18), 13.2, 13.3, 15.6, 18.69, 18.73, 19.4 (C-19), 21.1, 23.4, 24.2, 24.3, 24.4, 28.2, 29.7, 31.7, 31.8, 31.9, 35.6 (C-20), 35.7 (C-20), 35.8, 36.5 (C-10), 37.2, 39.8, 40.1, 42.3 (2C, incl. C-13), 50.1, 56.06, 56.10, 56.8, 71.8 (C-3), 118.0 (C-26), 118.6 (C-26), 121.7 (C-6), 136.2 (C-25), 136.5 (C-25), 140.8 (C-5). GC–MS (EI): *m/z* (%) *More volatile isomer:* Free alcohol: 398 (57, M^+), 383 (16, $M^+ - CH_3$), 381 (5, $M^{+\cdot}$-OH), 380 (15, $M^{+\cdot}$-H_2O), 365 (23), 341 (20), 313 (12), 299 (28), 281 (59), 271 (33), 255 (19), 231 (13), 213 (27), 199 (18), 185 (21), 173 (18), 161 (32), 145 (36), 133 (39), 125 (14), 119 (36), 105 (62), 93 (55), 83 (30), 81 (71), 69 (75), 55 (100), 41 (70). TMS ether: 470 (77, M^+), 455 (15, $M^+ - CH_3$), 400 (3), 381 (17, $M^{+\cdot}$-OTMS), 380 (61), 365 (16), 345 (3), 341 (58), 325 (2), 295 (3), 271 (11), 255 (14), 245 (6), 217 (11), 207 (6), 185 (5), 173 (6), 159 (16), 145 (24), 129 (100), 125 (5), 119 (21), 105 (28), 97 (6), 95 (34), 89 (2), 83 (13), 73 (97, TMS^+), 69 (51), 55 (57), 41 (13). *Less volatile isomer:* Free alcohol: 398 (47, M^+), 383 (15, $M^+ - CH_3$), 381 (6, $M^{+\cdot}$-OH), 380 (16, $M^{+\cdot}$-H_2O), 365 (20), 341 (20), 313 (11), 299 (25), 281 (24), 271 (39), 255 (20), 231 (13), 213 (29), 199 (20), 185 (18), 173 (15), 161 (25), 145 (34), 133 (29), 125 (15), 119 (38), 105 (43), 93 (45), 83 (24), 81 (56), 69 (78), 55 (100), 41 (73). TMS ether: 470 (73, M^+), 455 (13, $M^+ - CH_3$), 381 (10, $M^{+\cdot}$-OTMS), 380 (48), 373 (2), 365 (23), 345 (1), 341 (38), 329 (3), 313 (2), 281 (4), 271 (3), 259 (8), 243 (4),

215 (5), 207 (11), 185 (4), 173 (6), 159 (14), 145 (14), 129 (100), 125 (6), 121 (13), 107 (26), 97 (6), 95 (29), 89 (2), 83 (18), 73 (93, TMS^+), 69 (47), 55 (60), 41 (49). HRMS (ESI): Calcd for $C_{28}H_{46}NaO$ ($[M+Na]^+$): 421.3446. Obsd: 421.3434. HRMS (EI): TMS ether: Calcd for $C_{31}H_{54}OSi$ (M^+): 470.3944. Obsd: *More volatile isomer:* 470.3952, *less volatile isomer:* 470.3949.

4.2.9. (20*R*)-26-Methylcholest-5-en-3β-ol (6) and (20*R*)-26-Methylcholestan-3β-ol (22)

A mixture of **21** (9 mg, 17.5 μMol) and PtO_2 (3 mg, 13.2 μMol) in 1,4-dioxane and AcOH (50:1, 1 mL) was evacuated and purged twice with N_2 (g), then twice with H_2 (g). The reaction mixture was stirred under a H_2 atmosphere at room temperature for 16 h, and then filtered through a pad of Celite™ and silica gel, which was washed thoroughly with EtOAc. The filtrate was concentrated in vacuo and the residue was dissolved in CH_2Cl_2 (1 mL) and MeOH (1 mL). Concentrated aqueous HCl solution (32%, 20 μL) was added and the solution was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography (silica gel, 5–9% EtOAc in petroleum spirit 40–60) to afford a mixture comprised of approximately 80% **6** and 20% saturated analog **22** (7 mg, 17.5 μMol, quantitative over 2 steps, mixture of diastereomers at C-25) as a white solid. $[\alpha]_D^{24}$ −11.9 (*c* 0.10, $CHCl_3$). No spectral data is available for **6** or **22** in the literature. ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of diastereomers at C-25; contains ∼20% saturated steroid analog **22**): δ 0.63 (s, ∼0.7H, H_3-18 of saturated compound **22**), 0.66 (s, ∼2.3H, H_3-18 of **6**), 0.74–1.72 (m, 36H, incl. 0.89 [d, ∼2.5H, *J* = 6.6 Hz, H_3-21 of **6**], 0.99 [s, ∼2.6H, H_3-19 of **6**]), 1.73–1.87 (m, ∼3.5H), 1.90–2.04 (m, 2H), 2.15–2.32 (m, 2H), 3.44–3.62 (m, 1H, H-3), 5.33 (m, ∼0.8H, H-6 of **6**). ^{13}C NMR (100 MHz, $CDCl_3$) (mixture of diastereomers at C-25; contains ∼20% saturated steroid analog **22*** [tentative assignments]): δ 11.38, 11.44, 11.8, 12.1*, 12.3*, 18.65*, 18.69, 18.74, 19.2, 19.3, 19.4, 21.1, 21.3*, 23.5, 24.2*, 24.3, 28.2, 28.7, 29.4, 29.65, 29.68*, 31.5*, 31.7, 31.9, 32.1*, 34.45, 34.46, 35.46*, 35.51*, 35.75, 35.81, 36.2, 36.3, 36.5, 37.0*, 37.1, 37.18*, 37.25, 38.2*, 39.8, 40.0*, 42.3, 42.6*, 44.9*, 50.1, 54.4*, 56.1, 56.2, 56.27*, 56.30*, 56.5*, 56.8, 71.4* (C-3), 71.8 (C-3), 121.7 (C-6), 140.8 (C-5). GC–MS (EI): *m/z* (%) *(20R)-26-Methylcholest-5-en-3β-ol (**6**)*: Free alcohol: 400 (53, M^+), 385 (26, $M^+ - CH_3$), 383 (9, M^+-OH), 382 (34, $M^{+\cdot}$-H_2O), 367 (24), 341 (14), 315 (44), 289 (38), 281 (22), 273 (20), 255 (28), 231 (22), 213 (39), 199 (17), 185 (16), 173 (19), 159 (31), 145 (51), 131 (37), 119 (45), 105 (67), 91 (62), 85 (13), 81 (56), 71 (25), 67 (39), 57 (100), 43 (65). TMS ether: 472 (59, M^+), 457 (10, $M^+ - CH_3$), 415 (1), 383 (30, M^+-OTMS), 382 (97), 373 (1), 367 (45), 345 (5), 343 (94), 315 (5), 289 (8), 255 (4), 233 (4), 213 (9), 187 (3), 173 (13), 159 (21), 145 (22), 129 (100), 127 (1), 119 (24), 105 (26), 99 (2), 91 (19), 89 (3), 85 (10), 81 (27), 75 (63), 73 (85, TMS^+), 71 (15), 57 (88), 55 (40), 43 (40), 41 (30). *(20R)-26-Methylcholestan-3β-ol (**22**)*: Free alcohol: 402 (36, M^+), 387 (27, $M^+ - CH_3$), 385 (12, M^+-OH), 384 (8, $M^{+\cdot}$-H_2O), 369 (14), 315 (11), 276 (18), 257 (12), 233 (73), 215 (79), 193 (16), 165 (29), 147 (59), 133 (32), 121 (37), 107 (66), 95 (72), 85 (14), 81 (73), 71 (24), 67 (56), 57 (100), 43 (67). TMS ether: 474 (65, M^+), 459 (73, $M^+ - CH_3$), 417 (25), 389 (5), 385 (5, M^+-OTMS), 384 (24), 375 (2), 369 (32), 347 (1), 345 (6), 329 (4), 306 (18), 305 (17), 276 (8), 261 (3), 230 (6), 215 (51), 207 (12), 183 (6), 175 (6), 159 (7), 147 (14), 129 (16), 127 (2), 119 (15), 105 (19), 99 (1), 95 (19), 89 (1), 85 (11), 81 (25), 75 (100), 73 (44, TMS^+), 71 (18), 69 (29), 57 (56), 55 (31), 43 (30), 41 (29). HRMS (ESI): *(20R)-26-Methylcholest-5-en-3β-ol (**6**)*: Calcd for $C_{28}H_{48}NaO$ ($[M+Na]^+$): 423.3603. Obsd: 423.3593. *(20R)-26-Methylcholestan-3β-ol (**22**)*: Calcd for $C_{28}H_{50}NaO$ ($[M+Na]^+$): 425.3759. Obsd: 425.3742. HRMS (EI): *(20R)-26-Methylcholest-5-en-3β-ol (**6**)*: TMS ether: Calcd for $C_{31}H_{56}OSi$ (M^+): 472.4100. Obsd: 472.4109. *(20R)-26-Methylcholestan-3β-ol (**22**)*: TMS ether: Calcd for $C_{31}H_{58}OSi$ (M^+): 474.4257. Obsd: 474.4258.

4.2.10. (20*S*)-3β-(*tert*-Butyldimethylsilyloxy)-20-hydroxymethylpregna-5,16(17)-diene (24)[37,38]

A solution of freshly distilled $BF_3{\cdot}Et_2O$ in anhydrous CH_2Cl_2 (1% v/v, 0.18 mL, 15 μMol) was added dropwise to a solution of alkene (17*Z*)-**23** (∼92% [5% (17*E*)-**23**, 3% 20(22) alkene, 3β-(*tert*-butyldimethylsilyloxy)pregna-5,20(21)-diene], 58 mg, 0.14 mmol) and paraformaldehyde (5 mg) in anhydrous CH_2Cl_2 (20 mL) stirring under a N_2 atmosphere at room temperature. The reaction mixture was stirred for 5 min, and then saturated aqueous $NaHCO_3$ solution (10 mL) was added to quench the reaction. The mixture was extracted with CH_2Cl_2 (6 × 10 mL) and the combined organic extract was washed with brine (10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, 1–50% Et_2O in petroleum spirit 40–60) to afford **24** (46 mg, 0.10 mmol, 74%) as a pale yellow solid. Mp 144–146 °C. $[\alpha]_D^{25}$ −32.3 (*c* 0.79, $CHCl_3$). Synthesis of **24** has reportedly been achieved by this method previously,[37,38] but no experimental procedure or spectral data were reported. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.04 (s, 6H, $ROSi(CH_3)_2C(CH_3)_3$), 0.80 (s, 3H, H_3-18), 0.87 (s, 9H, $ROSi(CH_3)_2C(CH_3)_3$), 1.00–1.03 (m, 6H, incl. 1.02 [s, 3H, H_3-19], 1.02 [d, 3H, *J* = 6.9 Hz, H_3-21]), 1.19–1.44 (m, 4H), 1.47–1.74 (m, 6H), 1.78 (m, 2H), 1.87 (m, 1H), 1.99 (m, 1H), 2.09 (m, 1H), 2.16 (m, 1H), 2.26 (m, 1H), 2.38 (m, 1H, H-20), 3.47 (m, 1H, H-3), 3.55 (m, 1H, H_2-22), 5.31 (m, 1H, H-6), 5.42 (m, 1H, H-16). ^{13}C NMR (125 MHz, $CDCl_3$): δ −4.6 (2C, $ROSi(CH_3)_2C(CH_3)_3$), 16.2 (C-18), 18.1 (C-21), 18.2 ($ROSi(CH_3)_2C(CH_3)_3$), 19.3 (C-19), 20.7, 25.9 (3C, $ROSi(CH_3)_2C(CH_3)_3$), 30.5, 31.2, 31.6, 32.0, 34.9, 35.3 (C-20), 36.8 (C-10), 37.3, 42.8, 47.0 (C-13), 50.8, 57.4, 66.5 (C-22), 72.6 (C-3), 120.9 (C-6), 123.0 (C-16), 141.8 (C-5), 157.6 (C-17). GC–MS (EI): *m/z* (%) 443 (0.02, $M^{+\cdot}$-1), 429 (0.1, $M^+ - CH_3$), 427 (0.1, $M^{+\cdot}$-OH), 387 (19, M^+ -*t*Bu), 385 (1), 370 (1), 357 (7), 330 (1), 312 (1), 296 (1), 282 (1), 253 (5), 239 (1), 225 (1), 211 (2), 199 (2), 185 (3), 173 (5), 159 (10), 145 (8), 133 (8), 131 (6), 121 (9), 115 (3), 105 (17), 93 (15), 91 (19), 79 (15), 75 (100), 67 (14), 59 (11), 57 (10), 55 (20), 41 (41). Anal. Calcd for $C_{28}H_{48}O_2Si$: C, 75.61; H, 10.88. Found: C, 75.57; H, 10.86. HRMS (ESI): Calcd for $C_{28}H_{48}NaO_2Si$ ($[M+Na]^+$): 467.3321. Obsd: 467.3316.

4.2.11. (20*S*)-3β-(*tert*-Butyldimethylsilyloxy)-20-hydroxymethylpregn-5-ene (25)

A mixture of diene **24** (125 mg, 0.28 mmol) and PtO_2 (19 mg, 0.08 mmol) in EtOAc (20 mL) was degassed-purged twice with N_2 (g), then twice with H_2 (g). The reaction mixture was stirred under a H_2 atmosphere at room temperature for 4 h, and then was filtered through a pad of Celite™ and silica gel, which was washed thoroughly with additional EtOAc. The filtrate was concentrated in vacuo and the residue was purified by flash column chromatography (silica gel, 2–20% Et_2O in petroleum spirit 40–60) to afford **25** (108 mg, 0.24 mmol, 86%) as a white solid. Mp 151–152 °C, lit.[21] mp 153.5–155.5 °C. $[\alpha]_D^{25}$ −33.2 (*c* 0.43, $CHCl_3$). Synthesis of **25** via a different synthetic method has been reported,[21] while the complete NMR spectral data obtained here is consistent with the limited data available for **25** in the literature (^{1}H NMR),[21] and with data reported for a deuterium-labeled analog (^{1}H, ^{13}C).[54] ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.04 (s, 6H, $ROSi(CH_3)_2C(CH_3)_3$), 0.68 (s, 3H, H_3-18), 0.72–1.36 (m, 22H, incl. 0.86 [s, 9H, $ROSi(CH_3)_2C(CH_3)_3$], 0.98 [s, 3H, H_3-19], 1.03 [d, 3H, *J* = 6.6 Hz, H_3-21], 1.37–1.74 (m, 9H), 1.75–1.86 (m, 2H), 1.96 (m, 2H), 2.14 (m, 1H), 2.19–2.35 (m, 1H), 3.35 (dd, 1H, *J* = 10.5, 6.9 Hz, H-22), 3.45 (m, 1H, H-3), 3.61 (dd, 1H, *J* = 10.5, 3.3 Hz, H-22), 5.29 (m, 1H, H-6). ^{13}C NMR (125 MHz, $CDCl_3$): δ −4.6 (2C, $ROSi(CH_3)_2C(CH_3)_3$), 11.9 (C-18), 16.7 (C-21), 18.3 ($ROSi(CH_3)_2C(CH_3)_3$), 19.4

(C-19), 21.0, 24.4, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 27.7, 31.9, 31.9, 32.1, 36.6 (C-10), 37.4, 38.7 (C-20), 39.6, 42.4 (C-13), 42.8, 50.2, 52.4, 56.5, 68.0 (C-22), 72.6 (C-3), 121.1 (C-6), 141.6 (C-5). GC–MS (EI): *m/z* (%) 436 (1), 431 (1, M^+ –CH_3), 403 (1), 340 (12), 389 (12, M^+ -*t*Bu), 388 (8), 387 (3), 371 (1), 353 (1), 313 (1), 298 (1), 283 (1), 255 (1), 240 (1), 213 (2), 199 (2), 187 (3), 173 (3), 159 (12), 145 (6), 133 (6), 131 (5), 121 (6), 115 (2), 105 (13), 91 (15), 81 (19), 75 (100), 67 (15), 59 (10), 57 (13), 55 (32), 41 (50). Anal. Calcd for $C_{28}H_{50}O_2Si$: C, 75.27; H, 11.28. Found: C, 74.99; H, 11.50. HRMS (ESI): Calcd for $C_{28}H_{50}NaO_2Si$ ([M+Na]$^+$): 469.3478. Obsd: 469.3465.

4.2.12. (20*S*)-3β-(*tert*-Butyldimethylsilyloxy)-20-oxomethylpregn-5-ene (26)

PCC (98%, 703 mg, 3.20 mmol) was added to a solution of alcohol **25** (1.091 g, 2.44 mmol) and NaOAc (73 mg, 0.89 mmol) in anhydrous CH_2Cl_2 (20 mL) stirring under a N_2 atmosphere at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 42 h. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, 100% petroleum spirit 40–60 to 2% EtOAc in petroleum spirit 40–60) to afford aldehyde **26** (947 mg, 2.13 mmol, 87%) as a white solid. Mp 130–132 °C, lit.[55] mp 130–131 °C. $[\alpha]_D^{26}$ −24.6 (*c* 0.26, $CHCl_3$), lit.[55] $[\alpha]_D^{25}$ −42.4 (*c* 1.00, $CHCl_3$). Synthesis of **26** via a different procedure has been reported,[55] and the full ^{1}H and ^{13}C NMR spectral data obtained here (Supplementary data) is consistent with the data reported previously. GC–MS (EI): *m/z* (%) 444 (0.1, M^+), 429 (1, M^+ –CH_3), 388 (16), 387 (48, M^+ -*t*Bu and/or M^+ -C_3H_5O), 359 (8), 341 (2), 329 (5, M^+ -TBS), 313 (1, M^+ -OTBS), 311 (2), 295 (5), 253 (8), 239 (3), 225 (2), 213 (5), 199 (5), 185 (5), 159 (16), 145 (12), 133 (12), 131 (9), 119 (12), 115 (5), 107 (20), 93 (21), 81 (23), 75 (100), 67 (15), 58 (5), 57 (10), 55 (18), 41 (19). HRMS (ESI): Calcd for $C_{28}H_{48}NaO_2Si$ ([M+Na]$^+$): 467.3321. Obsd: 467.3318.

4.2.13. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-27-norcholesta-5,22-diene (27)

A solution of *n*-BuLi (0.9 M in hexanes, 1.0 mL, 0.90 mmol) was added dropwise to a solution of *n*-butyltriphenylphosphonium bromide (607 mg, 1.52 mmol) in anhydrous THF (4 mL) stirring under a N_2 atmosphere at −78 °C. The resulting orange solution was stirred at −78 °C for 30 min, then allowed to warm to room temperature and stirred for a further 30 min. The reaction mixture was re-cooled to −78 °C and a solution of aldehyde **26** (114 mg, 0.26 mmol) in anhydrous THF (3 mL) was added dropwise. The orange reaction mixture was allowed to warm to room temperature and stirred for a further 46 h. The solvent was then evaporated in vacuo and the residue was purified by flash column chromatography (silica gel, 100% petroleum spirit 40–60 to 2% EtOAc in petroleum spirit 40–60) to afford diene **27** (106 mg, 0.22 mmol, 87%) as a white solid. ^{13}C NMR and GC–MS analysis suggested that this consists of predominantly one isomer, tentatively assigned as having 22*E* stereochemistry based on ^{13}C NMR comparison.[40] Mp 122–124 °C. $[\alpha]_D^{24}$ −20.3 (*c* 0.28, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.04 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.70 (s, 3H, H_3-18), 0.79–1.27 (m, 25H, incl. 0.88 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.89 [obscured t, 3H, *J* = 7.3 Hz, H_3-26], 0.94 [d, 3H, *J* = 6.6 Hz, H_3-21]), 0.99 [s, 3H, H_3-19]), 1.27–1.59 (m, 8H), 1.59–1.75 (m, 2H), 1.79 (td, 1H, *J* = 13.2, 3.4 Hz), 1.86–2.10 (m, 4H), 2.15 (m, 1H), 2.25 (m, 1H), 2.41 (m, 1H, H-20), 3.46 (m, 1H, H-3), 5.06–5.26 (m, 2H, H-22 & H-23), 5.30 (m, 1H, H-6). ^{13}C NMR (125 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 12.1 (C-18), 13.9 (C-26), 18.2 (ROSi(CH_3)$_2$*C*(CH_3)$_3$), 19.4 (C-19), 20.8 (C-21), 21.1, 23.0, 24.3, 25.8, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 28.1, 29.7, 31.9, 32.1, 34.2 (C-20), 36.6 (C-10), 37.4, 39.7, 42.2 (C-13), 42.8, 50.3, 56.1, 56.9, 72.6 (C-3), 121.1 (C-6), 126.4 (C-23), 136.7 (C-22), 141.5 (C-5). GC–MS (EI): *m/z* (%) 484 (0.1, M^+), 469 (1, M^+ –CH_3), 428 (17), 427 (46, M^+ -*t*Bu), 409 (1), 353 (1, M^+ -OTBS), 351 (6), 329 (3), 281 (2), 255 (4), 231 (2), 207 (9), 159 (7), 145 (6), 133 (7), 131 (5), 119 (6), 115 (3), 105 (10), 97 (62), 75 (100), 69 (10), 67 (11), 57 (6), 55 (71), 43 (5), 41 (14). Anal. Calcd for $C_{32}H_{56}OSi$: C, 79.27; H, 11.64. Found: C, 79.12; H, 11.28.

4.2.14. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-27-norcholest-5-ene (28)

A mixture of diene **27** (91 mg, 0.19 mmol) and PtO_2 (17 mg, 70 μMol) in 1,4-dioxane (6 mL) and AcOH (0.12 mL) was degassed-purged twice with N_2 (g), then twice with H_2 (g). The reaction mixture was stirred under a H_2 atmosphere at room temperature for 18 h, and then filtered through a pad of Celite™ and silica gel, which was washed thoroughly with EtOAc. The filtrate was concentrated in vacuo to afford **28** (88 mg, 0.18 mmol, 96%) as a white solid. Mp 115–117 °C. $[\alpha]_D^{26}$ −14.4 (*c* 0.59, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.04 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.65 (s, 3H, H_3-18), 0.79–0.94 (m, 18H, incl. 0.88 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$]), 0.94–1.59 (m, 22H, incl. 0.98 [s, 3H, H_3-19]), 1.59–1.74 (m, 1H), 1.74–1.86 (m, 2H), 1.86–2.10 (m, 2H), 2.15 (m, 1H), 2.25 (m, 1H, H-20), 3.46 (m, 1H, H-3), 5.29 (m, 1H, H-6). ^{13}C NMR (125 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 11.8 (C-18), 14.1 (C-26), 18.2 (ROSi(CH_3)$_2$*C*(CH_3)$_3$), 18.7 (C-21), 19.4 (C-19), 21.1, 22.7, 24.3, 25.7, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 28.2, 31.90, 31.93, 32.1, 32.4, 35.7 (C-20), 35.9, 36.6 (C-10), 37.4, 39.8, 42.3 (C-13), 42.8, 50.2, 56.1, 56.8, 72.6 (C-3), 121.2 (C-6), 141.5 (C-5). GC–MS (EI): *m/z* (%) 486 (0.1, M^+), 471 (1, M^+ –CH_3), 430 (25), 429 (65, M^+ -*t*Bu), 411 (2), 355 (2, M^+ -OTBS), 353 (10), 339 (1), 289 (1), 275 (1), 255 (1), 233 (4), 207 (4), 159 (10), 145 (8), 133 (6), 131 (5), 119 (7), 115 (3), 105 (11), 99 (1), 95 (18), 75 (100), 71 (3), 69 (14), 67 (10), 57 (31), 55 (19), 43 (17), 41 (17). Anal. Calcd for $C_{32}H_{58}OSi$: C, 78.94; H, 12.01. Found: C, 79.21; H, 12.00.

4.2.15. (20*R*)-27-Norcholest-5-en-3β-ol (2)

Dowex 50W-X8(H) standard grade resin (cat) was added to a solution of TBS ether **28** (54 mg, 111 μMol) in a mixture of CH_2Cl_2 (2 mL) and MeOH (2 mL). The mixture was stirred at room temperature for 42 h, and then the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, 5–9% EtOAc in petroleum spirit 40–60) to afford sterol **2** (40 mg, 107 μMol, 97%) as a white solid. Mp 118–120 °C, lit.[56] mp (recrystallized from MeOH) 131–134 °C. $[\alpha]_D^{24}$ −14.5 (*c* 0.42, $CHCl_3$). The synthesis of **2** via a different synthetic route has been reported previously,[56] and the full ^{1}H NMR spectral data obtained here is consistent with the limited data available in the literature. ^{13}C NMR data has not been reported previously. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.65 (s, 3H, H_3-18), 0.73–1.72 (m, 32H, incl. 0.88 [apparent t, 3H, *J* = 7.5 Hz, H_3-26], 0.98 [s, 3H, H_3-19]), 1.72–1.88 (m, 3H), 1.97 (m, 2H), 2.25 (m, 2H), 3.50 (m, 1H, H-3), 5.33 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.8 (C-18), 14.1 (C-26), 18.7 (C-21), 19.4 (C-19), 21.1, 22.7, 24.3, 25.7, 28.2, 31.6, 31.9 (2C), 32.4, 35.7 (C-20), 35.9, 36.5 (C-10), 37.2, 39.8, 42.3 (2C, incl. C-13), 50.1, 56.1, 56.8, 71.8 (C-3), 121.7 (C-6), 140.8 (C-5). GC–MS (EI): *m/z* (%) Free alcohol: 372 (85, M^+), 357 (31, M^+ –CH_3), 355 (8, M^+-OH), 354 (43, M^+-H_2O), 339 (39), 312 (8), 287 (66), 273 (29), 261 (60), 255 (35), 231 (31), 213 (54), 199 (21), 173 (25), 159 (45), 145 (63), 133 (47), 119 (53), 105 (88), 91 (79), 81 (75), 67 (60), 57 (100), 43 (83). TMS ether: 444 (48, M^+), 429 (7, M^+ –CH_3), 355 (20, M^+-OTMS), 354 (78), 345 (1), 315 (90), 287 (3), 255 (14), 233 (14), 213 (9), 199 (5), 185 (5), 173 (9), 159 (17), 145 (24), 129 (100), 105 (27), 99 (1), 91 (24), 89 (1), 73 (93, TMS^+), 71 (4), 57 (51), 55 (37), 43 (39), 41 (28). Anal. Calcd for $C_{26}H_{44}O$: C, 83.80; H, 11.90. Found: C, 83.43; H, 11.70. HRMS (ESI): Calcd for $C_{26}H_{44}NaO$ ([M+Na]$^+$): 395.3290. Obsd: 395.3284. HRMS (EI): TMS ether: Calcd for $C_{29}H_{52}OSi$ (M^+): 444.3787. Obsd: 444.3781.

4.2.16. Ethyl (20*R*,22*E*)-3β-(*tert*-butyldimethylsilyloxy)chola-5,22-dien-24-oate (30)

A solution of aldehyde **26** (240 mg, 0.54 mmol) and ethyl 2-(triphenylphosphoranylidene)acetate[43] (**29**) (660 mg, 1.89 mmol) in anhydrous CH_2Cl_2 (20 mL) was heated at reflux under a N_2 atmosphere for 72 h. The reaction mixture was cooled to room temperature and concentrated in vacuo, and the residue was purified by flash column chromatography (silica gel, 100% petroleum spirit 40–60 to 2% EtOAc in petroleum spirit 40–60) to afford α,β-unsaturated ester **30** (259 mg, 0.50 mmol, 93%, >85% 22*E*-isomer) as a white solid. Mp 86–88 °C. $[\alpha]_D^{26}$ −19.0 (*c* 0.69, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of *E* and *Z* isomers): δ 0.04 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.69 (s, 3H, H_3-18), 0.74–1.11 (m, 20H, incl. 0.87 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.98 [s, 3H, H_3-19], 1.07 [d, 3H, *J* = 6.6 Hz, H_3-21]), 1.14–1.31 (m, 6H, incl. 1.26 [t, 3H, *J* = 7.2 Hz, $RCO_2CH_2CH_3$]), 1.34–1.87 (m, 8H), 1.95 (m, 2H), 2.14 (m, 1H), 2.24 (m, 2H), 3.45 (m, 1H, H-3), 4.06–4.21 (m, 2H, $RCO_2CH_2CH_3$, incl. 4.15 [q, *J* = 7.1 Hz] for major *E*-isomer), 5.29 (m, 1H, H-6), 5.67–5.77 (m, 1H, H-23, incl. 5.71 [d, *J* = 15.6 Hz] for *E*-isomer), 6.75–6.97 (m, 1H, H-22, incl. 6.81 [dd, *J* = 15.6, 8.9 Hz] for *E*-isomer). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 12.1 (C-18), 14.3 ($RCO_2CH_2CH_3$), 18.2 (ROSi(CH_3)$_2$C(CH_3)$_3$), 19.2, 19.4, 21.0, 24.3, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 28.1, 31.8, 31.9 (C-20), 32.1, 36.6 (C-10), 37.4, 39.6, 39.7, 42.7 (C-13), 42.8, 50.2, 54.9, 56.6, 60.1 ($RCO_2CH_2CH_3$), 72.6 (C-3), 118.9 (C-23), 121.0 (C-6), 141.5 (C-5), 154.7 (C-22 of *E*-isomer), 155.5 (C-22 of *Z*-isomer), 166.9 (C-24 of *Z*-isomer), 167.1 (C-24 of *E*-isomer). GC–MS (EI): *m/z* (%) 514 (0.2, M^+), 499 (2, M^+ –CH_3), 458 (32), 457 (95, M^+ -*t*Bu), 439 (2), 411 (2), 383 (3), 357 (4), 329 (3), 281 (3), 255 (7), 227 (3), 213 (5), 199 (6), 185 (5), 175 (14), 159 (19), 147 (15), 133 (14), 131 (11), 127 (2), 119 (14), 115 (6), 105 (22), 99 (8), 93 (23), 81 (26), 75 (100), 73 (36), 67 (15), 57 (8), 55 (21). Anal. Calcd for $C_{32}H_{54}O_3Si$: C, 74.65; H, 10.57. Found: C, 75.03; H, 10.25. HRMS (ESI): Calcd for $C_{32}H_{54}NaO_3Si$ ($[M+Na]^+$): 537.3740. Obsd: 537.3722.

4.2.17. Ethyl (20*R*)-3β-(*tert*-butyldimethylsilyloxy)chol-5-en-24-oate (31)

A mixture of α,β-unsaturated ester **30** (224 mg, 0.44 mmol) and PtO_2 (38 mg, 0.17 mmol) in 1,4-dioxane (6 mL) and AcOH (0.12 mL) was degassed-purged twice with N_2 (g), then twice with H_2 (g). The reaction mixture was stirred under a H_2 atmosphere at room temperature for 16 h, and then filtered through a pad of Celite™ and silica gel, which was washed thoroughly with EtOAc. The filtrate was concentrated in vacuo to afford **31** (218 mg, 0.42 mmol, 97%) as a white solid. Mp 90–92 °C. $[\alpha]_D^{26}$ −18.5 (*c* 0.59, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.02 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.64 (s, 3H, H_3-18), 0.72–2.03 (m, 39H, incl. 0.86 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.89 [d, 3H, *J* = 6.5 Hz, H_3-21]), 0.96 [s, 3H, H_3-19], 1.22 [t, 3H, *J* = 7.1 Hz, $RCO_2CH_2CH_3$]), 2.08–2.38 (m, 4H), 3.44 (m, 1H, H-3), 4.08 (q, 2H, *J* = 7.1 Hz, $RCO_2CH_2CH_3$), 5.28 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 11.8 (C-18), 14.2 ($RCO_2CH_2CH_3$), 18.2 (ROSi(CH_3)$_2$C(CH_3)$_3$), 18.3 (C-21), 19.4 (C-19), 21.0, 24.2, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 28.1, 31.0, 31.3, 31.9 (2C, incl. C-20), 32.1, 35.3, 36.5 (C-10), 37.3, 39.7, 42.3 (C-13), 42.8, 50.1, 55.7, 56.7, 60.1 ($RCO_2CH_2CH_3$), 72.6 (C-3), 121.1 (C-6), 141.5 (C-5), 174.2 (C-24). GC–MS (EI): *m/z* (%) 516 (0.2, M^+), 501 (2, M^+ –CH_3), 460 (28), 459 (84, M^+ -*t*Bu), 441 (3), 413 (2), 385 (1), 383 (3), 339 (9), 321 (7), 295 (3), 281 (1), 253 (2), 227 (3), 213 (7), 199 (7), 175 (12), 159 (21), 145 (14), 133 (14), 131 (11), 129 (8), 121 (15), 115 (5), 105 (20), 101 (11), 93 (21), 88 (11), 87 (1), 81 (26), 75 (100), 73 (35), 67 (16), 57 (9), 55 (25). Anal. Calcd for $C_{32}H_{56}O_3Si$: C, 74.36; H, 10.92. Found: C, 74.22; H, 11.18. HRMS (ESI): Calcd for $C_{32}H_{56}NaO_3Si$ ($[M+Na]^+$): 539.3896. Obsd: 539.3899.

4.2.18. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)chol-5-en-24-ol (32)

$LiAlH_4$ (95%, 24 mg, 0.60 mmol) was added portion-wise to a solution of ester **31** (200 mg, 0.39 mmol) in anhydrous THF (5 mL) stirring under a N_2 atmosphere at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 44 h. Sodium sulfate decahydrate (330 mg) was added to quench the reaction and the mixture was stirred for 10 min and then filtered through a pad of Celite™ and silica gel, which was washed thoroughly with EtOAc. The filtrate was concentrated in vacuo to afford alcohol **32** (180 mg, 0.38 mmol, 98%) as a white powder. Mp 156–158 °C, lit.[57] mp 164–165 °C for *ent*-**32**. $[\alpha]_D^{26}$ −11.2 (*c* 0.64, $CHCl_3$), lit.[57] $[\alpha]_D^{24}$ +28.4 (*c* 1.00, $CHCl_3$) for *ent*-**32**. The full ^{1}H and ^{13}C spectral data obtained here (Supplementary data) matches that reported for *ent*-**32**.[57] GC–MS (EI): *m/z* (%) 474 (0.04, M^+), 473 (0.2, $M^{+\cdot}$-1), 459 (1, M^+ –CH_3), 457 (0.2, $M^{+\cdot}$–OH), 419 (13), 417 (39, M^+ -*t*Bu), 399 (2), 387 (4), 343 (1), 341 (3), 325 (3), 283 (1), 269 (1), 255 (3), 243 (2), 229 (3), 215 (6), 199 (4), 175 (7), 159 (16), 145 (12), 133 (12), 131 (9), 121 (13), 115 (5), 105 (19), 95 (30), 87 (1), 81 (29), 75 (100), 69 (31), 67 (20), 59 (6), 57 (13), 55 (24), 45 (4), 41 (25). Anal. Calcd for $C_{30}H_{54}O_2Si$: C, 75.88; H, 11.46. Found: C, 75.69; H, 11.62. HRMS (ESI): Calcd for $C_{30}H_{54}NaO_2Si$ ($[M+Na]^+$): 497.3791. Obsd: 497.3783.

4.2.19. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-24-bromochol-5-ene (33)

CBr_4 (99%, 198 mg, 0.59 mmol) was added portion-wise to a solution of alcohol **32** (140 mg, 0.29 mmol), PPh_3 (99%, 168 mg, 0.63 mmol) and imidazole (102 mg, 1.50 mmol) in a mixture of anhydrous Et_2O (3 mL) and CH_3CN (1 mL) stirring under a N_2 atmosphere at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for 2.5 h, and then was re-cooled to 0 °C. MeOH (0.2 mL) was added to quench the reaction and the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, 100% petroleum spirit 40–60 to 2% Et_2O in petroleum spirit 40–60) to afford bromide **33** (151 mg, 0.28 mmol, 95%) as a white solid. Mp 108–110 °C. $[\alpha]_D^{26}$ −10.0 (*c* 0.16, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.66 (s, 3H, H_3-18), 0.74–2.05 (m, 38H, incl. 0.88 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.91 [d, 3H, *J* = 6.6 Hz, H_3-21]), 0.98 [s, 3H, H_3-19]), 2.14 (m, 1H), 2.24 (m, 1H), 3.35 (m, 2H, H_2-24), 3.46 (m, 1H, H-3), 5.29 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 11.8 (C-18), 18.2 (ROSi(CH_3)$_2$C(CH_3)$_3$), 18.7 (C-21), 19.4 (C-19), 21.0, 24.2, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 28.2, 29.6, 31.89 (C-20), 31.90, 32.1, 34.45, 34.50, 35.2, 36.6 (C-10), 37.4, 39.8, 42.3 (C-13), 42.8, 50.2, 55.8, 56.8, 72.6 (C-3), 121.1 (C-6), 141.5 (C-5). GC–MS (EI): *m/z* (%) 536/538 (0.2/0.1, M^+), 521/523 (1/1, M^+ –CH_3), 479/481 (33/38, M^+ -*t*Bu), 405/407 (7/3, M^+ -OTBS), 401 (7), 325 (8), 283 (3), 255 (4), 227 (3), 213 (5), 199 (4), 161 (8), 159 (20), 151 (3), 149 (6), 145 (14), 131 (9), 123 (5), 121 (10), 115 (5), 107/109 (15/14), 95 (30), 93 (18), 81 (23), 79 (13), 75 (100), 69 (28), 57 (10), 55 (26), 41 (22). Anal. Calcd for $C_{30}H_{53}BrOSi$: C, 67.01; H, 9.93. Found: C, 66.61; H, 9.68. HRMS (ESI): Calcd for $C_{30}H_{53}BrNaOSi$ ($[M+Na]^+$): 559.2947/561.2926. Obsd: 559.2943/561.2940.

4.2.20. (20*R*)-24-bromochol-5-en-3β-ol (10)

Dowex 50W-X8(H) standard grade resin (cat) was added to a solution of TBS ether **33** (11 mg, 20.5 μMol) in a mixture of CH_2Cl_2 (1 mL) and MeOH (1 mL). The mixture was stirred at room temperature for 90 h, and then the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, 5–9% EtOAc in *n*-hexane) to afford sterol **10** (8 mg, 18.9 μMol, 92%) as a white solid. The synthesis of **10** via a different synthetic route has been reported previously,[58] and the full ^{1}H NMR spectral data

obtained here is consistent with the very limited data available in the literature. ^{13}C NMR data has not been reported previously. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.66 (s, 3H, H_3-18), 0.73–2.34 (m, 35H, incl. 0.91 [d, 3H, J = 6.4 Hz, H_3-21]), 0.98 [s, 3H, H_3-19]), 3.36 (m, 2H, H_2-24), 3.51 (m, 1H, H-3), 5.33 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.8 (C-18), 18.7 (C-21), 19.4 (C-19), 21.1, 24.2, 28.2, 29.6, 31.6, 31.9, 34.5, 34.6, 35.1, 36.5, 36.6, 37.2, 39.7, 42.2, 42.3, 50.1, 55.8, 56.7, 71.8 (C-3), 121.7 (C-6), 140.7 (C-5). GC–MS (EI): *m*/*z* (%) Free alcohol: 422/424 (52/53, M^+), 409 (19), 407 (27), 405 (10), 404/406 (33/34, $M^{+\cdot}$-H_2O), 389/391 (33/33), 362/364 (7/9), 337/339 (49/46), 311/313 (42/40), 283 (17), 273 (32), 255 (46), 231 (37), 213 (57), 145 (80), 133 (51), 121 (50), 121 (61), 109 (43), 107 (98), 105 (100), 95 (94), 93 (80), 91 (89), 79/81 (81/86, Br^+), 69 (82), 55 (81), 41 (75). TMS ether: 494/496 (21/23, M^+), 479/481 (6/6, M^+ –CH_3), 429 (1), 405/407 (11/10, $M^{+\cdot}$-OTMS), 404/406 (32/33), 389/391 (19/15), 365/367 (54/40), 337 (1), 311 (5), 285 (11), 255 (7), 229 (2), 207 (7), 185 (7), 159 (16), 149/151 (6/5), 145 (20), 129 (83), 121 (15), 119 (22), 109 (13), 107 (22), 105 (27), 93/95 (21/28), 79/81 (22/22, Br^+), 73 (100, TMS^+), 69 (44), 55 (44), 41 (33). HRMS (ESI): Calcd for $C_{26}H_{44}NaO$ ([M+Na]$^+$): 445.2082/447.2062. Obsd: 445.2065/447.2070. HRMS (EI): Calcd for $C_{24}H_{39}BrO$ (M^+): 422.2184/424.2164. Obsd: 422.2186/424.2177.

4.2.21. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-24-(acetylthio)-chol-5-ene (34)

A solution of bromide **33** (52 mg, 97 μMol) and potassium thioacetate (98%, 32 mg, 275 μMol) in freshly distilled acetone (5 mL) was stirred under a N_2 atmosphere at room temperature for 23 h. The solvent was then evaporated and the residue was purified by flash column chromatography (silica gel, 1–2% EtOAc in petroleum spirit 40–60) to afford thioacetate **34** (49 mg, 92 μMol, 95%) as a white solid. Mp 90–92 °C. $[\alpha]_D^{26}$ −6.7 (*c* 0.50, $CHCl_3$). ^{1}H NMR (400 MHz, C_6D_6): δ 0.14 (d, 6H, J = 2.4 Hz, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.64 (s, 3H, H_3-18), 0.71–1.88 (m, 36H, incl. 0.91 [d, 3H, J = 6.6 Hz, H_3-21], 0.99 [s, 3H, H_3-19], 1.05 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$]), 1.90–2.03 (m, 5H, incl. 1.94 [s, 3H, RSCOCH_3), 2.41 (m, 1H), 2.52 (m, 1H), 2.83 (m, 2H, H_2-24), 3.65 (m, 1H, H-3), 5.41 (m, 1H, H-6). ^{13}C NMR (100 MHz, C_6D_6): δ −4.3 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 12.0 (C-18), 18.3 (ROSi(CH_3)$_2$C(CH_3)$_3$), 18.8 (C-21), 19.5 (C-19), 21.4, 24.5, 26.1 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 26.7, 28.4, 29.8, 30.2 (RSCOCH_3), 32.2 (C-20), 32.4, 32.7, 35.4, 35.7, 36.8 (C-10), 37.7, 40.1, 42.5 (C-13), 43.5, 50.6, 56.2, 56.9, 72.9 (C-3), 121.5 (C-6), 141.5 (C-5), 194.3 (C=O). GC–MS (EI): *m*/*z* (%) 532 (0.1, M^+), 517 (2, M^+ –CH_3), 476 (35), 475 (96, M^+ -*t*Bu), 457 (3, $M^{+\cdot}$-SAc), 433 (1), 417 (1, M^+ -TBS), 401 (5, M^+ -OTBS), 400 (2), 399 (5), 383 (1), 357 (2), 329 (2), 255 (3), 213 (5), 161 (9), 159 (13), 145 (11), 131 (8), 119 (32), 117 (13), 115 (6), 105 (17), 103 (8), 95 (20), 89 (2), 81 (18), 76 (8), 75 (100), 69 (16), 57 (7), 55 (15), 43 (98). HRMS (ESI): Calcd for $C_{32}H_{56}NaO_2SSi$ ([M+Na]$^+$): 555.3668. Obsd: 555.3668.

4.2.22. (20*R*)-25-Thia-27-norcholest-5-en-3β-ol (3)

A solution of sodium methoxide in anhydrous MeOH (0.46 M, 0.50 mL, 228 μMol) was added to a solution of thioacetate **34** (28 mg, 53 μMol) in a mixture of anhydrous MeOH (1 mL) and THF (1 mL) stirring under an Ar atmosphere at 0 °C. The mixture was allowed to warm to room temperature and stirred for 30 min. MeI (99%, 20 μL, 318 μMol) was added and the reaction mixture was stirred at room temperature for 2 h, and then concentrated under a stream of N_2 in order to evaporate excess MeI. The residue was re-dissolved in anhydrous THF (1 mL), and MeOH (0.5 mL) and concentrated aqueous HCl solution (32%, 10 μL) were added. The reaction mixture was stirred for 30 min, and then was concentrated under a stream of N_2. The residue was purified by flash column chromatography (silica gel, 5–17% EtOAc in petroleum spirit 40–60) to afford sterol **3** (14 mg, 36 μMol, 68% over 3 steps) as a pale yellow solid. Mp 113–115 °C, lit.[59] mp (recrystallized from hexane) 119–120 °C. $[\alpha]_D^{23}$ −13.1 (*c* 0.40, C_6D_6). Synthesis of **3** via a different synthetic route has been reported previously and only partial ^{1}H NMR data obtained in $CDCl_3$ has been reported.[59] ^{1}H NMR (500 MHz, C_6D_6): δ 0.67 (s, 3H, H_3-18), 0.76–1.86 (m, 28H, incl. 0.96 [d, 3H, J = 6.6 Hz, H_3-21], 0.98 [s, 3H, H_3-19]), 1.87 (s, 3H, H_3-26), 1.99 (m, 2H), 2.31 (m, 4H), 3.42 (m, 1H, H-3), 5.39 (m, 1H, H-6). ^{13}C NMR (125 MHz, C_6D_6): δ 12.0 (C-18), 15.4 (C-26), 18.9 (C-21), 19.5 (C-19), 21.4, 24.5, 26.1, 28.5, 32.1, 32.2 (C-20), 32.3, 35.1, 35.4, 35.8, 36.8 (C-10), 37.7, 40.1, 42.5 (C-13), 42.9, 50.5, 56.3, 56.9, 71.6 (C-3), 121.6 (C-6), 141.3 (C-5). GC–MS (EI): *m*/*z* (%) Free alcohol: 390 (65, M^+), 375 (39, M^+ –CH_3), 373 (24, $M^{+\cdot}$-OH), 372 (82, $M^{+\cdot}$-H_2O), 357 (42), 330 (10), 305 (37), 279 (33), 273 (28), 255 (52), 213 (77), 199 (25), 185 (21), 173 (26), 161 (55), 145 (74), 131 (47), 117 (100), 105 (91), 95 (83), 81 (86), 69 (75), 61 (60), 55 (73), 41 (67). TMS ether: 462 (35, M^+), 447 (15, M^+ –CH_3), 405 (4), 373 (15), 372 (75), 357 (21), 345 (3), 333 (64), 305 (5), 281 (8), 255 (9), 251 (9), 237 (4), 227 (4), 215 (5), 207 (24), 199 (9), 185 (6), 175 (6), 161 (13), 145 (20), 129 (69), 121 (18), 117 (15), 105 (8), 91 (24), 89 (1), 81 (20), 75 (79), 73 (100, TMS^+), 69 (26), 61 (20), 55 (23), 47 (12), 45 (13). HRMS (EI): TMS ether: Calcd for $C_{28}H_{50}OSiS$ (M^+): 462.3352. Obsd: 462.3349.

4.2.23. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-24-cyanochol-5-ene (35)

Potassium cyanide (62 mg, 952 μMol) was added to a solution of bromide **34** (140 mg, 260 μMol) in anhydrous DMF (4 mL) and the reaction mixture was stirred under a N_2 atmosphere at 80 °C for 2 h. The reaction mixture was then cooled to room temperature and saturated aqueous Na_2CO_3 solution (10 mL) was added to quench the reaction. The mixture was extracted with EtOAc (3 × 10 mL) and the combined organic extract was washed with saturated aqueous Na_2CO_3 solution (10 mL) and brine (10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, 5% EtOAc in petroleum spirit 40–60) to afford nitrile **35** (123 mg, 254 μMol, 98%) as a white solid. The full ^{1}H and ^{13}C spectral data obtained here (Supplementary data) matches that reported for *ent*-**35**.[57]

4.2.24. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-27-norcholesta-5,25-diene (37)

DIBAL-H (1.0 M in hexanes, 185 μL, 185 μMol) was added dropwise to a solution of nitrile **35** (68 mg, 141 μMol) in anhydrous Et_2O (4 mL) stirring under a N_2 atmosphere at −78 °C. The reaction mixture was stirred at −78 °C for 1 h, and then allowed to warm to room temperature and stirred for a further 4 h. Aqueous oxalic acid solution (5%, 10 mL) was added to quench the reaction and the mixture was stirred until clear phase separation was Obsd. The organic and aqueous layers were separated and the aqueous solution was extracted with EtOAc (3 × 10 mL). The combined organic extract was washed with saturated aqueous Na_2CO_3 solution (10 mL) and brine (10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to afford the aldehyde (20*R*)-3β-(*tert*-butyldimethylsilyloxy)-24-oxomethylchol-5-ene (**36**) (52 mg, 107 μMol, 76%) as a white solid that was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.65 (s, 3H, H_3-18), 0.73–1.85 (m, 36H, incl. 0.87 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.92 [d, 3H, J = 6.6 Hz, H_3-21]), 0.97 [s, 3H, H_3-19]), 1.89–2.01 (m, 2H), 2.10–2.19 (m, 1H), 2.19–2.29 (m, 1H), 2.37 (m, 2H, H_2-24), 3.46 (m, 1H, H-3), 5.29 (m, 1H, H-6), 9.74 (t, 1H, J = 1.8 Hz, H-25). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 11.8 (C-18), 18.3 (ROSi(CH_3)$_2$C(CH_3)$_3$), 18.6 (C-21), 18.7, 19.4 (C-19), 21.0, 24.2, 25.9

(3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 28.2, 31.9 (2C, incl. C-20), 32.1, 35.5, 35.6, 36.6 (C-10), 37.4, 39.8, 42.3 (C-13), 42.8, 44.3, 50.2, 55.8, 56.8, 72.6 (C-3), 121.1 (C-6), 141.6 (C-5), 202.9 (C-25). GC–MS (EI): *m/z* (%) 486 (0.2, M^+), 485 (0.3, $M^{+\cdot}$-1), 471 (3, M^+ –CH_3), 430 (30), 429 (89, M^+ -*t*Bu), 415 (1), 411 (3), 401 (11), 387 (1), 371 (1, M^+ -TBS), 355 (2, M^+ -OTBS), 353 (3), 337 (5), 295 (3), 281 (4), 267 (2), 255 (11), 241 (6), 227 (8), 213 (11), 199 (10), 185 (9), 173 (12), 159 (30), 145 (20), 133 (20), 131 (17), 121 (19), 115 (6), 105 (28), 99 (5), 95 (34), 81 (44), 75 (100), 71 (3), 67 (19), 57 (11), 55 (29), 43 (11), 41 (13).

A solution of *n*-BuLi (1.1 M in hexanes, 0.15 mL, 165 μMol) was added dropwise to a solution of methyltriphenylphosphonium bromide (95 mg, 266 μMol) in anhydrous THF (2 mL) stirring under a N_2 atmosphere at −78 °C. The resulting orange solution was stirred at −78 °C for 40 min, then allowed to warm to room temperature and stirred for a further 10 min. The reaction mixture was re-cooled to −78 °C and a solution of aldehyde **36** (25 mg, 51 μMol) in anhydrous THF (1 mL) was added dropwise. The orange reaction mixture was allowed to warm to room temperature and stirred for a further 12 h. The solvent was then evaporated and the residue was filtered through a pad of silica gel that was washed thoroughly with additional EtOAc. The filtrate was concentrated in vacuo to afford diene **37** (25 mg, 51 μMol, quantitative) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.04 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.65 (s, 3H, H_3-18), 0.73–1.87 (m, 36H, incl. 0.87 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.90 [d, 3H, J = 6.5 Hz, H_3-21], 0.98 [s, 3H, H_3-19]), 1.90–2.07 (m, 4H), 2.10–2.18 (m, 1H), 2.20–2.31 (m, 1H), 3.46 (m, 1H, H-3), 4.87–5.02 (m, 2H, H_2-26), 5.29 (m, 1H, H-6), 5.79 (m, 1H, H-25). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 11.8 (C-18), 18.3 (ROSi(CH_3)$_2$*C*(CH_3)$_3$), 18.7 (C-21), 19.4 (C-19), 21.1, 24.3, 25.3, 25.4, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 28.2, 31.92 (C-20), 31.94, 32.1, 34.2, 35.5, 35.6, 36.6 (C-10), 37.4, 39.8, 42.3 (C-13), 42.8, 50.2, 56.1, 56.8, 72.6 (C-3), 114.1 (C-26), 121.2 (C-6), 139.3 (C-25), 141.6 (C-5). GC–MS (EI): *m/z* (%) 484 (0.2, M^+), 469 (2, M^+ –CH_3), 429 (8), 428 (36), 427 (100, M^+ -*t*Bu), 409 (4), 353 (2, M^+ -OTBS), 351 (11), 281 (2), 255 (3), 235 (3), 207 (4), 159 (12), 145 (8), 135 (7), 131 (6), 121 (7), 115 (3), 105 (11), 97 (6), 95 (19), 81 (19), 75 (79), 69 (7), 67 (10), 57 (4), 55 (21), 41 (7). HRMS (ESI): Calcd for $C_{32}H_{56}NaOSi$ ([M+Na]$^+$): 507.3998. Obsd: 507.3984.

4.2.25. (20*R*)-27-Norcholesta-5,25-dien-3β-ol (7)

Dowex 50W-X8(H) standard grade resin (cat) was added to a solution of TBS ether **37** (7 mg, 14.4 μMol) in a mixture of CH_2Cl_2 (1 mL) and MeOH (1 mL). The reaction mixture was stirred at room temperature for 90 h, and then the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, 5–9% EtOAc in *n*-hexane) to afford sterol **7** (5 mg, 13.5 μMol, 93%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.66 (s, 3H, H_3-18), 0.72–2.36 (m, 34H, incl. 0.90 [d, 3H, J = 6.4 Hz, H_3-21], 0.99 [s, 3H, H_3-19]), 3.51 (m, 1H, H-3), 4.86–5.02 (m, 2H, H_2-26), 5.33 (m, 1H, H-6), 5.78 (m, 1H, H-25). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.8 (C-18), 18.7 (C-21), 19.4 (C-19), 21.1, 24.3, 25.4, 28.2, 31.6, 31.9, 34.2, 35.4, 35.6, 36.5, 36.6, 37.2, 39.8, 42.25, 42.31, 50.1, 56.0, 56.8, 71.8 (C-3), 114.1 (C-26), 121.7 (C-6), 139.3 (C-25), 140.7 (C-5). GC–MS (EI): *m/z* (%) Free alcohol: 370 (63, M^+), 355 (28, M^+ –CH_3), 353 (10, $M^{+\cdot}$-OH), 352 (33, $M^{+\cdot}$-H_2O), 337 (31), 300 (10), 285 (48), 273 (17), 271 (45), 255 (27), 231 (30), 213 (43), 199 (17), 185 (15), 173 (24), 159 (43), 145 (57), 133 (43), 119 (46), 107 (72), 105 (73), 97 (24), 95 (77), 81 (85), 69 (31), 67 (54), 55 (100), 41 (43). TMS ether: 442 (52, M^+), 427 (13, M^+ –CH_3), 400 (2), 353 (15, $M^{+\cdot}$-OTMS), 352 (48), 337 (32), 313 (28), 284 (6), 255 (7), 207 (10), 159 (11), 145 (15), 129 (76), 119 (17), 107 (20), 105 (20), 97 (14), 91 (30), 89 (1), 75 (64), 73 (100, TMS^+), 69 (18), 55 (94), 41 (37). HRMS (ESI): Calcd for $C_{26}H_{42}NaO$ ([M+Na]$^+$): 393.3133. Obsd: 393.3122. HRMS (EI): TMS ether: Calcd for $C_{29}H_{50}OSi$ (M^+): 442.3631. Obsd: 442.3630.

4.2.26. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-27-norcholest-5-en-25-ol (38)

Methylmagnesium bromide (3.0 M in Et_2O, 20 μL, 60 μMol) was added dropwise to a solution of aldehyde **36** (15 mg, 31 μMol) (see preparation of **37**) in anhydrous THF (3 mL) stirring under a N_2 atmosphere at −78 °C. The reaction mixture was stirred at −78 °C for 1 h, then allowed to warm to room temperature and stirred for 12 h. Saturated aqueous NH_4Cl solution (10 mL) was added to quench the reaction and the mixture was extracted with Et_2O (3 × 10 mL). The combined organic extract was washed with brine (10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, 20% Et_2O in petroleum spirit 40–60) to afford alcohol **38** (14 mg, 27 μMol, 90%, 1:1 mixture of diastereomers at C-25) as a white solid. Synthesis of **38** via a different synthetic route has been reported previously,[50] and the full ^{1}H and ^{13}C NMR spectral data obtained here is consistent with the limited data available in the literature. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.65 (s, 3H, H_3-18), 0.73–1.86 (m, 41H, incl. 0.87 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.90 [d, 3H, J = 6.5 Hz, H_3-21], 0.98 [s, 3H, H_3-19], 1.17 [d, 3H, J = 6.2 Hz, H_3-26]), 1.89–2.04 (m, 2H), 2.10–2.18 (m, 1H), 2.19–2.35 (m, 1H), 3.46 (m, 1H, H-3), 3.78 (m, 1H, H-25), 5.29 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$) (mixture of diastereomers at C-25): δ −4.6 (2C, ROSi(CH_3)$_2$C(CH_3)$_3$), 11.8 (C-18), 18.3 (ROSi(CH_3)$_2$*C*(CH_3)$_3$), 18.7 (C-21), 19.4 (C-19), 21.1, 22.16, 22.24, 23.5, 23.6, 24.3, 25.9 (3C, ROSi(CH_3)$_2$C(CH_3)$_3$), 28.2, 31.90 (C-20), 31.93, 32.1, 35.5 (br), 35.69, 35.70, 35.88, 35.94, 36.6 (C-10), 37.4, 39.8, 42.3 (C-13), 42.8, 50.2, 56.00, 56.01, 56.8, 68.2 (C-25), 68.3 (C-25), 72.6 (C-3), 121.1 (C-6), 141.6 (C-5). GC–MS (EI): *m/z* (%) 489 (2), 487 (2, M^+ –CH_3), 476 (2), 459 (1), 446 (22), 445 (65, M^+ -*t*Bu), 443 (3), 429 (4), 428 (7), 427 (20), 415 (1), 401 (2), 387 (2, M^+ -OTBS), 371 (1), 369 (7), 353 (16), 341 (2), 329 (3), 311 (3), 297 (2), 281 (6), 255 (9), 241 (5), 227 (5), 213 (9), 203 (6), 187 (6), 173 (11), 159 (25), 147 (21), 133 (14), 131 (10), 121 (17), 115 (5), 105 (21), 95 (35), 87 (2), 81 (35), 75 (100), 73 (38), 69 (16), 67 (14), 59 (4), 57 (14), 55 (30), 45 (15).

4.2.27. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-25-chloro-27-norcholest-5-ene (39)

Triethylamine (70 μL, 502 μMol) and methanesulfonyl chloride (99%, 9 μL, 115 μMol) were added sequentially to a solution of alcohol **38** (25 mg, 50 μMol) in anhydrous CH_2Cl_2 (2 mL) stirring under a N_2 atmosphere at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then allowed to warm to room temperature. H_2O (5 mL) was added to quench the reaction and the mixture was extracted with EtOAc (3 × 10 mL). The combined organic extract was washed with brine (10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude mesylate was dissolved in anhydrous THF (3 mL) and LiCl (30 mg, 708 μMol) was added. The reaction mixture was heated at reflux for 1 h, and then cooled to room temperature and filtered through a pad of silica gel, which was washed thoroughly with EtOAc. The filtrate was concentrated under reduced pressure to afford chloride **39** (26 mg, 50 μMol, quantitative, 1:1 mixture of diastereomers at C-25) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.04 (s, 6H, ROSi(CH_3)$_2$C(CH_3)$_3$), 0.65 (s, 3H, H_3-18), 0.73–1.87 (m, 41H, incl. 0.87 [s, 9H, ROSi(CH_3)$_2$C(CH_3)$_3$], 0.91 [d, 3H, J = 6.5 Hz, H_3-21], 0.98 [s, 3H, H_3-19], 1.48 [br d, 3H, J = 6.5 Hz, H_3-26]), 1.90–2.04 (m, 2H), 2.10–2.18 (m, 1H), 2.20–2.31 (m, 1H), 3.46 (m, 1H, H-3), 4.00 (m, 1H, H-25), 5.29 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$) (mixture of diastereomers at C-25): δ −4.6 (2C, ROSi(CH_3)$_2$C

$(CH_3)_3$), 11.8 (C-18), 18.3 (ROSi$(CH_3)_2C(CH_3)_3$), 18.6 (C-21), 18.7 (C-21), 19.4 (C-19), 21.1, 23.1, 23.2, 24.3, 25.3, 25.5, 25.9 (3C, ROSi$(CH_3)_2C(CH_3)_3$), 28.2, 31.91 (C-20), 31.92, 32.1, 35.3, 35.4, 35.6, 35.7, 36.6 (C-10), 37.4, 39.8, 40.8, 40.9, 42.3 (C-13), 42.8, 50.2, 55.97, 55.99, 56.8, 58.97 (C-25), 59.01 (C-25), 72.6 (C-3), 121.1 (C-6), 141.6 (C-5). GC–MS (EI): *m/z* (%) 520/522 (0.4/0.1, M^+), 505/507 (2/1, M^+ –CH_3), 464/466 (30/11), 463/465 (85/35, M^+ -*t*Bu), 445 (2), 429 (13), 427 (21), 415 (1), 389/391 (3/1, M^+ -OTBS), 387 (5), 353 (32), 311 (3), 297 (2), 283 (4), 267 (4), 255 (13), 241 (7), 227 (7), 213 (12), 199 (8), 187 (8), 173 (14), 159 (34), 145 (23), 133/135 (23/13), 131 (13), 119 (15), 115 (6), 107 (20), 105 (23), 95/97 (38/13), 93 (22), 91 (16), 81 (35), 79 (14), 77 (7), 75 (100), 69 (17), 65 (17), 65 (1), 63 (1), 57 (15), 55 (36).

4.2.28. (20*R*)-25-Chloro-27-norcholest-5-en-3β-ol (8)

Dowex 50W-X8(H) standard grade resin (cat) was added to a solution of TBS ether **39** (13 mg, 24.9 μMol) in a mixture of CH_2Cl_2 (1 mL) and MeOH (1 mL). The mixture was stirred at room temperature for 90 h, and then the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, 5–9% EtOAc in *n*-hexane) to afford sterol **8** (9 mg, 22.1 μMol, 89%, 1:1 mixture of diastereomers at C-25) as a white solid. Synthesis of **8** via a different synthetic route has been reported previously,[60] but no NMR spectral data was reported. ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of diastereomers at C-25): δ 0.66 (s, 3H, H_3-18), 0.73–2.38 (m, 37H, incl. 0.91 [d, 3H, J = 6.8 Hz, H_3-21]), 0.99 [s, 3H, H_3-19], 1.48 [br d, 3H, J = 6.4 Hz, H_3-26]), 3.51 (m, 1H, H-3), 4.00 (m, 1H, H-25), 5.33 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$) (mixture of diastereomers at C-25): δ 11.9 (C-18), 18.6 (C-21), 18.7 (C-21), 19.4 (C-19), 21.1, 23.16, 23.21, 24.3, 25.3, 25.5, 28.2, 31.6, 31.9 (2C, incl. C-20), 35.3, 35.4, 35.6, 35.7, 36.5 (C-10), 37.2, 39.8, 40.8, 40.9, 42.25, 42.33, 50.1, 55.97, 55.99, 56.7 (br), 59.00, 59.03, 71.8 (C-3), 121.7 (C-6), 140.7 (C-5). GC–MS (EI): *m/z* (%) Free alcohol: 406/408 (73/26, M^+), 393 (9), 391 (27), 389 (12), 388/390 (40/15, $M^{+\cdot}$-H_2O), 373/375 (39/14), 346 (8), 321 (58), 295 (58), 273 (30), 255 (43), 231 (34), 213 (64), 199 (22), 161 (47), 159 (46), 147 (40), 145 (63), 133 (45), 123 (22), 121 (41), 119 (48), 109 (37), 107 (76), 105 (72), 95 (73), 93 (58), 91 (60), 81 (76), 79 (54), 67 (47), 55 (100), 41 (36). TMS ether: 478/480 (16/6, M^+), 463/465 (7/3, M^+ –CH_3), 443 (1, $M^{+\cdot}$-Cl), 442 (3, $M^{+\cdot}$-HCl), 415 (1), 401 (1), 389/391 (14/6, $M^{+\cdot}$-OTMS), 388 (54), 373 (26), 349 (63), 345 (1), 313 (5), 267 (10), 228 (5), 207 (14), 185 (7), 159 (26), 133/135 (12/8), 129 (67), 95 (37), 91/93 (31/21), 89 (1), 77/79 (12/7), 75 (57), 73 (100, TMS^+), 63/65 (5/2), 55 (75), 41 (31). HRMS (ESI): Calcd for $C_{26}H_{43}ClNaO$ ([M+Na]$^+$): 429.2900/431.2871. Obsd: 429.2878/431.2872. HRMS (EI): TMS ether: Calcd for $C_{29}H_{51}ClOSi$ (M^+): 478.3397/480.3368. Obsd: 478.3397/480.3416.

4.2.29. (20*R*)-3β-(*tert*-Butyldimethylsilyloxy)-25-bromo-27-norcholest-5-ene (40)

Triethylamine (40 μL, 287 μMol) and methanesulfonyl chloride (99%, 5 μL, 64 μMol) were added sequentially to a solution of alcohol **38** (14 mg, 28 μMol) in anhydrous CH_2Cl_2 (1 mL) stirring under a N_2 atmosphere at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then allowed to warm to room temperature. H_2O (5 mL) was added to quench the reaction and the mixture was extracted with EtOAc (3 × 10 mL). The combined organic extract was washed with brine (10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude mesylate was dissolved in anhydrous THF (3 mL) and LiBr (30 mg, 345 (mol) was added. The reaction mixture was heated at reflux for 1 h, and then cooled to room temperature and filtered through a pad of silica gel, which was washed thoroughly with EtOAc. The filtrate was concentrated under reduced pressure to afford bromide **40** (16 mg, 28 μMol, quantitative, 1:1 mixture of diastereomers at C-25) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.03 (s, 6H, ROSi$(CH_3)_2C(CH_3)_3$), 0.65 (s, 3H, H_3-18), 0.73–1.86 (m, 41H, incl. 0.87 [s, 9H, ROSi$(CH_3)_2C(CH_3)_3$], 0.91 [d, 3H, J = 6.5 Hz, H_3-21], 0.98 [s, 3H, H_3-19], 1.68 & 1.69 [2 overlapping d, 3H, J = 6.6 Hz, H_3-26]), 1.90–2.01 (m, 2H), 2.11–2.17 (m, 1H), 2.20–2.28 (m, 1H), 3.46 (m, 1H, H-3), 4.11 (m, 1H, H-25), 5.29 (m, 1H, H-6). ^{13}C NMR (125 MHz, $CDCl_3$) (mixture of diastereomers at C-25): δ –4.6 (2C, ROSi$(CH_3)_2C(CH_3)_3$), 11.8 (C-18), 18.3 (ROSi$(CH_3)_2C(CH_3)_3$), 18.6 (C-21), 18.7 (C-21), 19.4 (C-19), 21.0, 24.27, 24.30, 25.9 (3C, ROSi$(CH_3)_2C(CH_3)_3$), 26.4, 26.6, 28.2, 31.89 (C-20), 31.92, 32.1, 35.2, 35.3, 35.5 (br), 35.6, 35.7, 36.6 (C-10), 37.4, 39.8, 41.5, 41.7, 42.3 (C-13), 42.8, 50.2, 52.0 (C-25), 52.1 (C-25), 55.96, 55.98, 72.6 (C-3), 121.1 (C-6), 141.6 (C-5). GC–MS (EI): *m/z* (%) 564/566 (0.2/0.2, M^+), 549/551 (1/1, M^+ –CH_3), 508/510 (17/17), 507/509 (48/51, M^+ -*t*Bu), 433/435 (2/2, M^+ -OTBS), 429 (22), 427 (33), 353 (36), 311 (4), 283 (3), 271 (2), 255 (11), 241 (6), 227 (5), 213 (10), 199 (7), 187 (7), 177/179 (3/4), 173 (12), 159 (30), 151 (4), 149 (8), 145 (20), 137 (5), 135 (12), 133 (21), 131 (12), 123 (10), 121 (15), 115 (6), 107/109 (18/19), 105 (21), 95 (34), 81 (32), 79 (13), 75 (100), 69 (18), 57 (15), 55 (32).

4.2.30. (20*R*)-25-Bromo-27-norcholest-5-en-3β-ol (9)

Dowex 50W-X8(H) standard grade resin (cat) was added to a solution of TBS ether **40** (10 mg, 17.7 μMol) in a mixture of CH_2Cl_2 (1 mL) and MeOH (1 mL). The mixture was stirred at room temperature for 90 h, and then the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, 5–9% EtOAc in *n*-hexane) to afford sterol **9** (7 mg, 15.5 μMol, 88%, 1:1 mixture of diastereomers at C-25) as a white solid. Synthesis of **9** via a different synthetic route has been achieved previously,[61] and the full ^{1}H NMR spectral data obtained here is consistent with the limited data reported. ^{13}C NMR data has not been reported previously. ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of diastereomers at C-25): δ 0.66 (s, 3H, H_3-18), 0.73–2.38 (m, 37H, incl. 0.91 [d, 3H, J = 6.4 Hz, H_3-21]), 0.99 [s, 3H, H_3-19], 1.68 & 1.69 [2 overlapping d, 3H, J = 6.8 Hz, H_3-26]), 3.50 (m, 1H, H-3), 4.12 (m, 1H, H-25), 5.33 (m, 1H, H-6). ^{13}C NMR (100 MHz, $CDCl_3$) (mixture of diastereomers at C-25): δ 11.9 (C-18), 18.6 (C-21), 18.7 (C-21), 19.4 (C-19), 21.1, 24.27, 24.32, 26.4, 26.6, 28.2, 31.6, 31.9 (2C, incl. C-20), 35.2, 35.3, 35.6, 35.7, 36.5 (C-10), 37.2, 39.8, 41.6, 41.7, 42.27, 42.34, 50.1, 52.0, 52.1, 55.97, 55.99, 56.7 (br), 71.8 (C-3), 121.7 (C-6), 140.7 (C-5). GC–MS (EI): *m/z* (%) Free alcohol: 450/452 (30/29, M^+), 437 (10), 435 (14), 433 (6), 432/434 (18/18, $M^{+\cdot}$-H_2O), 417/419 (14/15), 392 (4), 365/367 (25/25), 339/341 (23/22), 311 (7), 285 (7), 273 (27), 271 (21), 255 (35), 231 (30), 213 (46), 199 (16), 179 (6), 177 (9), 159/161 (43/42), 151 (7), 149 (25), 147 (39), 145 (61), 131 (39), 123 (21), 121 (41), 119 (50), 109 (50), 107 (75), 105 (78), 95 (72), 93 (60), 91 (67), 81 (78), 79 (61), 67 (49), 55 (100), 41 (47). TMS ether: 522/524 (28/26, M^+), 507/509 (11/9, M^+ –CH_3), 488 (5), 460 (2), 449 (2), 433/435 (21/15, $M^{+\cdot}$-OTMS), 432/434 (35/39), 415 (2), 393/395 (46/49), 373 (3), 367 (10), 345 (2), 341 (13), 314 (9), 281/283 (16/14), 253/255 (13/13), 229 (8), 207 (54), 177/179 (5/5), 165 (19), 149/151 (6/5), 135/137 (10/11), 129 (100), 121/123 (5/4), 107 (28), 91 (31), 89 (1), 79/81 (3/3, Br^+), 75 (61), 73 (94, TMS^+), 55 (81), 40 (36). HRMS (ESI): Calcd for $C_{26}H_{43}BrNaO$ ([M+Na]$^+$): 473.2395/475.2375. Obsd: 473.2376/475.2386. HRMS (EI): Calcd for $C_{26}H_{42}BrO$ (M^+): 450.2497/452.2477. Obsd: 450.2499/452.2498.

4.3. UV–vis spectrophotometry

Absolute and difference spectra were recorded using 1 cm path length quartz cuvettes on a dual-beam Cary UV–vis spectrophotometer as described previously.[11] Equilibrium dissociation constants were obtained by measuring the concentration-dependent spin-shifts and were fitted as described elsewhere.[11]

4.4. Conversion of sterols 2–13 to ketones 2k–13k

Sterols **2–13** were converted to the respective ketones **2k–13k** (cholest-4-en-3-one forms for **2k–10k**) by incubation with 0.005 U/μL units of cholesterol oxidase and 5 μg catalase in 50 mM potassium phosphate (pH 7.5, 0.5 mL) for 3 h at 25 °C. For GC–MS analysis, the reaction mixtures were quenched by the addition of 1 N HCl (0.5 mL), then were rapidly mixed and extracted with hexanes (2 × 5 mL). The combined organic extracts were dried over anhydrous $MgSO_4$ (~0.5 g), filtered and then dried under a gentle stream of N_2. In order to derivatize any remaining sterols as their trimethylsilyl (TMS) ethers, the residues were suspended in 50 μL hexanes and then were treated with 70 μL BSTFA and incubated at 37 °C for 2 h prior to GC–MS analysis. For HPLC assays, the reaction mixtures were quenched by the addition of acetonitrile containing 0.2% (v/v) formic acid (0.5 mL). The organic phases were analyzed directly without derivatization. The steroids for further enzymatic assays were mixed with the appropriate reagents in situ and were then processed for either HPLC and/or GC–MS analyzes as described above.

4.5. Enzymatic evaluation of compounds

Cytochrome P450 activity assays and product characterization for each of the sterol analogs with the three enzymes (CYP124A1, CYP125A1, or CYP142A1) were conducted by incubating 250 nm P450 with substrate (50 μM), 5 μg catalase, 50 μg/mL ferredoxin, 0.16 U/mL ferredoxin-$NADP^+$ reductase, 1 mM glucose-6-phosphate, 2.4 U/mL glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$, and 800 μM $NADP^+$ in 50 mM potassium phosphate (pH 7.5, 0.5 mL) at 25 °C for 1 h. The reaction mixtures were quenched and the product mixtures derivatised as TMS ethers and analyzed by GC–MS as described above. Control reactions omitted either the cytochrome P450 enzyme or the NADPH. For assays conducted using the ketone derivatives of the sterols, HPLC analysis was also performed using diode array detection at 240 nm.

4.6. Time-dependent inhibition

27-Norcholesta-5,25-dienol (**7**) was tested with each of the P450 enzymes as a time-dependent inhibitor by pre-incubating 50 μM compound **7** with 5 μM P450 in 50 mM potassium phosphate (pH 7.5, 0.5 mL) containing ferredoxin, ferredoxin-$NADP^+$ reductase, catalase, and the NADPH-regenerating system, as described for catalytic assays. The control pre-incubation reactions were identical but instead contained only the MβCD buffer used to dissolve the steroids. Over time, portions of the pre-incubated enzyme/inhibitor or enzyme/control reactions were diluted twenty-fold into fresh buffer (0.5 mL) containing 50 μM cholest-4-en-3-one (**1k**), spinach ferredoxin, ferredoxin-$NADP^+$ reductase and 1 mM NADPH. These reactions were allowed to proceed for 10 min at 25 °C before being terminated and analyzed for residual catalytic activity by HPLC. Residual activity percentages for each time point were calculated by taking the ratio of enzyme activity towards cholest-4-en-3-one (**1k**) remaining for the enzyme pre-incubated with compound **7** versus the control reaction and multiplying by 100.

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Supplementary data

Supplementary data (characterization of synthetic compounds **14**, (20*E*)-**15**, (20*Z*)-**15**, (17*Z*)-**23**, **26**, **32**, and **35**, and intermediates in the synthesis of **15** and **23** from **14**, ligand binding spectra, mass spectra, and NMR spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.003.

References and notes

1. World Health Organization 2009, Facts Sheets on Tuberculosis. Available at http://www.who.int/tb/publications/factsheets/en/index.html (Accessed 23 January, 2012).
2. Ouellet, H.; Johnston, J. B.; Ortiz de Montellano, P. R. *Trends Microbiol.* **2011**, *19*, 530.
3. Lamb, D. C.; Jackson, C. J.; Warrilow, A. G. S.; Manning, N. J.; Kelly, D. E.; Kelly, S. L. *Mol. Biol. Evol.* **2007**, *24*, 1714.
4. Pearson, A.; Budin, M.; Brocks, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15352.
5. Gatfield, J.; Pieters, J. *Science* **2000**, *288*, 1647.
6. Miner, M. D.; Chang, J. C.; Pandey, A. K.; Sassetti, C. M.; Sherman, D. R. *Ind. J. Exp. Biol.* **2009**, *47*, 407.
7. Pandey, A. K.; Sassetti, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 4376.
8. Mohn, W. W.; van der Geize, R.; Stewart, G. R.; Okamoto, S.; Liu, J.; Dijkhuizen, L.; Eltis, L. D. *J. Biol. Chem.* **2008**, *283*, 35368.
9. Brzostek, A.; Dziadek, B.; Rumijowska-Galewicz, A.; Pawelczyk, J.; Dziadek, J. *FEMS Microbiol. Lett.* **2007**, *275*, 106.
10. Ouellet, H.; Guan, S.; Johnston, J. B.; Chow, E. D.; Kells, P. M.; Burlingame, A. L.; Cox, J. S.; Podust, L. M.; de Montellano, P. R. *Mol. Microbiol.* **2010**, *77*, 730.
11. Johnston, J. B.; Ouellet, H.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **2010**, *285*, 36352.
12. Driscoll, M. D.; McLean, K. J.; Levy, C.; Mast, N.; Pikuleva, I. A.; Lafite, P.; Rigby, S. E.; Leys, D.; Munro, A. W. *J. Biol. Chem.* **2010**, *285*, 38270.
13. Rosloniec, K. Z.; Wilbrink, M. H.; Capyk, J. K.; Mohn, W. W.; Ostendorf, M.; van der Geize, R.; Dijkhuizen, L.; Eltis, L. D. *Mol. Microbiol.* **2009**, *74*, 1031.
14. McLean, K. J.; Lafite, P.; Levy, C.; Cheesman, M. R.; Mast, N.; Pikuleva, I. A.; Leys, D.; Munro, A. W. *J. Biol. Chem.* **2009**, 35524.
15. Capyk, J. K.; Kalscheuer, R.; Stewart, G. R.; Liu, J.; Kwon, H.; Zhao, R.; Okamoto, S., ; Jacobs, W. R., Jr.; Eltis, L. D.; Mohn, W. W. *J. Biol. Chem.* **2009**, *284*, 35534.
16. Griffin, J. E.; Gawronski, J. D.; Dejesus, M. A.; Ioerger, T. R.; Akerley, B. J.; Sassetti, C. M. *PLoS Pathog.* **2011**, 7.
17. Johnston, J. B.; Ouellet, H.; Podust, L. M.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **2011**, *507*, 86.
18. McLean, K. J.; Dunford, A. J.; Neeli, R.; Driscoll, M. D.; Munro, A. W. *Arch. Biochem. Biophys.* **2007**, *464*, 228.
19. Van der Geize, R.; Yam, K.; Heuser, T.; Wilbrink, M. H.; Hara, H.; Anderton, M. C.; Sim, E.; Dijkhuizen, L.; Davies, J. E.; Mohn, W. W.; Eltis, L. D. *Proc. Natl. Acad. Sci. U.S.A.* **1947**, *2007*, 104.
20. Johnston, J. B.; Kells, P. M.; Podust, L. M.; Ortiz de Montellano, P. R. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 20687.
21. Dygos, J. H.; Desai, B. N. *J. Org. Chem.* **1979**, *44*, 1590.
22. Hazra, B. G.; Joshi, P. L.; Bahule, B. B.; Argade, N. P.; Pore, V. S.; Chordia, M. D. *Tetrahedron* **1994**, *50*, 2523.
23. Drew, J.; Letellier, M.; Morand, P.; Szabo, A. G. *J. Org. Chem.* **1987**, *52*, 4047.
24. Sondheimer, F.; Mechoulam, R. *J. Am. Chem. Soc.* **1957**, *79*, 5029.
25. Pettit, G. R.; Green, B.; Dunn, G. L.; Sunder-Plassmann, P. *J. Org. Chem.* **1970**, *35*, 1385.
26. Bergmann, E. D.; Rabinovitz, M.; Levinson, Z. H. *J. Am. Chem. Soc.* **1959**, *81*, 1239.
27. Schmit, J. P.; Piraux, M.; Pilette, J. F. *J. Org. Chem.* **1975**, *40*, 1586.
28. McMorris, T. C.; Schow, S. R. *J. Org. Chem.* **1976**, *41*, 3759.
29. Drew, J.; Gowda, G.; Morand, P.; Proulx, P.; Szabo, A. G.; Williamson, D. *J. Chem. Soc., Chem. Commun.* **1985**, 901.
30. Nuzillard, J.-M.; Boumendjel, A.; Massiot, G. *Tetrahedron Lett.* **1989**, *30*, 3779.
31. Ikan, R.; Markus, A.; Bergmann, E. D. *J. Org. Chem.* **1971**, *36*, 3944.
32. Kametani, T.; Katoh, T.; Tsubuki, M.; Honda, T. *J. Am. Chem. Soc.* **1986**, *108*, 7055.
33. Kametani, T.; Katoh, T.; Tsubuki, M.; Honda, T. *Chem. Pharm. Bull.* **1987**, *35*, 2334.
34. Kametani, T.; Katoh, T.; Fujio, J.; Nogiwa, I.; Tsubuki, M.; Honda, T. *J. Org. Chem.* **1982**, *1988*, 53.
35. Narwid, T. A.; Cooney, K. E.; Uskokovic, M. R. *Helv. Chim. Acta* **1974**, *57*, 771.
36. Hershberg, E. B.; Oliveto, E. P.; Gerold, C.; Johnson, L. *J. Am. Chem. Soc.* **1951**, *73*, 5073.
37. Izzo, I.; Di Filippo, M.; Napolitano, R.; De Riccardis, F. *Eur. J. Org. Chem.* **1999**, *1999*, 3505.
38. Deng, L.; Wu, H.; Yu, B.; Jiang, M.; Wu, J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2781.
39. Batcho, A. D.; Berger, D. E.; Davoust, S. G.; Wovkulich, P. M.; Uskoković, M. R. *Helv. Chim. Acta* **1981**, *64*, 1682.
40. Izgu, E. C.; Burns, A. C.; Hoye, T. R. *Org. Lett.* **2011**, *13*, 703.

41. Kurek-Tyrlik, A.; Marczak, S.; Michalak, K.; Wicha, J. *Synlett* **2000**, *2000*, 547.
42. Kurek-Tyrlik, A.; Marczak, S.; Michalak, K.; Wicha, J.; Zarecki, A. *J. Org. Chem.* **2001**, *66*, 6994.
43. Xiao, Y.; Liu, P. *Angew. Chem., Int. Ed.* **2008**, *47*, 9722.
44. *Cytochrome P450*; Ortiz de Montellano, P. R., Ed.Structure, Mechanism, and Biochemistry; Kluwer Academic/Plenum Publishers: New York, 2005.
45. Jefcoate, C. R. *Methods Enzymol.* **1978**, *52*, 258.
46. Ouellet, H.; Lang, J.; Couture, M.; Ortiz de Montellano, P. R. *Biochemistry* **2009**, *48*, 863.
47. McCauliff, L. A.; Xu, Z.; Storch, J. *Biochemistry* **2011**, *50*, 7341.
48. Ortiz de Montellano, P. R. *Chem. Rev.* **2010**, *110*, 932.
49. Rittle, J.; Green, M. T. *Science* **2010**, *330*, 933.
50. Satsangi, R. K.; Mott, G. E. *Analyst* **1992**, *117*, 953.
51. Ferraboschi, P.; Rezaelahi, S.; Verza, E.; Santaniello, E. *Tetrahedron: Asymmetry* **1998**, *9*, 2193.
52. Dauben, W. G.; Bradlow, H. L. *J. Am. Chem. Soc.* **1950**, *72*, 4248.
53. Kircher, H. W.; Rosenstein, F. U. *J. Org. Chem.* **1982**, *47*, 1722.
54. Ciuffreda, P.; Casati, S.; Bollini, D.; Santaniello, E. *Steroids* **2003**, *68*, 193.
55. Matsuya, Y.; Yamakawa, Y.-i.; Tohda, C.; Teshigawara, K.; Yamada, M.; Nemoto, H. *Org. Lett.* **2009**, *11*, 3970.
56. Sato, Y.; Sonoda, Y. *Chem. Pharm. Bull.* **1982**, *30*, 628.
57. Westover, E. J.; Covey, D. F. *Steroids* **2006**, *71*, 484.
58. Morisaki, M.; Shibata, M.; Duque, C.; Imamura, N.; Ikekawa, N. *Chem. Pharm. Bull.* **1980**, *28*, 606.
59. Acuna-Johnson, A. P.; Oehlschlager, A. C.; Pierce, A. M.; Pierce, H. D., Jr.; Czyzewska, E. K. *Bioorg. Med. Chem.* **1997**, *5*, 821.
60. Ryer, A. I.; Gebert, W. H.; Murrill, N. M. *J. Am. Chem. Soc.* **1953**, *75*, 491.
61. Mason, J. I.; Arunachalam, T.; Caspi, E. *Biochim. Biophys. Acta Lipids Lipid Metab.* **1983**, *752*, 265.