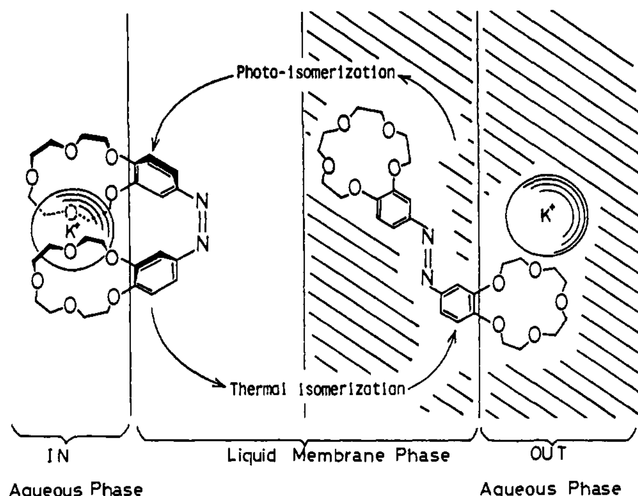
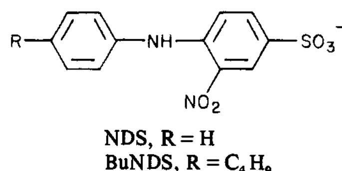


Figure 5. Schematic representation of light-driven ion transport.

the order of their hydrophobicity). The results are summarized in Table III.



NDS, R = H
BuNDS, R = C_4H_9

The rate of ion transport is strongly dependent on the anion present.[31] We also observed that the transport rate is markedly affected by the hydrophobicity of the anion. Under dark conditions, the transport rate of BuNDS·K^+ is greater by a factor of more than 300 than that of NDS·K^+. The transport of picrate·K^+ is 7 times faster than that of BuNDS·K^+. The differences are rationalized in terms of the hydrophobicity of counteranions. Interestingly, the photoirradiation brought about two contrasting influences on the transport rate: the transport of picrate·K^+ and BuNDS·K^+ was decelerated by 1.9-fold and 1.7-fold, respectively, whereas that of NDS·K^+ was markedly accelerated on photoirradiation (17-fold). As described in the introduction, there are two possible rate-limiting steps in an ion-transport system: the rate-limiting step for very stable complexes is at the ion-release site while that for less stable complexes is at the ion-complexation site. The situation is applicable to the effect of counteranions. Probably, the increase in hydrophobicity of counteranions changes the rate-limiting step from the ion-complexation site to the ion-release site. The effect of photoirradiation in Table III is rationalized if one accepts that the rate-limiting step for picrate·K^+ and BuNDS·K^+ is at the ion-release site and that for NDS·K^+ is at the ion-complexation site. In the transport of picrate·K^+ and BuNDS·K^+, photoisomerized *cis*-**1** would further suppress the release of K^+ to the OUT aqueous phase. However, if ion complexation from the IN aqueous phase is rate limiting, it would facilitate the extraction of NDS·K^+, resulting in an enhancement in the overall transport velocity. These results demonstrate that the acceleration of ion transport by light occurs only when the ion transport in the rate-limiting step is modified by light.

Table III shows, however, that there is an exception. We found that when one-fifth of the *o*-dichlorobenzene phase in contact with the IN aqueous phase is partially photoirradiated, the transport of picrate·K^+ is accelerated. As expected, photoisomerized *cis*-**1** rapidly extracts K^+ from the IN aqueous phase, and while moving in the liquid membrane to the ion-relase site, it would isomerize thermally to *trans*-**1**. Hence, the concentratiuon of K^+ in the liquid membrane phase can be enhanced without perturbing the situation in the ion-release site. The enhanced ion concentration would necessarily lead to the acceleration of K^+ transport (Figure 5). This finding implies that the dilemma which may occur in the ion-transport system can be broken by changing the binding ability of ion carrier by light.

Concluding Remarks. The present system demonstrated that, in principle, ion extraction and ion transport through a liquid membrane can be controlled by light. The novel phenomena are attained because **1** combines within a molecule an antenna (azobenzene) which acts as a photoresponsive trigger and a functional group (crown ether) which causes a subsequent event. We expect that this concept might lead to a more efficient control of ion extraction, ion transport, and other effects by switching the light source on and off.

Acknowledgment. We thank Professors T. Kunitake and M. Takagi for helpful discussions. We also thank Professor K. Higashi for bringing some references on the dipole moment of azobenzene to our attention.

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Minor and Trace Sterols in Marine Invertebrates. 19.[1] Isolation, Structure Elucidation, and Partial Synthesis of 24-Methylene-25-ethylcholesterol (Mutasterol): First Example of Sterol Side-Chain Bioalkylation at Position 25

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Abstract: A new C_{30} sterol—mutasterol—has been isolated as a minor component of the sterol fraction from the Caribbean sponge *Xestospongia muta*. Its structure (**5**) (24-methylene-25-ethylcholesterol) has been deduced from spectroscopic data and confirmed by partial synthesis. The side chain contains an acyclic, quaternary carbon—hitherto unknown among sterols—and its biosynthetic origin is discussed. The sterol composition is compared with that of other *Xestospongia* species collected at different locations.

Sterols have been found in all forms of life, from the higher animals and plants down to the protista at the bottom of the evolutionary hierarchy.[3] The only exceptions seem to be found among bacteria.[4] In higher animals and plants only a few major

(1) For Part 18 in this series, see: Kokke, W. C. M. C.; Fenical, W.; Bohlin, L.; Djerassi, C. *Comp. Biochem. Physiol.*, in press.

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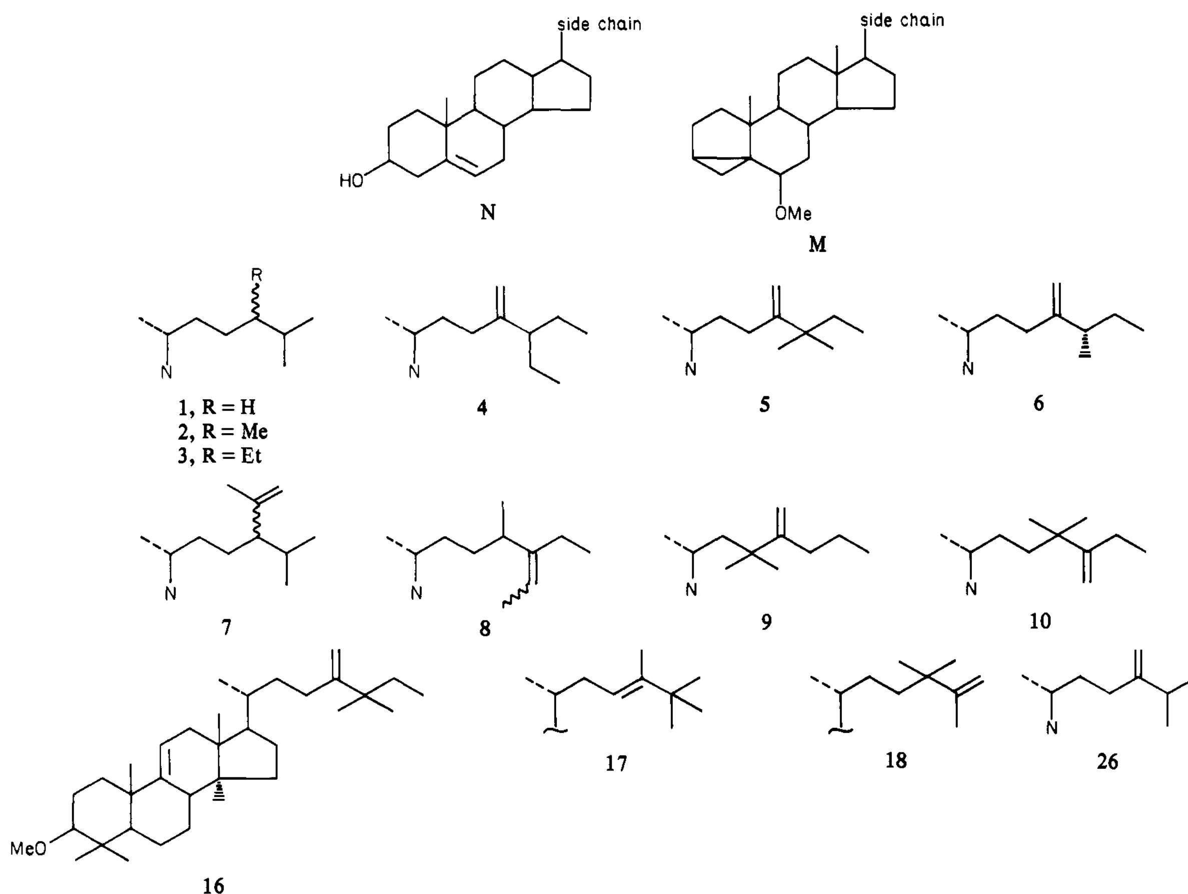
Chart I



sterols are found, typified by cholesterol (**1**) and its 24-methyl (**2**) and 24-ethyl (**3**) derivatives. This has been interpreted[5] in terms of a finer "tuning" of the membrane function during evolution, leading to a closer fit of the sterol into the system and hence allowing less flexibility for the sterol structure. Among lower organisms, on the other hand, the steric requirements are less strict, and sometimes even a sterollike molecule may play the same role.[6]

The last decade has shown lower invertebrates, almost exclusively of marine origin, to be a rich source of sterols with new structures. Until now, more than 120 new sterols have been characterized from marine organisms, many without terrestrial counterparts.[7] Discoveries of unusual sterols as major constituents can give information on the phylogenetic significance of the sterol structure and can also shed some light on the complex structure–function role of the sterol in the membranes. These major sterols can also be used in labeling experiments for biosynthetic studies. New sterols present in minor and trace amounts may offer important clues to biosynthetic or dietary pathways for the major components.

Recently,[8] we have examined a sample of a deep sea sponge collected at Lighthouse Reef, Belize. It was first identified as *Xestospongia muta*, but a reexamination has shown it to be another *Xestospongia* species, not yet determined. It contained as the major sterol (71%) xestosterol (**4**)—a C_{30} sterol with an abnormally long side chain. The virtual absence of "conventional" sterols and its relatively high titer suggest that xestosterol may be a membrane constituent. This interesting observation prompted us to examine another sponge of the same genus, *X. muta*, collected at Barbados. The new specimen contained no xestosterol (**4**), but analysis led to the discovery of a new sterol, now named mutasterol (**5**), which proved to be the first naturally occurring sterol with an acyclic quaternary carbon atom in the side chain. Finally, still another sponge from the same genus, *Xestospongia exigua*, collected in the South China Sea, has been analyzed for its sterol content and found to be completely different.

Results and Discussion

Argentic TLC of the sterol acetates from *X. muta* from Barbados followed by conversion to free sterols and GC–MS analysis of the fractions showed that at least 18 sterols (cf. *Table* I) were present in this sample. The main sterol was 24(28)-dehydroaplysterol (**6**).[9] Isolation on reverse-phase high-performance liquid chromatography (LC) yielded four minor C_{30} sterols with $M^+ = m/z$ 426, but none of them was identical with xestosterol (**4**). The amount of one of them was too small to allow structure determination. Two of them were recently described compounds—24-isopropenylcholesterol (**7**)[10] and verongulasterol (**8**).[11] The fourth sterol proved to be unknown and was named mutasterol.

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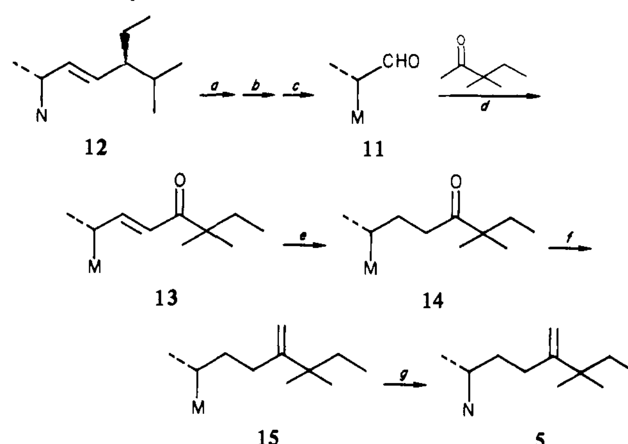
Table I. Sterols of *Xestospongia muta* and *X. exigua*

structure[a]		mobility[b]		M^+	% sterol fraction	
		LC[c]	GC	*m/z*	*X. muta*	*X. exigua*
	27	0.60	0.65	370		0.4
	28	0.71	0.95	384	1.9	3.2
	29	0.74	0.95	384		
	26	0.80	1.32	398		51.4
	30	0.82	1.16	398	9.0	7.4
	31	0.88	1.16	398		
	32	0.83	1.67	412	trace	trace
	33	0.91	1.72	412		8.3
	6	0.91	1.72	412	39.3	
	34	0.93	1.76	412	trace	
	7	0.95	1.88	426	trace	
	1	1.00	1.00	386	17.1	13.5
	35	1.07	1.00	388		
	12	1.02	1.42	412	3.9	trace
	8	1.02	2.16	426	9.7	
	2	1.07	1.32	400	3.3	trace
	5	1.07	2.32	426	3.2	
	3	1.12	1.61	414	9.7	5.9
	36	1.22	1.70	414	trace	
	37	1.23	1.34	402	trace	trace

[a] All structures have a Δ^5 nucleus except 35 and 37, which have a 5α-H structure. [b] Cholesterol is taken as the standard (1.00). [c] High-performance liquid chromatography.

Its mass spectrum was very similar to that of xestosterol (**4**), which suggested a Δ^5-3β-ol nucleus and a 24(28)-double bond in the side chain (*m/z* 314 is the base peak).[12] A 24-methylene group was deduced from the 360-MHz ^{1}H NMR spectrum, which showed two broad singlets at 4.75 and 4.78 ppm in $CDCl_3$. The methyl region of the spectrum revealed a doublet at δ 0.953 (3 H, J = 6.43 Hz), a triplet at δ 0.703 (3 H, J = 7.3 Hz)—pointing to the presence of an ethyl substituent—and two singlets at δ 0.684 (3 H) and 1.005 (9 H). In C_6D_6 the latter singlet was split into two singlets at δ 0.949 (3 H) and 1.069 (6 H), respectively. Two structures consistent with these data are **5** and **9**, the difference being the position of the isochronous methyl groups. However, a quartet centered at 1.4 ppm (2 H) could also be seen, which collapsed to a singlet when the triplet was irradiated and therefore can be assigned to an ethyl group attached to a quaternary carbon. These data exclude **9** as a possible candidate. A structure such as **10**, otherwise consistent with the NMR data, is ruled out on mass spectral grounds, since such a compound would be expected[12] to display an *m/z* 328 rather than *m/z* 314 peak. The only structure consistent with both NMR and mass spectral data is **5**, 24-methylene-25-ethylcholesterol (25,26-dimethylergosta-5,24(28)-dien-3β-ol).

Scheme I. Synthetic Route to Mutasterol



[a] TsCl-pyridine. [b] KOAc/MeOH. [c] O_3/CH_2Cl_2, 1% pyridine, -78 °C. [d] *n*-BuLi, *i*-Pr_2NH. [e] H_2, 5% Pd(C). [f] $Ph_3PCH_3^+Br^-$, *n*-BuLi, THF. [g] TsOH, H_2O in *p*-dioxane.

To confirm the structure we synthesized **5** by the route outlined in Scheme I. The aldehyde **11**, with the Δ^5-double bond protected as an *i*-ether, was obtained from stigmasterol (**12**) by known procedures.[13] Aldol condensation at -70 °C with 3,3-dimethyl-2-pentanone[14] in the presence of lithium diisopropylamide yielded the enone **13** in poor yield. However, it was easily separated by chromatography from the crude reaction mixture, since the enone is UV active and can be spotted on TLC. The aldol condensation is a shorter route for attaching a side chain to the aldehyde **11** than the conventional Wittig condensation used frequently in the generation of sterols with unsaturated side chains.[15] A problem is the strong basic conditions used, which might cause epimerization at C-20. Chromatographic properties and NMR spectral parameters, however, are quite different for enones epimeric at C-20, and by comparison with known compounds,[16] it could be shown that the product had retained its configuration at C-20.

Catalytic hydrogenation gave the ketone **14** in quantitative yield. A Wittig reaction with methyltriphenylphosphonium bromide in THF followed by deprotection of the *i*-ether of **15** yielded the desired product **5**. Injection on capillary GC gave the same retention time as the natural compound, the high-resolution mass spectra were virtually superimposable, and the 360-MHz ^{1}H NMR spectra were identical in both $CDCl_3$ and C_6D_6.

The structure of mutasterol (**5**) is unusual in the sense that it contains an acyclic, quaternary carbon in the side chain. This

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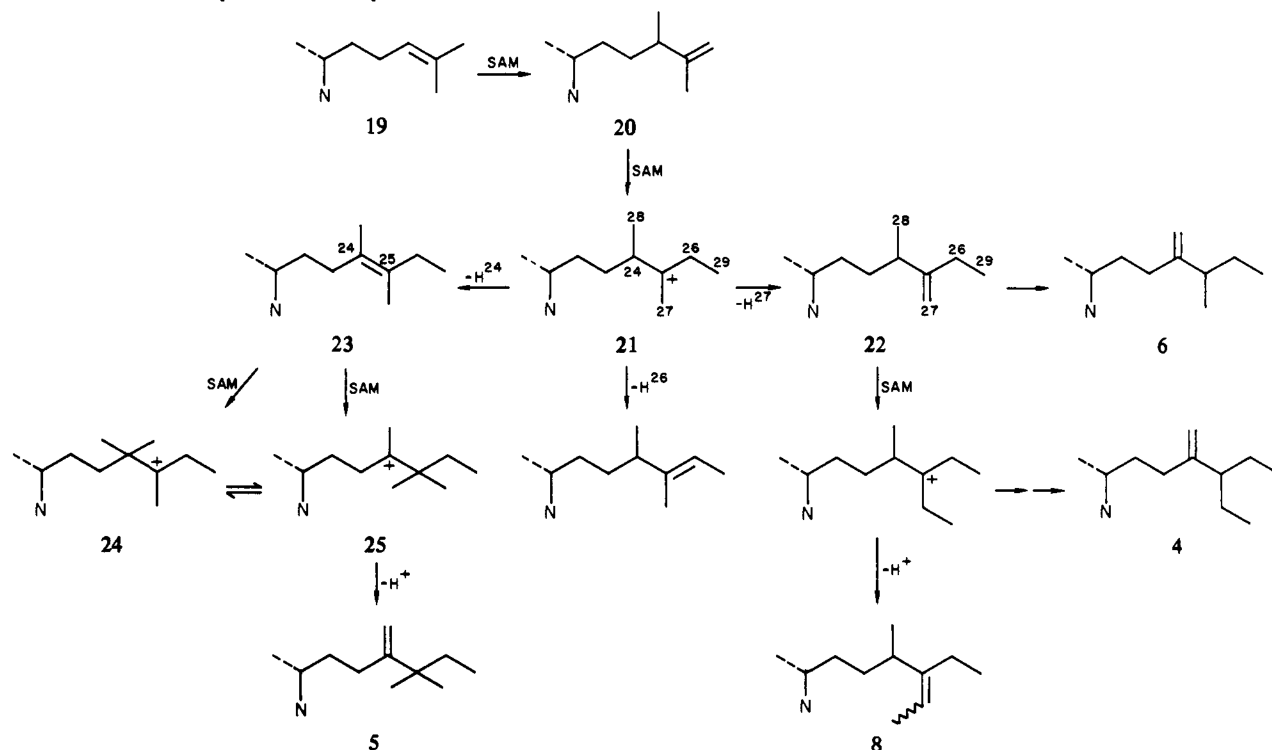
Scheme II. Possible Biosynthetic Pathways for Mutasterol and Related Unusual Sterols



is the first example of such a side chain in a sterol, but a few have been encountered among triterpenoids. Bosistoin (**16**),[17a] also named *O*-methylpertyol,[17b] is a triterpene found in two higher plants and contains the same side chain as mutasterol (**5**). The side chain **17**, found in another terpene,[17c] also contains a quaternary center at C-25, whereas **18**, first seen in cycloneolitsin,[17d] represents a class of side chains with the quaternary center at C-24.

It is not clear whether sponge sterols are synthesized de novo by the sponge, result from modification of dietary sterols, or are dietary constituents. The opinion now seems to be that sponges are not capable of de novo synthesis.[18] If instead we assume that the sponge modifies dietary sterols, desmosterol (**19**) could serve as a starting material, since it is an important biosynthetic intermediate in many systems, e.g., as a precursor to cholesterol and 24-alkylated side chains.[18,19] The biosynthetic pathways outlined in Scheme II could explain several of the extended-side-chain sterols found in this sponge. Biomethylation via *S*-adenosylmethionine (SAM) and deprotonation would lead to **20** (codisterol[20] or its recently encountered[10] 24-epimer), which can undergo a second biomethylation to the carbonium ion **21**. Deprotonation can now occur in three ways, from C-24, C-26, and C-27. If **21** is deprotonated at C-27, 25(27)-dehydroaplysterol (**22**) is produced which can undergo biological hydrogenation–dehydrogenation[21] to 24(28)-dehydroaplysterol (**6**) or another biomethylation at C-27 followed by deprotonation to give verongulasterol (**8**)—both sterols having been found in this sample (cf. Table I). The same 25(27)-dehydroaplysterol (**22**) is also believed to be the precursor to xestosterol (**4**).[8] Products from deprotonation at C-26 have not been found, but deprotonation from C-24 to give the desmosterol homologue **23** has now been demonstrated indirectly since bioalkylation with SAM is now only possible at C-24 or C-25. The former leads to the carbonium ion **24** while attack at C-25 generates **25**. If enzymatically induced methyl migration operates, then these two tertiary carbonium ions are interchangeable. Proton loss from **25** will then yield mutasterol (**5**).

Mutasterol, comprising only 3% of the sterol fraction, probably does not play any important role in the membranes of this sponge. 24(28)-Dehydroaplysterol (**6**), on the other hand, being the most abundant sterol (39%), may very well serve as such a constituent.

An examination of the sponge *Xestospongia exigua* from the South China Sea yielded 24(28)-methylenecholesterol (**26**) as the main sterol (51%) together with other conventional sterols (cf. Table I). No sterols with alkylation at C-26/C-27 could be found, not even at trace levels.

The puzzling question still remains why three species of the same genus produce so completely different sterol mixtures. Are there physical differences in the environment, such as water temperature, salinity, pH, and trace elements, that might change the course of the biosynthesis? Or do these sponges have symbiotic relations to zooxanthellae, responsible for the sterol production, and the differences in sterol content merely reflect different symbionts?[1] In our opinion the most likely possibility is the incorporation of a different dietary precursor in these three locations.

We hope to undertake experiments that will shed some light on the intriguing questions of the biosynthetic origin of novel marine sterols and of their role in the complex structure–function role of sterols in cellular membranes.

Experimental Section

General Methods. Analytic GC was carried out on a Hewlett-Packard 402A chromatograph equipped with a flame-ionization detector and a "U"-shaped glass column (1.80 m × 4-mm i.d.) containing 3% OV-17 on GCQ (carrier gas He, oven temperature 260 °C) or a Carlo Erba capillary GC (Fractovap 4160) equipped with a flame-ionization detector and a capillary glass column (15 m × 0.3-mm i.d.) containing SE-52 (carrier gas H_2, oven temperature 260 °C).

Preparative high-performance liquid chromatography (LC) was performed on a Whatman Partisil M9 10/50 ODS-2 reverse-phase column (50 cm × 1 cm), using a Haskel Model 28303 pump and a Waters Associates dual-cell refractometer detector. Absolute methanol was used as eluent (flow rate 1.5 mL min^{-1}, pressure 500 psi).

Combined GC–MS analysis was performed on a Varian MAT 44 GC–MS system using a spiral glass column (2 m × 2.7-mm i.d.), containing 3% OV-17 on GCQ (carrier gas He, temperature 260 °C).

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High-resolution mass spectra were recorded on a Varian MAT 711 double-focusing spectrometer, equipped with a PDP-11/45 computer for data acquisition and reduction.

^{1}H NMR spectra were recorded on a Bruker HXS-360 (360 MHz) or a Varian XL-100 (100 MHz) spectrometer. Me_4Si was used as internal standard and $CDCl_3$ as solvent unless otherwise stated.

Melting points were measured on a Thomas-Hoover Unimelt capillary melting point apparatus, and specific rotations were obtained on a Rudolph Research Autopol III polarimeter.

Extraction and Isolation of Free Sterols. A frozen sample (900 g) of the sponge *Xestospongia muta* (class Demospongiae, order Haplosclerida), collected at Barbados, was homogenized in a Waring blender and extracted according to the Bligh and Dyer procedure[22] ($CHCl_3$/$MeOH$/H_2O). The chloroform layer was evaporated under reduced pressure, yielding a crude extract (14.3 g), which was applied to a silica gel column (1000 g). The column was successively washed with hexane, hexane–toluene (3:2), hexane–ether (1:1), and ether–methanol (1:1). Fractions of 500 mL were collected and examined on TLC. Free sterols (0.566 g) were obtained from the hexane–ether (1:1) fractions 3–13. Sterol acetates formed by acetylation with pyridine–acetic anhydride were separated into five fractions by argentic TLC, using hexane–toluene (3:2) as solvent. Each fraction was eluted with ether and saponified in NaOH/MeOH, before being subjected to LC. Fraction 5 contained 24(28)-dehydroaplysterol (**6**) and mutasterol (**5**).

A dry sample (30 g) of *X. exigua*, collected in the South China Sea, off the Province Kuang-xi (China), was extracted with acetone in a Soxhlet extractor for 24 h to yield, after evaporation, 500 mg of a crude extract. Chromatography on silica (see above) gave the sterol fraction (56 mg), which was subjected to preparative LC for separation of the different sterols. Identification of the sterols described in Table I was performed by mass spectrometric and 360-MHz NMR spectral means and by comparison of chromatographic mobility with appropriate reference compounds.

(22*E*)-25,26-Dimethyl-6β-methoxy-3α,5-cyclocholest-22-en-24-one (13). The aldol condensation procedure of Eyley and Williams was followed.[23] *n*-Butyllithium (7.2 mL of 1.6 M solution in hexane, 11.5 mmol) was added to a stirred solution of diisopropylamine (1.11 g, 11.0 mmol) in dry THF (10 mL) under nitrogen at 0 °C. 3,3-Dimethylpentan-2-one[14] (1.14 g, 10.0 mmol) in dry THF (10 mL) was added dropwise at 0 °C during 20 min. The solution was stirred at 0 °C for 1 h, and cooled to −70 °C, and a solution of the crude aldehyde **11**[13] (4 g, 10 mmol) in dry THF (50 mL) was added dropwise. The temperature was raised to −20 °C (1 h), lowered to −70 °C overnight, and kept at −20 °C for another 2 h. Glacial acetic acid (1.5 mL) was added and the solution brought to room temperature. Ether (500 mL) and water (250 mL) were added and the organic layer was separated and washed with dilute hydrochloric acid, sodium bicarbonate solution, and water. Evaporation of the solvent gave a crude product, which was dissolved in THF (60 mL) containing 1.5 N hydrochloric acid (8 mL). After 3 h at room temperature the mixture was diluted with ether and the organic phase was separated, washed with sodium bicarbonate solution and water, and dried over sodium sulfate. Evaporation of the solvent yielded the crude product (5 g). Chromatography on silica with hexane–ether as eluent with increasing amount of ether gave fractions which were checked on TLC for UV-active (254 nm) spots. Further purification of some of the fractions on LC gave a white solid (273 mg, 6%): mp 98–99 °C; ^{1}H NMR (100 MHz) δ 0.755 (3, s, C-18 Me), 0.778 (3, t, J = 7.5 Hz, C-29 Me), 1.026 (3, s, C-19 Me), 1.080 (3, d, J = 6.5 Hz, C-21 Me), 1.098 (6, s, C-27/C-28 Me), 3.322 (3, s, C-6 OMe), 6.37 (1, d, J = 15.5 Hz, C-23 H), 6.78 (1, dd, J = 8.3, 15.5 Hz, C-22 H); mass spectrum, m/z (relative intensity) 440.3660 (M^+, $C_{30}H_{48}O_2$, 89; calcd 440.3654), 425 (32), 408 (74), 385 (56), 369 (23), 337 (61), 309 (42), 255 (42), 253 (27), 154 (100). The peaks at m/z 408 and 385 are characteristic for this type of *i*-ether and originate from loss of methanol ($M^+ - 32$) and ring-A cleavage ($M^+ - 55$), respectively.[24]

25,26-Dimethyl-6β-methoxy-3α,5-cyclocholestan-24-one (14). The enone 13 (159 mg, 0.36 mmol) was dissolved in ethyl acetate (25 mL), and 5% palladium-on-carbon (75 mg) was added. The solution was kept under an atmosphere of hydrogen for 45 min and then checked by TLC. The UV-active spot had disappeared and a new spot with a slightly longer retention time could be seen [silica, hexane–ether(9:1)]. The catalyst was removed by filtration on a short column with Florisil. Evaporation of the solvent left an oil which crystallized on standing: yield 159.5 mg (100%); mp 88–89 °C (from MeOH); ^{1}H NMR (360 MHz), δ 0.708 (3, s, C-18 Me), 0.778 (3, t, J = 7.5 Hz, C-29 Me), 0.903 (3, d, J = 6.5 Hz, C-21 Me), 1.015 (3, s, C-19 Me), 1.088 (6, s, C-27/C-28 Me), 3.318 (3, s, C-6 OMe); mass spectrum m/z 442 (M^+, $C_{30}H_{50}O_2$).

25,26-Dimethyl-6β-methoxy-3α,5-cycloergost-24(28)-ene (15). Methyltriphenylphosphonium bromide (623.6 mg, 1.75 mmol) was suspended in anhydrous THF (30 mL) under nitrogen. A 1.6 M solution (1.09 mL, 1.75 mmol) of *n*-butyllithium in hexane was added and the solution was heated to gentle reflux (1 h) and cooled to room temperature. The ketone **14** (159.5 mg, 0.360 mmol) in dry THF (5 mL) was added and the solution left at reflux for 30 h (starting material absent by TLC). After cooling, the solution was poured into ether and washed with water, and the organic phase was dried over magnesium sulfate. Evaporation of the solvent left a brown tar, which was chromatographed on silica gel [hexane–ether(1:1)]. TLC showed two spots, and these compounds could be separated on silica [hexane–ether (95:5)]. GC showed one peak with some base-line noise; yield 97 mg (61%); mass spectrum, m/z 440 (M^+, $C_{31}H_{52}O$). NMR showed a new peak around 4.8 ppm (2 H).

25,26-Dimethylergosta-5,24(28)-dien-3β-ol (24-Methylene-25-ethylcholesterol, Mutasterol) (5). The *i*-ether **15** (92 mg, 0.209 mmol) was dissolved in *p*-dioxane (25 mL) and water (5 mL) and *p*-toluenesulfonic acid (25 mg) was added. The solution was heated under reflux for 1 h (starting material absent by TLC) and, after cooling, poured into ether. The organic phase was washed with water, sodium bicarbonate solution, and water and dried over magnesium sulfate. The solvent was evaporated to leave colorless crystals (66.3 mg, 74%), which after recrystallization from MeOH gave the following: mp 146–148 °C; $[\alpha]_D^{25.0}$ −26° (*c* 0.29, $CHCl_3$). Gas chromatography (SE-52, capillary GC) showed only one peak, retention time 8.40 min. Subsequent coinjection of natural mutasterol still showed only one peak. ^{1}H NMR (360 MHz) δ 0.686 (3, s, C-18 Me), 0.705 (3, t, J = 7.5 Hz, C-30 Me), 0.954 (3, d, J = 6.6 Hz, C-21 Me), 1.006 (9, s, C-19/C-27/C-29 Me), 1.369 (2, q, J = 7.5 Hz, C-26 H), 3.51 (1, m, C-3 H), 4.757 (1, s, C-28 H), 4.786 (1, s, C-28 H), 5.35 (1, br, C-6 H); mass spectrum, m/z (relative intensity) 426.3883 (M^+, $C_{30}H_{50}O$, 9; calcd 426.3862), 411 (4), 408 (4), 314 (100), 300 (10), 299 (13), 281 (13), 271 (16).

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