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Biosynthetic Studies of Marine Lipids. 22.[1] Calysterol: Some Aspects of Sterol Cyclopropene Biosynthesis in the Sponge *Calyx nicaeensis*

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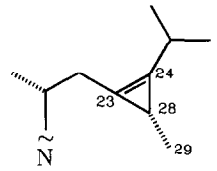
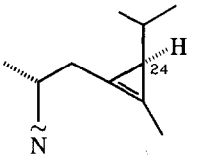
Abstract—Calysterol (**1**) was shown not to be biosynthesized by direct dehydrogenation of its cyclopropane analog, dihydrocalysterol (**5**). Loss of H-28 from **5** during the biosynthesis of **1** was demonstrated, which implies that either 24-*H*-isocalysterol (**2**) or (23*S*)-23*H*-isocalysterol (**4**) is the direct dehydrogenation product of **5**. A novel synthesis of **5** is described which allowed facile introduction of tritium at C-28.

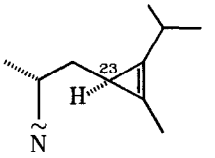
Introduction

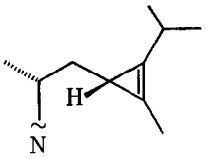
Calysterol (**1**),[2,3] the principal sterol in the Mediterranean sponge *Calyx nicaeensis*, possesses one of the most intriguing functionalities—a cyclopropene ring—in the side chain. Subsequent to the isolation of calysterol, two other isomeric cyclopropenes, **2**[4] and **3**,[3] as well as the cyclopropane, dihydrocalysterol (**5**),[3,5] were later isolated from the same sponge. More recently,[6] another cyclopropene, (23*S*)-23*H*-isocalysterol (**4**), was isolated from the Caribbean sponge *Calyx podatypa*.

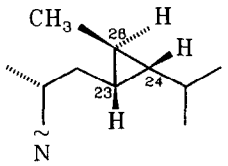
The biosynthesis of these unusual sterols has been the focus of two studies.[7,8] Feeding experiments using radiolabeled dihydrocalysterol (**5**) demonstrated that calysterol (**1**) and its isomers, **2** and **3**, are produced by dehydrogenation of **5**.[8] The details of the biosynthetic steps from **5** to calysterol and its isomers are largely unknown. Two alternative pathways are possible: (i) calysterol might be produced directly by dehydrogenation of **5**; or (ii) one of the other isomers—particularly **2** or **4**—is initially produced from **5** which then isomerizes to calysterol and the other cyclopropenes. In this report, we describe the synthesis and results of feeding experiments of [3-^{3}H]- and [28-^{3}H]-labeled dihydrocalysterol which unambiguously ruled out the first of these two pathways.

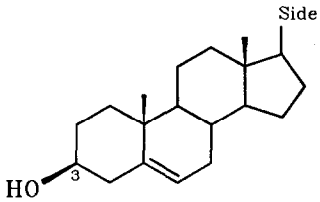












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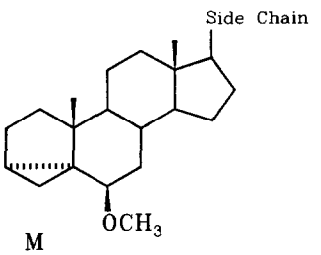

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Results and Discussion

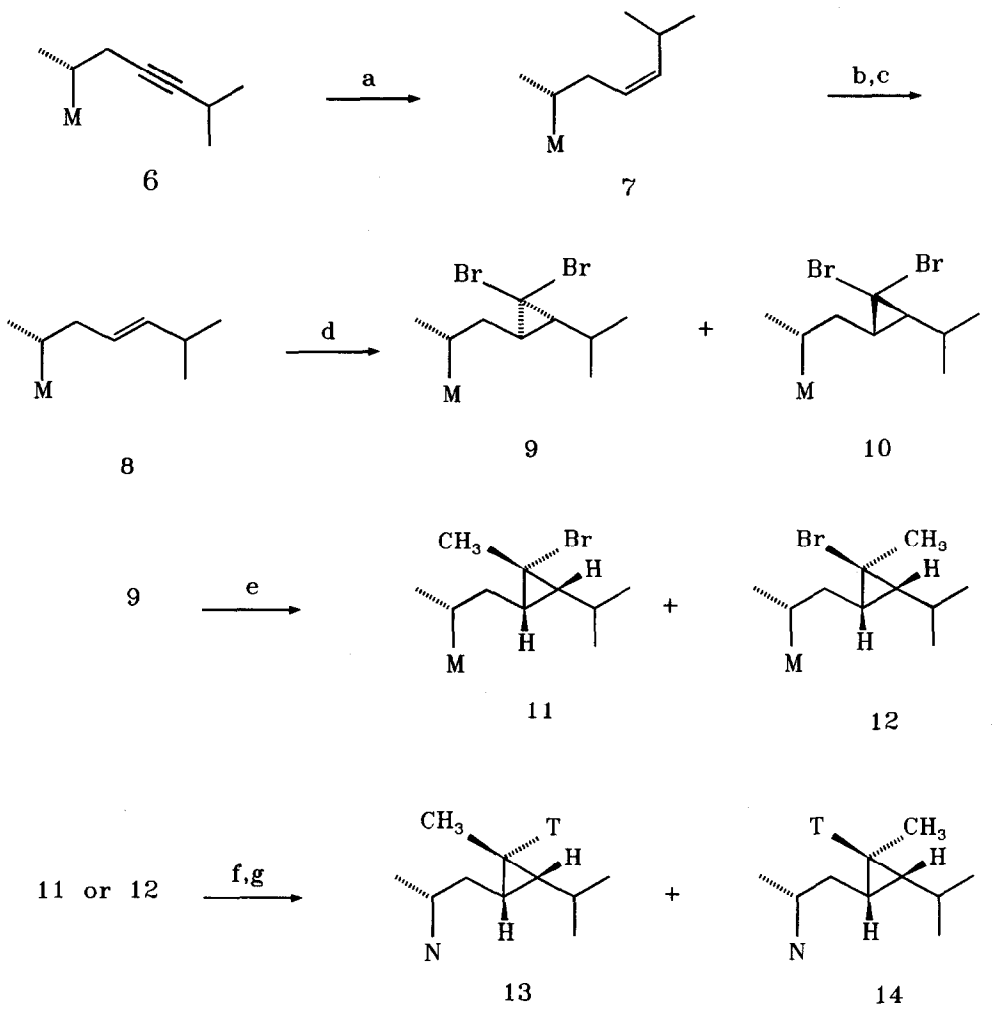
In order to test whether calysterol is directly produced from dihydrocalysterol (**5**) or by isomerization of one of the other cyclopropenes (**2**–**4**), we set out to synthesize **5**, labeled with tritium at C-28 (**13**). Depending on whether or not the label was lost during the transformation **5** → **1**, the question whether or not **1** is produced by direct dehydrogenation of **5** would be answered.

Synthesis of [28-^{3}H]-dihydrocalysterol (13). An ideal synthesis of **13** should allow introduction of tritium at C-28 at the latest stage possible in the synthetic scheme. Although a partial synthesis of the correct isomer of dihydrocalysterol (**5**) has recently been reported,[5] it does not lend itself to our present objective. Our approach to the synthesis of **13** is shown in Scheme I.

The *E*-olefin **8** was prepared in excellent stereochemical purity by reduction of the corresponding alkyne (**6**)[9,10] followed by inversion of the resulting *Z*-olefin (**7**) using the method of Vedejs and Fuchs.[11,12] Dibromocarbene addition[13] to **8** resulted in a 3:2 mixture of two (out of four possible) isomeric dibromocyclopropanes, **9** and **10**, which were separated by HPLC. The assignment of the stereochemistry of **9** and **10** was made by comparison of their high-field NMR spectra with their known dichloro analogs.[14] In particular, the C-18 protons of the (23*R*, 24*R*)-isomer, **9**, are shifted downfield compared to that of the (23*S*, 24*S*)-isomers, **10**.

Monolithiation of **9** with *n*-butyllithium in THF/HMPA at −95 °C in the presence of methyl iodide[15,16] afforded the bromomethylcyclopropanes, **11** and **12** (1:3 ratio). This

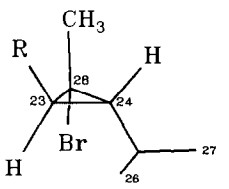
Scheme I.



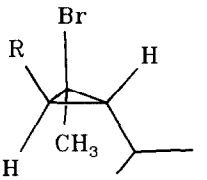
Reagents: (a) H_2, P-2 nickel cat., ethylenediamine, EtOH, 3 h. (b) *m*-$ClC_6H_4CO_3H$, CH_2Cl_2, 30 min. (c) Ph_2PLi, THF, 18 h, then MeI, 30 min. (d) $CHBr_3$, 50% aq. NaOH, $PhCH_2(Et_3)N^+ Cl^-$ (cat.), 18 h. (e) *n*-BuLi, HMPA/THF, MeI, −95 °C, 2.5 h. (f) Me_3SnCl, NaB^3H_4, EtOH, 70 °C, 16 h. (g) TsOH (cat.), dioxane–H_2O, 90 °C, 30 min.

reaction should be conducted at −95° since at higher temperatures the intermediate lithio derivative loses LiBr and the resulting carbene rearranges to the corresponding allene.[15,16] Separation of **11** and **12** was achieved by repeated injection on HPLC.

The stereochemical assignment of **11** and **12** was based on deshielding of the cyclopropane proton *cis* to the bromo group in the NMR spectra of **11** and **12** (*cf.* structures



11a



12a

11a and **12a**). In **11**, H-24 appears as a dd (4 lines) at −0.050 ppm, while, in **12**, H-23 appears as a dt (6 lines) at 0.279 ppm. The other cyclopropane proton in each isomer is deshielded by the *cis* bromo group and is overlapping with signals at δ higher than 0.82 ppm. A similar, albeit smaller, deshielding effect due to the *cis* bromo group was also observed for C-26 and C-27 in **11** compared to those in **12**.

Replacement of the bromo group in **11** with tritium was accomplished by reaction with tritium-enriched trimethyltin hydride—generated *in situ* by reaction of trimethyltin chloride with [^{3}H]-sodium borohydride.[17,18] Unfortunately, this reaction was not stereospecific: a 1:1 mixture of **5** and its 28-epimer was obtained regardless of which bromocyclopropane (**11** or **12**) was used as a starting material. The lack of stereospecificity in such a reaction is not surprising in view of the rapid inversion of the intermediate cyclopropyl radical.[19]

The separation of the desired 28-labeled dihydrocalysterol (**13**) from its 28-epimer (**14**) by HPLC proved difficult (especially on very small scale), so we decided to use the mixture as such in our feeding experiment. The presence of the unnatural 28-epimer (**14**) was immaterial to our results (see below).

The dihydrocalysterol (**5**) obtained in this synthesis showed an NMR spectrum identical to the naturally-occurring sterol. In addition, its 28-epimer (**14**) showed an NMR pattern very close to that of the corresponding stanol obtained by catalytic hydrogenation of **4**.[20]

Feeding experiments. Since loss of tritium from the [28-^{3}H]-labeled **5** would result in a 'negative' experiment (*i.e.*, the product would be non-radioactive), we decided to feed a mixture of [3-^{3}H]- and [28-^{3}H]-labeled **5** with the intention of performing a suitable

degradation to locate the label in the product.

Analysis of the sponge sterols after feeding with 3,28- doubly-labeled **5** showed that all the cyclopropene sterols (**1–3**) were radioactive—a result consistent with our previous experiment[8] (Table I). In order to determine whether the label at C-28 in calysterol (**1**) was still retained, we converted **1** to the corresponding Δ^5-3-ketone using Pfitzner-Moffatt[21] oxidation, under which conditions the cyclopropene ring is not affected.[1] The resulting ketone was 'cold' indicating that calysterol (**1**) was labeled only at C-3 and thus loss of tritium at C-28 had taken place during the biosynthesis from **5**.

Table I. Results of feeding [3,28-$^3H_2$]-dihydrocalysterol in *Calyx nicaeensis*[a].

	Recovered Sterol			
	1	**2**	**3**	**5**
RRT[b]	1.00	0.95	1.07	1.33
Weight (mg)	28.1	2.4	18.3	1.2
Radioactivity (dpm)	276,900	585,500	195,900	1,117,000
Percent incorporation[c]	7.7%	16.3%	5.5%	31.1%

[a] A mixture of 20μCi each of [3-^{3}H]- and [28-^{3}H]-dihydrocalysterol was fed. [b] HPLC relative retention time (calysterol= 1.00). [c] based on recovered radioactivity.

The loss of the label at C-28 clearly indicates that calysterol (**1**) is not produced by direct dehydrogenation of dihydrocalysterol (**5**). In addition, based on the established[5] stereochemistry of **5**, (23*R*)-23*H*-isocalysterol (**3**) cannot be produced directly from **5**. Thus, the primary product of the dehydrogenation of **5** should be either **2** or **4**.

It is important to note that stereochemistry of dehydrogenation of **5** → **2** (*cis*) is opposite to that of **5** → **4** (*trans*). The only known similar biosynthetic dehydrogenation of a cyclopropane to a cyclopropene (in the biosynthesis of sterculic acid) proceeds with *cis* stereochemistry.[22] It is also interesting to note that **4** does not exist in *C. nicaeensis*, but is the major sterol in the related sponge *C. podatypa*.[6]

In conclusion, by feeding [3,28-$^3H_2$]-labeled dihydrocalysterol, it was demonstrated that calysterol (**1**) is not produced by direct dehydrogenation of dihydrocalysterol (**5**). but rather by isomerization of another cyclopropene: **2** or **4**. Loss of the 28-tritium label due to formation of an exocyclic Δ^{28}- methylenecyclopropane appears to us less likely. Further experiments are needed to unravel the details of the biosynthesis of these intriguing sterols.

Experimental

General Methods. High performance liquid chromatography (HPLC) was carried out on a Waters Associates HPLC system (M 6000 pump, UK6 injector, R403 differential refractometer) using two Altex Ultrasphere ODS 5-μm columns (25 cm × 10 mm, i.d.) connected in series. Either methanol or a mixture of acetonitrile–methanol–ethyl acetate (22:9:7) was used as the mobile phase at a flow rate of 3 mL/min. Gas liquid chromatographic analysis was performed on a Carlo Erba series 4160 gas chromatograph equipped with 25 m × 0.32 mm SE-54 capillary column and a flame ionization detector.

^{1}H NMR spectra were recorded on a Varian XL-400 spectrometer operating at 400 MHz and were referenced to residual solvent resonance ($CHCl_3$ at 7.260 ppm). Low resolution mass spectra were recorded on a Hewlett-Packard 5970 series mass spectrometer system with a model 5890A GC for sample introduction and a Hewlett-Packard 9133 system for data acquisition. High resolution mass spectra were recorded on an AEI MS-30 instrument by a direct probe inlet system at the University of Minnesota mass spectrometry service laboratory. Radioactivity was determined with a Beckmann LS 7500 liquid scintillation counter. [^{3}H]-Sodium borohydride was purchased from ICN Biomedicals Inc. THF was dried by distillation over sodium wire and HMPA was vacuum distilled from CaH_2 prior to use.

(23*Z*)-6β-Methoxy-3α,5-cyclo-5α-cholest-23-ene (7). (The use of Brown's P-2 nickel catalyst[23] offers more reproducible results compared to the literature procedure.[9]) To a solution of nickel acetate (0.76 g, 3.0 mmol) in ethanol (5 mL), was added a solution of sodium borohydride (0.085 g) in ethanol (5 mL) while stirring. The apparatus was purged with hydrogen gas and ethylenediamine (0.3 mL) was added to the black suspension followed by a solution of the acetylene **6**[9,10] (3.2 g, 8 mmol) in ethanol (50 mL). After stirring for 3 h under hydrogen, the mixture was filtered over celite and the filtrate was concentrated under reduced pressure. The residue was diluted with water and extracted with ether (3 × 200 mL). The organic layer was dried ($MgSO_4$) and the solvent was removed under reduced pressure to afford the olefin **7** (3.3 g, 95%); physical and spectroscopic characteristics were identical to those reported in the literature.[9]

(23*E*)-6β-Methoxy-3α,5-cyclo-5α-cholest-23-ene (8). To a solution of the *Z*-olefin **7** (0.40 g, 1.0 mmol) in dichloromethane (50 mL), was added *m*-chloroperbenzoic acid (258 mg, 1.5 mmol). The mixture was stirred for 30 min (TLC monitoring) after which it was diluted with dichloromethane and washed with concentrated solutions of sodium bisulfite, sodium bicarbonate, and finally with brine. After drying ($MgSO_4$) and removal of the solvent under reduced pressure, the resulting mixture of diastereomeric epoxides

was added to a solution of lithium diphenylphosphide, prepared by reaction of Ph_2PCl (0.383 mL, 1.1 mmol) with lithium wire,[12] in THF (10 mL). The reaction mixture was stirred under argon for 18 h after which methyl iodide (0.1 mL) was added. After stirring for an additional 30 min, the mixture was diluted with water and extracted with hexane (3 × 40 mL). The hexane extract was washed with water, dried ($MgSO_4$), and evaporated under reduced pressure. The residue was purified by chromatography on silica gel using 0–1% ethyl acetate in hexane as an eluent to yield **8**[14] (310 mg, 77%); stereochemical purity > 97% by GC; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.325 (m, 2H, C-23 and C-24), 3.318 (s, 3H, OCH_3), 2.770 (m, 1H, C-6), 1.014 (s, 3H, C-19), 0.962 (d, 6H, C-26 and C-27, J = 6.8 Hz), 0.887 (d, 3H, C-21, J = 6.5 Hz), 0.713 (s, 3H, C-18), 0.647 (t, 1H, C-4β, J = 4.4 Hz), 0.430 (dd, 1H, C-4α, J = 5.2, 7.9 Hz); mass spectrum, m/z (relative intensity) 398 (17, M^+), 383 (16), 366 (17), 343 (27), 314 (6), 283 (18), 253 (8), 245 (5), 227 (10), 215 (7), 189 (4), 159 (9), 133 (8), 121 (13), 119 (11), 107 (17), 95 (13), 69 (100); Exact mass: calcd for $C_{28}H_{46}O$: 398.3549; found: 398.3530.

Dibromocarbene addition to the alkene 8. A mixture of the alkene **8**[14] (200 mg, 0.50 mmol), bromoform (0.25 mL), 50% aqueous NaOH (1 mL), benzyltriethylammonium chloride (4 mg), and ethanol (0.02 mL), was vigorously stirred for 18 h. The mixture was then diluted with water (50 mL) and extracted with hexane (3 × 40 mL). The organic layer was washed with brine, dried ($MgSO_4$), and evaporated under reduced pressure. The residue was chromatographed on silica gel using 0–1% EtOAc in hexane. The colorless mixture was then further fractionated by HPLC using methanol as a solvent to afford **9** followed by **10** (ratio, 3:2; yield, 54%).

(23*R*,24*R*)-23,24-(Dibromomethylene)-6β-methoxy-3α,5-cyclo-5α-cholestane (9). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.322 (s, 3H, OCH_3), 2.768 (m, 1H, C-6), 1.133 (d, 3H, C-21, J = 6.4 Hz), 1.064 (d, 3H, C-26/27, J = 6.4 Hz), 1.029 (d, 3H, C-27/26, J = 6.4 Hz), 1.022 (s, 3H, C-19), 0.756 (s, 3H, C-18), 0.647 (t, 1H, C-4β, J = 4.4 Hz), 0.430 (dd, 1H, C-4α, J = 5.2, 7.9 Hz); mass spectrum, m/z (relative intensity) 572, 570, 568 (5, M^+), 555 (15), 538 (10), 515 (33), 355 (8), 313 (8), 283 (15), 267 (13), 253 (100), 227 (23), 213 (26), 199 (15), 187 (13), 173 (21), 159 (41), 145 (44), 133 (36), 121 (54), 107 (51), 95 (56), 81 (59), 75 (56); Exact mass: calcd. for $C_{29}H_{46}O\,^{79}Br\,^{81}Br$: 570.1895; found: 570.1937.

(23*S*,24*S*)-23,24-(Dibromomethylene)-6β-methoxy-3α,5-cyclo-5α-cholestane (10). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.323 (s, 3H, OCH_3), 2.770 (m, 1H, C-6), 1.136 (d, 3H, C-21, J = 6.6 Hz), 1.068 (d, 3H, C-26/27, J = 6.1 Hz), 1.027 (d, 3H, C-27/26, J = 6.2 Hz), 1.020 (s, 3H, C-19), 0.725 (s, 3H, C-18), 0.647 (t, 1H, C-4β, J = 4.4 Hz), 0.430 (dd, 1H, C-4α, J = 5.2, 7.9 Hz); mass spectrum, similar to **9**.

Monolithiation and methylation of 9. A mixture of **9** (16 mg, 0.032 mmol), methyl iodide (14 mg, 0.096 mmol), dry THF (0.7 mL), and HMPA (0.3 mL), was cooled to −95 °C (toluene–liquid nitrogen bath) under argon atmosphere. A solution of *n*-butyllithium in hexane (1.6 *M*, 0.1 mL) was then injected slowly while stirring at −95 °C. After 2.5 h, the mixture was allowed to warm slowly to −50 °C when it was quenched by the addition of few drops of water. The mixture was then diluted with water (5 mL) and extracted with hexane (3 × 4 mL). The organic layer was washed with brine, dried ($MgSO_4$), and evaporated under a stream of nitrogen. The residue was chromatographed on silica gel using 0–1% EtOAc in hexane as an eluent. The mixture of products was then fractionated by HPLC using methanol as a solvent to afford **12** followed by **11** (ratio, 3:1; yield, 52%).

(23*R*,24*R*,28*R*)-28-Bromo-23,28-cyclostigmast-5-en-3β-ol (12). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.320 (s, 3 H, OCH_3), 2.772 (m, 1 H, C-6), 1.787 (s, 3 H, C-29), 1.046 (d, 3 H, C-21, J = 6.7 Hz), 1.020 (s, 3 H, C-19), 1.012 (d, 3 H, C-26/27, J = 6.5 Hz), 0.971 (d, 3 H, C-27/26, J = 6.4 Hz), 0.744 (s, 3 H, C-18), 0.647 (t, 1 H, C-4β, J = 4.4 Hz), 0.430 (dd, 1 H, C-4α, J = 5.0, 8.0 Hz), 0.279 (dt, 1 H, C-23, J = 5.2, 7.5 Hz); mass spectrum, *m/z* (relative intensity) 506, 504 (1 , M^+), 491 (5), 474 (2), 451 (10), 449 (10), 424 (2), 409 (3), 393 (8), 369 (5), 283 (28), 253 (100), 227 (13), 213 (16), 159 (30), 137 (41), 121 (33), 109 (56), 94 (62), 80 (39); Exact mass: calcd. for $C_{30}H_{49}O\,^{81}Br$: 506.2946; found: 506.2922; calcd. for $C_{30}H_{49}O\,^{79}Br$: 504.2967; found: 504.2976.

(23*R*,24*R*,28*S*)-28-Bromo-23,28-cyclostigmast-5-en-3β-ol (11). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.322 (s, 3 H, OCH_3), 2.771 (m, 1 H, C-6), 1.731 (s, 3 H, C-29), 1.195 (d, 3 H, C-26/27, J = 6.4 Hz), 1.045 (d, 3 H, C-21, J = 6.6 Hz), 1.023 (d, 3 H, C-27/26, J = 6.5 Hz), 1.021 (s, 3 H, C-19), 0.727 (s, 3 H, C-18), 0.647 (t, 1 H, C-4β, J = 4.3 Hz), 0.429 (dd, 1 H, C-4α, J = 5.2, 7.9 Hz), −0.050 (dd, 1 H, C-24, J = 7.0, 9.7 Hz); mass spectrum, similar to **12**.

[28-^{3}H]-Dihydrocalysterol (13). To a mixture of the bromocyclopropane **11** (18 mg, 0.035 mmol), trimethyltin chloride (13 mg, 0.065 mmol), and air-free ethanol (1 mL), was added [^{3}H]-sodium borohydride (90 mCi, spec. activity, 1.4 Ci/mmol) in ethanol (0.5 mL). The mixture was heated (70 °C) under argon atmosphere for 16 h. The solvent was then removed under a stream of nitrogen and the residue was chromatographed on silica gel using 0–1% ethyl acetate in hexane as an eluent. The product was separated from unreacted starting material by HPLC using methanol as solvent. The *i*-methyl ether was then deprotected by heating (90 °C) for 0.5 h with a catalytic amount of *p*-toluenesulfonic acid in 80% aqueous dioxane (1 mL). The solvent was evaporated and the labeled sterol (610 μCi) was obtained by chromatographing the residue on silica gel using

1–2% ethyl acetate in hexane. The same mixture of products was obtained by using **12** instead of **11**. Cold materials were obtained using $NaBH_4$.

(23*S*,24*S*,28*R*)-23,28-cyclostigmast-5-en-3β-ol (dihydrocalysterol) (5) ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.350 (m, 1H, C-6), 3.530 (m, 1H, C-3), 1.019 (d, 3H, C-21, J = 6.6 Hz), 1.010 (s, 3H, C-19), 0.988 (d, 3H, C-29, J = 6.1 Hz), 0.931 (d, 3H, C-27/26, J = 6.8 Hz), 0.927 (d, 3H, C-26/27, J = 6.9 Hz), 0.686 (s, 3H, C-18), 0.440 (m, 2H, C-28 and C-23), −0.128 (m, 1H, C-24).

(23*S*,24*S*,28*S*)-23,28-cyclostigmast-5-en-3β-ol (14) ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.350 (m, 1H, C-6), 3.530 (m, 1H, C-3), 1.034 (d, 3H, C-29, J = 6.3 Hz), 1.009 (s, 3H, C-19), 0.997 (d, 6H, C-27/26 and C-21, J = 6.7 Hz), 0.932 (d, 3H, C-26/27, J = 6.8 Hz), 0.681 (s, 3H, C-18), 0.340, 0.150, 0.072 (m, 1H each, cyclopropane protons).

Feeding experiments. The precursor was administered to *C. nicaeensis* using the technique described previously.[24] The sponge was incubated for 21 days before it was collected for analysis. The air-dried samples were extracted 4 times with dichloromethane. The extract was concentrated under reduced pressure with minimal exposure to heat and was fractionated on an open silica-gel column (eluent, hexane-ether, 3:1). The sterol fractions (R_f = cholesterol by TLC) were combined and evaporated under reduced pressure. The total sterol mixture was fractionated by reverse phase HPLC using a mixture of acetonitrile–methanol–ethyl acetate (22:9:7). Individual fractions were reinjected as many times as necessary to get pure sterols having constant specific activity.

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