Biosynthetic Studies of Marine Lipids. 25.[1] Biosynthesis of $\Delta^{9(11)}$- and Δ^{7}-Sterols and Saponins in Sea Cucumbers

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Radiolabeling experiments in the sea cucumbers *Bohadschia argus*, *Holothuria mexicana*, *Holothuria arenicola*, and *Stichopus californicus* show that the only sterols synthesized de novo are lanost-9(11)-en-3β-ol (**20**), 4α,14α-dimethyl-5α-cholest-9(11)-en-3β-ol (**19**), and 14α-methylcholest-9(11)-en-3β-ol (**18**). Squalene is first cyclized to lanosta-9(11),24-dien-3β-ol or parkeol (**21**). The $\Delta^{9(11)}$-sterols then arise by sequential $\Delta^{24,25}$-bond reduction, loss of the 4α-methyl group, and finally loss of the remaining 4-methyl group. None of the sea cucumber saponins, however, were radioactive. The synthesis of Δ^{7}-sterols in *Stichopus californicus* is shown to occur by modification of Δ^{5}-sterols of probable dietary origin. Thus, cholesterol (**16**), 24-methylenecholesterol (**28**), and isofucosterol (**29**) are transformed into their Δ^{7} analogues. This process most likely occurs via a $\Delta^{5,7}$-diene intermediate. Sea cucumbers were shown also to possess the ability to introduce a double bond into a saturated nucleus.

Sea cucumbers (Holothuroidae) are members of the invertebrate phylum Echinodermata, the chordates' closest invertebrate relatives.[2] They have been shown to contain mainly Δ^{7}-sterols, for example, 5α-cholest-7-en-3β-ol (**1**) (Figure 1).[3] Δ^{5}-Sterols such as cholesterol (**16**) and stanols such as 5α-cholestanol (**12**) have also been identified.[4,5] More recently, $\Delta^{9(11)}$-sterols with a 14α-methyl substituent have been isolated from holothurians.[6] The structures of three such compounds are shown in Figure 1 (**18–20**). In addition, sea cucumbers are known to contain appreciable quantities of toxic saponins or holothurins,[7] two examples of which are shown in Figure 2. Very few studies describing sterol and saponin biosynthesis in sea cucumbers have been reported. We now describe experiments designed to explore the biosynthetic origin of these three classes of sea cucumber isoprenoids.

Previous studies on sterol and saponin biosynthesis in sea cucumbers report conflicting results. For example, Nomura et al.[8] administered labeled acetate to *Stichopus japonicus* and found labeled squalene but no label in the lanosterol or in the sterols. Other researchers have contradicted this result. Voogt and Over[5] and Goad[9] reported incorporation of labeled acetate and mevalonate into the sterols of five species of sea cucumber. Goad observed that 5α-cholest-7-en-3β-ol (**1**) is the major radioactive product in *Cucumaria elongata* and *C. lactea*.[9] Voogt and Over[5] in their conclusion, however, do point out that the specific activity of the sterols was low, suggesting that biosynthesis of these sterols is not as important as conversion from Δ^{5}-sterols. Sheikh and Djerassi[10] reported conversion of acetate and lanosterol to Δ^{5}- and Δ^{7}-sterols in *Stichopus californicus*. The latter claim was particularly surprising as the lanosterol was labeled in the C-3 position, and during loss of the 4-methyl groups, any tritium atom at position 3 ought to be lost. In addition, Sheikh et al. showed that cholesterol (**16**) and 5α-cholest-7-en-3β-ol (**1**) could be interconverted. [3-^{3}H]Cholesterol (**16**) and 5α-cholest-7-en-3β-ol (**1**) were also reported to be converted to 5α-cholestanol (**12**). This indicated that conversion of the Δ^{7} to the Δ^{5} double bond was not occurring via formation of a Δ^{4}-3-keto intermediate. This mechanism has been described in starfish.[11] Lastly, Sheikh and Djerassi observed 24-alkylation of cholesterol to furnish both 24-methyl and 24-ethyl Δ^{5}- and Δ^{7}-sterols.[10]

There is some evidence for de novo synthesis of saponins. Murthy and Der Marderosian[12] obtained very low incorporation of [^{14}C]-labeled mevalonate into the crude holothurins from *Stichopus badionotus*. Elyakov et al. also obtained low incorporation of labeled acetate into stichopogenin A4 obtained by hydrolysis of the saponins from *Stichopus japonicus*.[13] Kelecom et al.[14] showed that labeled acetate was incorporated into the saponins from *Thelenota ananas*. The incorporation, however, was very low (5×10^{-2}%). Sheikh and Djerassi found that the efficiency of conversion of tritium-labeled lanosterol (**24**) into stichopogenin A4 obtained by hydrolysis of holothurins from *Stichopus californicus* was about 200 times higher than that of labeled acetate, suggesting that de novo biosynthesis of the aglycons proceeds via lanosterol.[15] Our present studies, however, did not detect any holothurins in this sea cucumber, which is consistent with the obser-

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(6) (a) Goad, L. J.; Garneau, F.-X.; Simard, J.-L.; ApSimon, J. W.; Girard, M. *Tetrahedron Lett.* **1985**, *26*, 3513. (b) Kalinovskaya, N. I.; Kalinovskii, A. I.; Kuznetsova, T. A.; Stonik, V. A.; Elyakov, G. B. *Dokl. Akad. Nauk SSSR* **1984**, *278*, 630. (c) Ambia, K. B. M.; Goad, L. J.; Hkycko, S.; Garneau, F.-X.; Belanger, J.; ApSimon, J. W. *Comp. Biochem. Physiol.* **1987**, *86B*, 191. (d) Goad, L. J.; Garneau, F.-X.; Simard, J. L.; ApSimon, J. W.; Girard, M. *Comp. Biochem. Physiol.* **1986**, *84B*, 189. (e) Findlay, J. A.; Daljeet, A.; Matsoukas, J.; Moharir, Y. E. *J. Nat. Prod.* **1984**, *47*, 560.

(7) (a) Stonik, V. A.; Elyakov, G. B. In *Bioorganic Marine Chemistry*; Springer-Verlag: Berlin, 1988; Vol. 2, p 43. (b) Burnell, D. J.; ApSimon, J. W. In *Marine Natural Products Chemistry, Chemical and Biological Perspectives*; Scheuer, P. J., Ed.; Academic Press: New York, 1983; Vol. V, p 287. (c) In the animal kingdom, steroidal glycosides have recently been found in the ophiuroid *Ophioderma longicaudum*: Riccio, R.; D'Auria, M. V.; Minale, L. *J. Org. Chem.* **1986**, *51*, 533. They are also found rarely in Alyconarians (Kobayashi, M.; Kiyota, Y.; Orita, S.; Kyogoku, Y.; Kitagawa, I. *Tetrahedron Lett.* **1984**, *25*, 3731) and some fish (Tachibana, K.; Sakaitanai, M.; Nakanishi, K. *Science* **1984**, *226*, 703).

(8) Nomura, T.; Tsuchiya, Y.; Andrê, D.; Barbier, M. *Nippon Suissan Gakkaishi* **1969**, *35*, 299.

(9) Goad, L. J.; Rubinstein, I.; Smith, A. G. *Proc. R. Soc. London, Ser. B* **1972**, *180*, 223.

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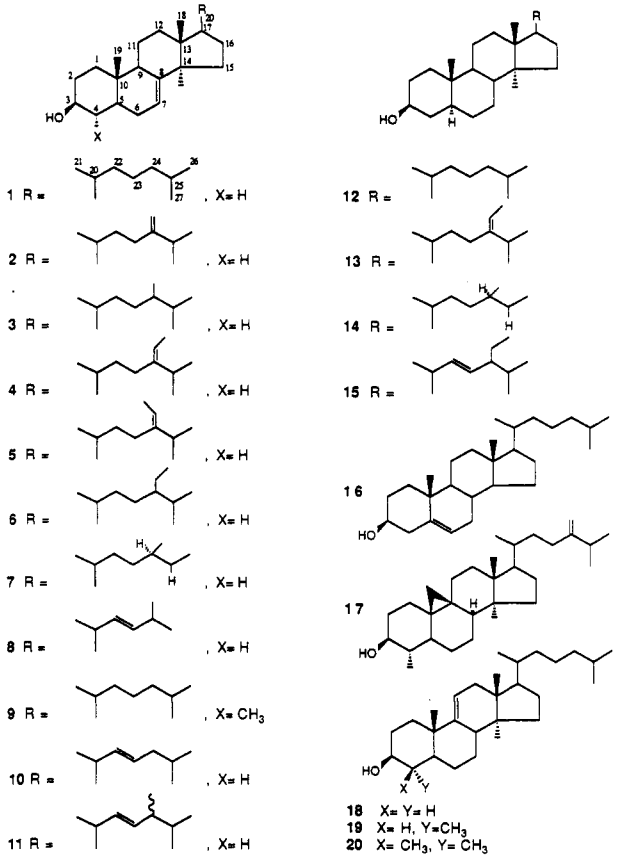

Figure 1. Sterols isolated from *Holothuria arenicola, Bohadschia argus, Holothuria mexicana,* and *Stichopus californicus.*

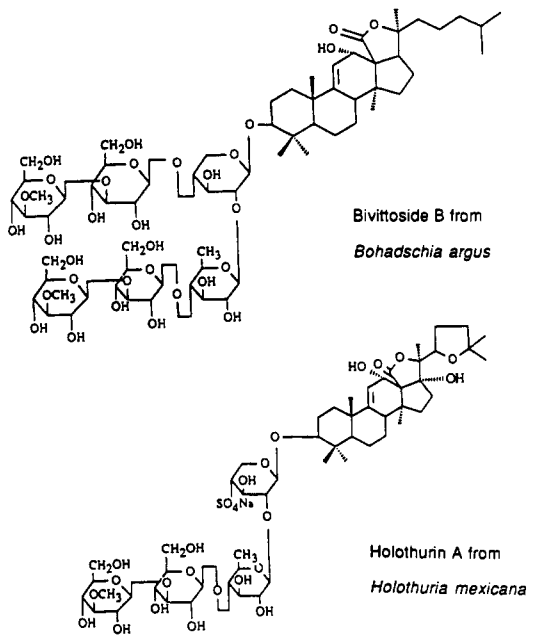

Figure 2. Saponins from *Bohadschia argus* and *Holothuria mexicana.*

vation that *Stichopus californicus,* like other sea cucumbers found off the California coast, is not toxic.[16a] However, it is conceivable that the presence of such toxins is seasonable, since Soviet investigators[16b] did encounter toxins in *Stichopus japonicus.*

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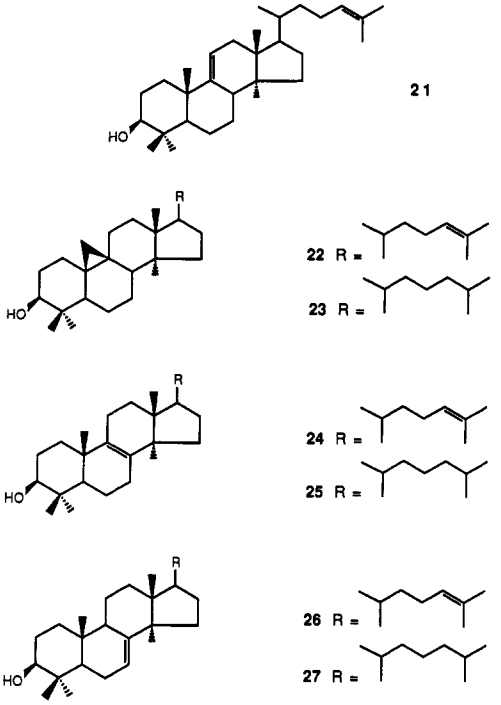

Figure 3.

Results and Discussion

Holothuria arenicola, Bohadschia argus, Holothuria mexicana, and *Stichopus californicus* were examined for sterols and saponins. The sterols were identified by ^{1}H NMR, GC and low-resolution GC–MS, and comparison with literature values. The saponins were identified by ^{13}C NMR and comparison to literature values.

Most of the Δ^7-sterols, Δ^5-sterols, and saturated sterols have previously been reported in starfish and sea cucumbers (Figure 1).[3a,c,17] The biosynthetically intriguing sterol 14α-methylcholest-9(11)-en-3β-ol (**18**) was isolated from all the sea cucumbers studied. 4α,14α-Dimethyl-5α-cholest-9(11)-en-3β-ol (**19**) and the $\Delta^{9(11)}$ analogue of 24,25-dihydrolanosterol, lanost-9(11)-en-3β-ol (**20**), were also detected (Figure 1). These compounds have been isolated from sea cucumbers by other researchers.[6] The structures of the saponins bivittoside B from *Bohadschia argus*[18a] and holothurin A from *Holothuria mexicana*[18b] are shown in figure 2. *Stichopus californicus* was found not to contain steryl sulfates, while the other sea cucumbers were not examined by us for such components.

The 14α-methyl sterol nucleus is rarely encountered; most of the naturally occurring sterols in organisms lack carbon substituents at C-4 or C-14 and possess an alkyl substituent or no substituent at C-24.[19] There are some reports, however, of the isolation of sterols containing the 14α-methyl group.[20] The biogenesis of 14α-methyl

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Table I. Radiolabel Incorporation Experiments in *Stichopus californicus*[a]

sterol	precursor: 20-μCi [24-^{3}H]-cycloartenol (22)	25-μCi [24-^{3}H]-lanosterol (24)	11-μCi [24-^{3}H]-lanosta-7,24-dien-3β-ol (26)	10-μCi [24-^{3}H]-parkeol (21)	25-μCi (*RS*)-[5-^{3}H]-mevalonate	50-μCi [3-^{3}H]-squalene
lanosterol (24)	cold	5.5×10^5 dpm	not examined	cold	cold	cold
lanost-8-en-3β-ol (25)	cold	2.7×10^4 dpm	not examined	cold	cold	cold
parkeol (21)	cold	1.0×10^4 dpm	1.3×10^4 dpm	1.1×10^6 dpm	1.8×10^6 dpm	8.3×10^4 dpm
lanost-9(11)-en-3β-ol (20)	cold	4.0×10^3 dpm	4.4×10^3 dpm	2.1×10^5 dpm	7.3×10^5 dpm	1.3×10^6 dpm
cycloartenol (22)	2×10^6 dpm	cold	cold	cold	cold	cold
24,25-dihydrocycloartenol (23)	4.8×10^4 dpm	cold	cold	cold	cold	cold
cycloeucalenol (17)	1.9×10^5 dpm	cold	cold	cold	cold	cold
lanost-7-en-3β-ol (27)	cold	cold	1.3×10^5 dpm	cold	cold	cold
lanosta-7,24-dien-3β-ol (26)	cold	cold	1.3×10^6 dpm	cold	cold	cold
14α-methylcholest-9(11)-en-3β-ol (18)	cold	cold	cold	2.4×10^4 dpm	2.3×10^4 dpm	3.8×10^4 dpm
4α,14α-dimethyl-5α-cholest-9(11)-en-3β-ol (19)	cold	cold	cold	2.0×10^5 dpm	1.4×10^5 dpm	5.8×10^5 dpm
Δ^7-sterols	cold	cold	cold	cold	cold	cold
radioactivity recovd (%)	2.0×10^6 dpm (4.5%)	5.4×10^5 dpm (1.4%)	1.26×10^6 dpm (5.2%)	1.1×10^6 dpm (3.5%)	not examined	not examined

[a] All fractions were purified by HPLC followed by argentic TLC to constant activity.

$\Delta^{9(11)}$-sterols has intrigued researchers over the years. Itoh et al.[21] have suggested that lanosta-9(11),24-dien-3β-ol (**21**) (Figure 3) may be formed via cycloartenol (**22**) ring opening in shea fat. They refer to early work on the HCl-catalyzed isomerization of cycloartenol as a chemical precedent for such a reaction but provide no experimental support.[22]

The mechanism by which 14-demethylation occurs in lanosterol has been described[23] and leads to formation of the $\Delta^{8,14}$-diene. Goad et al.[6d] suggest that in creatures in which 14α-dimethyl-5α-cholest-9(11)-en-3β-ol (**18**) accumulates, squalene oxide cyclizes to lanosta-9(11),24-dien-3β-ol (**21**) as well as lanosterol (**24**). Since formation of the $\Delta^{8,14}$-diene via the loss of the 14α-methyl group is not possible in the case of **21**, sequential loss of the two methyl groups at C-4 would lead to formation of **18**. Lanosterol, on the other hand, would then serve as a precursor to 4-demethyl sterols such as 5α-cholest-7-en-3β-ol (**1**) (Figure 4). The same authors point out that since holothurins with $\Delta^{9(11)}$ bonds are found in many sea cucumbers, parkeol (**21**) might serve as a precursor in these animals as indicated in Figure 4.[6d] In contrast, Akhila et al.[24] suggest that 5α-stigmast-9(11)-en-3β-ol, a $\Delta^{9(11)}$-sterol lacking the 14α-methyl group but containing a 24-ethyl group, may arise by cyclization of squalene oxide to parkeol (**21**) but point out that formation of cycloartenol could also explain their results.

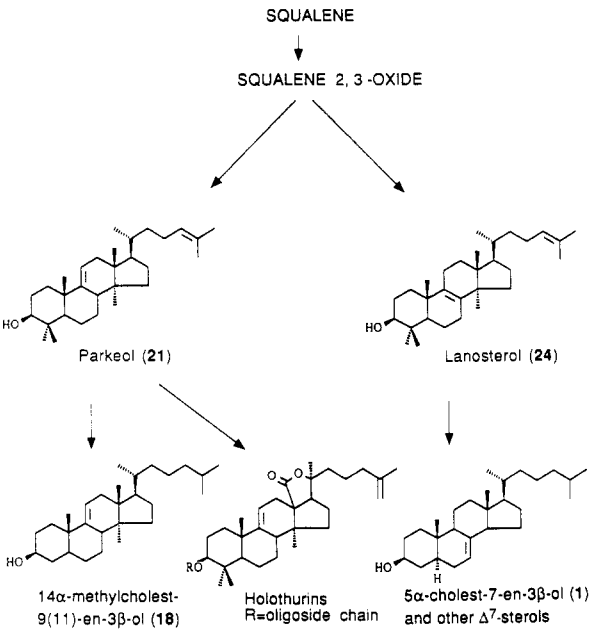

Figure 4. Hypothetical scheme for formation of sterols and saponins in sea cucumbers.

Biosynthesis of $\Delta^{9(11)}$-Sterols. In order to the study the origin of the $\Delta^{9(11)}$-sterols, [24-^{3}H]lanosterol (**24**), [24-^{3}H]cycloartenol (**22**), [24-^{3}H]parkeol (lanosta-9(11),24-dien-3β-ol, **21**), and [24-^{3}H]lanosta-7,24-dien-3β-ol (**26**) (Figure 3) were fed to the local sea cucumber *Stichopus californicus*.[25] The radiolabeled compounds (*RS*)-[5-$^3H_2$]mevalonate and [3-^{3}H]squalene were also synthesized and fed to *Stichopus californicus*. The synthesis of the [24-^{3}H]parkeol (**21**) is described in detail in the Experimental Section. [24-^{3}H]-Labeled cycloartenol (**22**), lanosterol (**24**), and lanosta-7,24-dien-3β-ol (**26**) were prepared in an analogous manner.

The results are summarized in Table I. Examination of the mevalonate and squalene experiments gave interesting results. Addition of carrier cycloartenol (**22**) and lanosterol (**24**) to the tetracyclic triterpene fraction followed by separation by reverse-phase HPLC showed that no radioactivity had been incorporated into cycloartenol. The lanosterol peak was hot, however, with most of the radioactivity in the second half of the peak. This corresponds to the HPLC retention time of cold parkeol (**21**), but it should be noted that lanosta-7,24-dien-3β-ol (**26**) has a very similar retention time. To determine which of the tetracyclic triterpenes were labeled, this peak was collected and evaporated to dryness. Cold **21** and **26** were added, and the mixture was acetylated and separated by argentation TLC. Only parkeol (**21**) was found to be labeled. Examination of the lanost-9(11)-en-3β-ol (**20**), 4α,14α-dimethyl-5α-cholest-9(11)-en-3β-ol (**19**), and 14α-methyl-

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Table II. Biosynthetic Experiments in *Stichopus californicus*

	precursor				
toxin or sterol	20-μCi [3-^{3}H]-cholesterol (16)	20-μCi [3-^{3}H]-5α-cholest-7-en-3β-ol (1)	20-μCi [3-^{3}H]-5α-cholestan-3β-ol (12)	20-μCi [3-^{3}H]-24-methylenecholesterol (28)	20-μCi [3-^{3}H]-isofucosterol (29)
toxins	none detected	none detected	none detected	none detected	none detected
14α-methyl-$\Delta^{9(11)}$-sterol (18)	cold	cold	cold	cold	cold
Δ^5- and saturated sterols	5α-cholestanol, cold	5α-cholestanol, cold	5α-cholestanol, 8.4×10^5 dpm	24-methylenecholesterol, 1.1×10^6 dpm	isofucosterol, 1.2×10^6 dpm
	cholesterol, 2.1×10^6 dpm	cholesterol, 1.3×10^4 dpm	cholesterol, 3.7×10^3 dpm		
Δ^7-sterols	5α-cholest-7-en-3β-ol, 1.0×10^4 dpm	5α-cholest-7-en-3β-ol, 8.2×10^5 dpm	5α-cholest-7-en-3β-ol, 8.4×10^5 dpm	24-methylene-5α-cholest-7-en-3β-ol, 1.2×10^4 dpm	24-*trans*-ethylidene-5α-cholest-7-en-3β-ol, 1.7×10^4 dpm
radioactivity recovd (%)	2.1×10^6 dpm (4.7%)	8.2×10^5 dpm (2.0%)	8.4×10^5 dpm (2.0%)	1.1×10^6 dpm (2.4%)	1.2×10^6 dpm (2.6%)

cholest-9(11)-en-3β-ol (**18**) peaks from reverse-phase HPLC, followed by acetylation and argentation TLC, showed that the radioactivity was indeed associated with these compounds and not due to other nuclear double-bond isomers.

When [24-^{3}H]parkeol (**21**) was fed to *Stichopus californicus*, compounds **18–20** were also labeled. This was found to be the case in the other three sea cucumbers studied. Incorporation experiments with cycloartenol (**22**), lanosterol (**24**), and lanosta-7,24-dien-3β-ol (**26**) gave several interesting results. Isolation of the tetracyclic triterpenes from the cycloartenol experiment and addition of cold carrier cycloartenol (**22**), 24,25-dihydrocycloartenol (**23**), and lanosterol (**24**) before separation by reverse-phase HPLC showed that no cyclopropyl ring opening to lanosterol had occurred. 24,25-Dihydrocycloartenol (**23**) was labeled, however, as was cycloeucalenol (**17**). None of the $\Delta^{9(11)}$-sterols were radioactive.

Injection of the tetracyclic triterpene fraction from a lanosterol incorporation after addition of cold cycloartenol and lanosterol on reverse-phase HPLC showed that cycloartenol was cold. The lanost-9(11)-en-3β-ol (**20**) fraction was hot, however, possibly due to coelution of 24,25-dihydrolanosterol (**25**). This fraction, therefore, was acetylated after addition of cold 24,25-dihydrolanosterol and subjected to argentic TLC. The two compounds were well separated after three consecutive elutions with solvent: R_f(3β-acetoxylanost-9(11)-ene) 0.5, R_f(3β-acetoxylanost-8-ene) 0.6. Surprisingly, although almost 90% of the radioactivity was associated with the Δ^8 compound, the 3β-acetoxylanost-9(11)-ene fraction was also hot. This suggested that there had been some isomerization of the Δ^8 bond to the $\Delta^{9(11)}$ position. Hence, the lanosterol fraction was also acetylated after addition of cold lanosta-9(11),24-dien-3β-ol (**21**) and further separated on argentic TLC: R_f(3β-acetoxylanosta-9(11),24-diene) 0.2, R_f(lanosteryl acetate) 0.4. Radiolabeled lanosta-9(11),24-dien-3β-ol was found to be present together with the recovered lanosterol. The extent of isomerization, however, is very low, and 4α,14α-dimethyl-5α-cholest-9(11)-en-3β-ol (**19**) was not labeled nor was the 14α-methyl $\Delta^{9(11)}$-sterol (**18**). Isomerization to parkeol (**21**) was also observed in the lanosta-7,24-dien-3β-ol incorporation, and some radioactivity was observed in lanost-9(11)-en-3β-ol (**20**). In addition, lanost-7-en-3β-ol (**27**) was found to display radioactivity.

Biosynthesis of Δ^7-Sterols. All the experiments on the origin of the conventional Δ^7-sterols were done in *Stichopus californicus* and are summarized in Table II. The structures of the radiolabeled precursors used in the incorporation experiments are shown in Figure 5. The results described above showed that these sterols were not

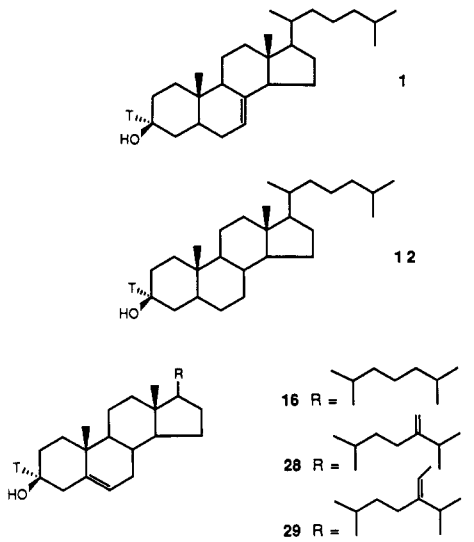

Figure 5. [3-^{3}H]-Labeled Δ^5-sterols, Δ^7-sterols, and saturated sterols used to study the interconversion of these nuclei in *Stichopus californicus*.

synthesized de novo, and thus it was thought that they may arise by nuclear double-bond migration. For example, 24-methylenecholesterol (**28**) may be converted to its Δ^7 analogue (**2**) and isofucosterol (**29**) into its Δ^7 analogue (**4**).

From Table II, it can be seen that [^{3}H]-labeled cholesterol, 24-methylenecholesterol, and isofucosterol are all transformed to their Δ^7 analogues. That this process is readily reversible is implicated by the conversion of lathosterol (**1**) to cholesterol (**16**). Cholestanol (**12**) is also transformed to cholesterol and lathosterol, thus showing that double-bond introduction can occur in addition to migration.

Biosynthesis of Saponins. Unfortunately in both of the sea cucumbers that were found to contain saponins, *B. argus* and *H. mexicana*, none of the administered precursors (lanosterol, parkeol, or cycloartenol) were incorporated. The possibility that transportation of these lipophilic precursors to the site of saponin biosynthesis was not occurring was addressed by using [5-$^3H_2$]-labeled mevalonate, but once again with no success.

The experiments described above enable several conclusions to be drawn. First, only parkeol (**21**), the $\Delta^{9(11)}$ isomer of lanosterol, is converted by the sea cucumber to lanost-9(11)-en-3β-ol (**20**), 4α,14α-dimethyl-5α-cholest-9(11)-en-3β-ol (**19**), and 14α-methylcholest-9(11)-en-3β-ol (**18**). Lanosterol (**24**) and cycloartenol (**22**), the usual cyclization products of squalene oxide, are not transformed to any of these $\Delta^{9(11)}$ compounds. No lanosterol or cycloartenol was detected in the sea cucumbers, in contrast with

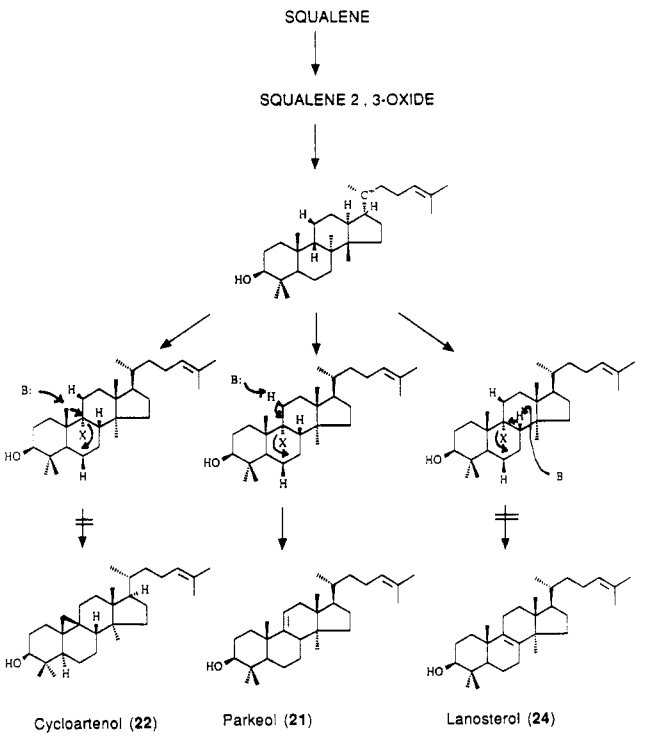

Figure 6. Cyclization of squalene to parkeol (21) in holothurians.

the observations of Nomura et al., who reported these compounds in *Stichopus japonicus*.[4] When mevalonate and squalene are fed, labeled parkeol (**21**) is isolated, but no labeled lanosterol (**24**) or cycloartenol (**22**). A third possible product of squalene oxide cyclization, lanosta-7,24-dien-3β-ol (**26**), is also cold. These results imply that squalene oxide is cyclizing directly to the $\Delta^{9(11)}$ isomer of lanosterol, as indicated in Figure 6. The cationic product of squalene is thought to be stabilized by a nucleophilic group (X) from the enzyme, squalene cyclase.[26] Abstraction of the 11β proton by a basic group on the enzyme together with displacement of the group X would lead to formation of parkeol (**21**). Reduction of the Δ^{24} double bond would then form lanost-9(11)-en-3β-ol (**20**), from which the 14α-methyl group is not lost. Presumably this is because such loss is not facilitated by the $\Delta^{9(11)}$ double bond, now homoallylic rather than allylic as in lanosterol (**24**). Alternatively, the sea cucumbers may lack the cytochrome P450 dependent oxidase system required for C-14 demethylation as suggested by Goad et al.[6d] Thus 4α-demethylation leads to formation of 4α,14α-dimethyl-5α-cholest-9(11)-en-3β-ol (**19**), and subsequent removal of the remaining 4-methyl group results in formation of 14α-methylcholest-9(11)-en-3β-ol (**18**). The only de novo sterols synthesized by the sea cucumber are lanost-9(11)-en-3β-ol (**20**), 4α,14α-dimethyl-5α-cholest-9-(11)-en-3β-ol (**19**), and 14α-dimethylcholest-9(11)-en-3β-ol (**18**). This is in marked contrast to the results reported by Goad[9] in *Cucumaria elongata* in which 5α-cholest-7-en-3β-ol (**1**) is the only product from a [2-^{14}C]mevalonate incorporation experiment. The sterols were separated by GC but the conditions did not give base-line separation of sterols, and the 14α-methyl $\Delta^{9(11)}$-sterol (**18**) which is often found in sea cucumbers was not detected. One explanation for Goad's result is that **18**, if present, may have a similar retention to that of **1** under his GC conditions and that it, and not 5α-cholest-7-en-3β-ol, is labeled.

The sea cucumbers examined do not contain any lanosterol, nor does squalene cyclize to produce lanosterol in vivo. Although some lanosterol (**24**) is isomerized to form parkeol (**21**), which is subsequently converted to labeled lanost-9(11)-en-3β-ol (**20**), no other sea cucumber sterols show measurable radioactivity. Presumably **20** once formed could then be transformed to 14α-methylcholest-9(11)-en-3β-ol (**18**) and 4α,14α-dimethyl-5β-cholest-9-(11)-en-3β-ol (**19**). The isomerization to **20**, however, is so low that under our experimental conditions there was no detectable radioactivity in these two sterols. The product of Δ^{24}-bond reduction, 24,25-dihydrolanosterol (**25**), is found to have activity. Lanosta-7,24-dien-3β-ol (**26**) gave similar results. Once again, isomerization to the $\Delta^{9(11)}$ bond occurs and [^{3}H]-labeled **21** and **20** are isolated. There was no detectable radioactivity in **18** and **19**. Lanost-7-en-3β-ol (**27**), the product of $\Delta^{24,25}$-bond reduction of lanosta-7,24-dien-3β-ol (**26**), was also found to have activity.

The isolation of cycloeucalenol (**17**) from *Stichopus californicus* was surprising since cycloartenol and its metabolites are not generally found in animals. An exception is *Asterias rubens* from which cycloartenol (**22**) and 24,25-dihydrocycloartenol (**23**) have been isolated.[27,28] [3-^{3}H]-Labeled cycloartenol was found not to be converted to lanosterol, but it was reduced to 24,25-dihydrocycloartenol. Similar results have been obtained in rat liver homogenates.[27] It was, however, surprising that the sea cucumber can alkylate and remove the 4α-methyl group from cycloartenol to form **17**, because this is typically a process that occurs in plants.[29] Perhaps *Stichopus californicus* harbors a plant or algal symbiont that is responsible for this transformation.

The incorporation experiments with [3-^{3}H]cholesterol (**16**), [3-^{3}H]5α-cholest-7-en-3β-ol (**1**), and [3-^{3}H]5α-cholestan-3β-ol (**12**) confirm Sheikh's observation[9] that **16** and **1** are readily interconverted. This process does not proceed via formation of the Δ^4-3-ketone as in starfish,[11] since the tritium atom at C-3 is retained. Direct reduction to the saturated nucleus is probably not occurring, since 5α-cholestan-3β-ol (**12**) has no activity when either [^{3}H]-labeled **1** or **16** is fed. This leaves the $\Delta^{5,7}$-diene as the probable intermediate. 24-Methylenecholesterol and isofucosterol are probably converted to their Δ^7 analogues via the same mechanism. [^{3}H]5α-Cholestan-3β-ol (**12**), however, is readily transformed to both cholesterol and 5α-cholest-7-en-3β-ol, thus indicating that direct introduction of a double bond into a saturated nucleus can occur. This has been demonstrated in sea stars.[9,11,30]

The fact that neither the toxins of *Bohadschia argus* nor those of *Holothuria mexicana* were labeled by any of the precursors used is puzzling. Since the $\Delta^{9(11)}$-sterols were all labeled after feeding either [^{3}H]-labeled parkeol (**21**) or mevalonate, it was thought that one explanation for this result might be that these precursors are efficiently transported to the site of sterol biosynthesis but not to the site of saponin synthesis. Dissection of an individual *B. argus* cucumber and separation into three fractions were informative. The three fractions studied were the body wall, the cuvier glands, and the coelomic fluid and internal organs. These fractions were examined for the presence of $\Delta^{9(11)}$-sterol and saponins, and all the fractions were

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found to contain both groups of compounds. Of course, it is still possible that the toxins are synthesized at a specific site in the cucumber and then distributed around the body and that the labeled precursor cannot be transported to the specific cells that synthesize saponins. Another possible explanation is that the biosynthesis of the saponins is slow and that the incorporation experiments (10 days) were not run long enough. This is unlikely, since reference to the saponin biosynthetic experiments in starfish shows that high uptake was obtained in just 48 h.[31] One other explanation was provided by Garson in a recent review article.[32] She discusses the results of feeding experiments on tetrodotoxin, which occurs in a range of marine animals such as pufferfish, newts, and the blue-ring octopus and is a product of bacterial metabolism. Feeding ^{14}C-labeled citrulline, arginine, glucose, and acetate to newts did not produce labeled tetrodotoxin, although metabolism of these precursors was demonstrated to have occurred by their incorporation into sterols and amino acids. The toxin may be produced only in response to aggression or to developmental needs, and thus not continuously. It is possible that this is true of the sea cucumber toxins as well.

Experimental Section

General Methods. High-performance liquid chromatography (HPLC) was carried out with Waters Associates equipment (M6000A and M45 pumps and R401 differential refractometers) as well as Spectra Physics pumps (SP8810) fitted with a Rheodyne sample injection valve (Model 7010). Altex Ultrasil-Si normal-phase columns (10-mm i.d. × 25 cm) were used to separate various steroid nuclei both from the sea cucumber extractions and for purification of reagents prior to radiolabeling. Ethyl acetate (6%) in hexane or 2% ethyl acetate in hexane was used as the mobile phase. Further purification was achieved by using two Altex Ultrasphere ODS columns (10-mm i.d. × 25 cm) connected in series. For separation of sterols or sterol acetates, the mobile phase used was either methanol, an acetonitrile/methanol/ethyl acetate (22:8:6) mixture, or 97% methanol in water containing 30 mM silver nitrate. For purification of the saponins from the cucumbers, aqueous solutions of 27% water in methanol or 50% water in methanol were used as the mobile phase. The flow rate was 3 mL/min except when the eluant was 50% water in methanol. The large proportion of water resulted in high backpressures, and thus a flow rate of 2.3 mL/min was chosen. The purity of the HPLC sterol fractions was ascertained with a Carlo Erba Model 4160 gas chromatograph with a flame ionization detector and an HP Ultra 2 capillary column (0.32-mm i.d. × 25 cm) with 0.52-mm film thickness. GC–MS analyses were performed on a Carlo Erba Model 4160 gas chromatograph with on-column injection (column: 30 m × 0.33 mm Durabond DB-5; carrier gas: helium at 0.6 bar; column temperature: 130–280 °C at 6 °C/min. Low-resolution mass spectra were recorded by Annemarie Wegmann-Szente using a Ribermag R-10-10 quadrupole instrument using the SADR (simultaneous acquisition and data reduction) program (source temperature: 115–120 °C). High-resolution mass spectra were recorded on an AEI MS-30 instrument with a direct probe inlet system at the University of Minnesota mass spectrometry service laboratory. The source temperature was 200 °C, the accelerating voltage was 4 kV, and resolution was 3000. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Varian XL-400 spectrometer operating at 400 MHz. All ^{1}H spectra were run in $CDCl_3$ and referenced to residual chloroform at 7.259 ppm. ^{13}C spectra were run in deuteriopyridine and referenced to the 123.5 ppm resonance of pyridine. ^{13}C attached proton test spectra[33] were obtained with the pulse sequences supplied in the manufacturer's software. Melting points were determined on a Thomas-Hoover Unimelt capillary melting point apparatus and are uncorrected.

Column chromatography was performed with Grace grade 62 silica gel, 60–200 mesh (Schoofs Inc.). TLC work was carried out with silica-coated aluminum, Kieselgel 60 F254, 0.2 mm (Merck Chemical Co.). Ceric sulfate[34] or berberine chloride was used as spray reagent.

Solvents used were reagent grade or purified by distillation before use. Radioactivity was determined with a Beckman LZ 7500 liquid scintillation counter through the courtesy of Professor R. Simoni of the Biology Department. Specific rotations were measured in chloroform at room temperature with an Autopol III automatic polarimeter (Rudolph Research): $[\alpha]^{25}$ (percent concentration, solvent).

Collection and Storage of Sea Cucumbers. *Bohadschia argus* was collected by Jane Fromont from the John Brewer Reef, Great Barrier Reef, Australia, at a depth of 7 m. The individuals were freeze-dried and sent by mail to Stanford University. *Stichopus californicus* was collected by Russell Kerr and Max Hoberg near Hopkins Marine Station, Pacific Grove, CA, at a depth of 30 m. The individuals from San Diego were collected by trawling at a depth of 30–40 m. They were promptly frozen in liquid nitrogen. The Puerto Rican sea cucumber *Holothuria mexicana* was collected by Russell Kerr at a depth of 10 m from Ahogado Reef, La Paguerra, S.W. Puerto Rico. *Holothuria arenicola* was collected by Russell Kerr and Vance Vicente at the same site at a depth of 1–2 m. They were frozen in a −40 °C freezer and shipped by overnight mail to Stanford.

The frozen sea cucumbers were stored in the freezer and the freeze-dried individuals were kept in the refrigerator until ready for extraction.

Extraction of Cucumbers. Soxhlet extraction of a dissected specimen of *Stichopus californicus* (300 g) with 95% ethanol in water was found to yield 2 mg of sterol after 48 h. Longer extraction times were not found to increase the total yield. This method was then adopted for the other cucumbers. The thawed cucumber was chopped up and placed in the Soxhlet funnel, and the remaining coelomic fluid was added to the extraction round-bottom flask. We found this method more convenient than the literature procedure.[35]

Column Chromatography of the Crude Extracts. The ethanol extract from *Stichopus californicus* was evaporated to dryness. The remaining solid was extracted 5–6 times with ether, and this solution was evaporated to dryness to afford 1 g of material. *Holothuria arenicola* yielded much less material (0.5 g). With *Bohadschia argus* and *Holothuria mexicana*, one-fifth of the ethanolic extracts was evaporated to dryness to leave 1 g of residual material. Typically 1 g of material was applied to a column containing 30 g of silica gel. The column was eluted with 200 mL of 2.5:1 hexane/ether, and fractions were collected in 20-mL aliquots. A brown/yellow band containing an oily material always came through the column in fraction 1. The sterols eluted in fractions 3–8 and these were combined and evaporated under reduced pressure.

The residue remaining after the ether extractions of *B. argus* and *H. mexicana* was treated with portions of 20 mL of chloroform/methanol/water (65:35:10), and the solution was evaporated to dryness. Typically one-tenth of the total ethanolic extract was used. The solid was taken up in a few milliliters of the chloroform/methanol/water solution and a column run with the same solvent as eluant. The mass of solid material loaded on a 30 g silica column was between 5 and 10 g. Most of this fraction, however, constituted salts since the total yield of saponins after chromatography was only 250 mg from an individual *B. argus* and 40 mg from a large individual of *H. mexicana*. The column was eluted with about 200 mL of solvent, and the saponins were found in fractions 4–10 (R_f for *B. argus* saponins 0.4 and R_f for *H. mexicana* saponins 0.7 by TLC using 65:35:10 chloroform/methanol/water).

HPLC of Sterol and Saponin Mixture. The sterol mixture was initially fractionated by HPLC on an Ultrasil-Si normal-phase column using 6% ethyl acetate/hexane as the mobile phase.[36]

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This allowed separation of the various nuclei: $\Delta^{9(11)}$, Δ^{0}, Δ^{5}, and Δ^{7}. Reverse-phase HPLC using methanol and 2 ODS columns connected in series yielded various sterol fractions. The purity of each HPLC fraction was judged by GC analysis and if necessary, the fractions were subjected to further fractionation using acetonitrile/methanol/ethyl acetate (22:8:6) or 97% methanol in water containing 30 mM silver nitrate. The pure sterols were identified by ^{1}H NMR, low-resolution MS, and comparison to literature values.

The saponin mixture from *B. argus* was purified by injection of 10 mg on a reverse-phase column using 27% water in methanol as the mobile phase. The major saponin had a retention time, at a flow rate of 3 mL/min, of 2 h. The saponin was identified as bivittoside B by comparison of ^{13}C attached proton test spectra to literature spectra.[18a] The saponin mixture from *H. mexicana* was purified by injection of 5 mg on a reverse-phase column using 50% methanol in water. Holothurin A had a retention time of 25 min at a flow rate of 2.3 mL/min. No other saponins were detected. Once again, the saponin was characterized by ^{13}C NMR and comparison to literature values.[18b]

Incorporation Experiments. The precursor was taken up in 1 mL of 70% ethanol in water and administered to the holothurian by injection into the coelomic cavity. Approximately 0.2 mL was injected at each of five sites around the body using a sterile disposable syringe (Stylex, 3 cm^3, 21 × 1.6 cm), taking care to position the needle tip about 1.5 cm below the body surface. It was hoped this way to minimize the chances of injection into the gut, which runs through the middle of the body. The animal was maintained in aerated sea water for 10 days in an aquarium before sacrifice. Examination of two 20-day incorporation experiments with [24-^{3}H]-labeled parkeol (**21**) and [24-^{3}H]-labeled 24-methylenecholesterol (**28**) did not result in substantially better conversion to sterols, although periods of <10 days were not examined. Thus the animals were sacrificed after 10 days.

Preparation of Labeled Precursors. (*RS*)-[5-$^3H_2$]Sodium mevalonate was prepared from (*RS*)-[5-$^3H_2$]mevalonolactone obtained from ICN Radiochemicals by the method of Cornforth and Cornforth.[37] Briefly 25 μCi was taken in a 1-mL ampule; a freshly prepared solution (1 mL) of 1 mM sodium hydroxide was added, and the solution was warmed for 15 min.

[3-^{3}H]Squalene was synthesized by Laurent Wünsche in our laboratory.[38a]

5α-Cholestan-3-one was purchased from Aldrich and purified by reverse-phase HPLC using methanol as the mobile phase until GC analysis indicated the presence of only one compound. A 3-mg sample of the purified material was taken up in 1 mL of 2-propanol, and 10 mCi of $NaBT_4$ was added. The vial was sealed with Parafilm, and the reaction was allowed to proceed for 24 h. Examination of the reaction by TLC after this time showed that no starting material remained (20% ethyl acetate in hexane used as eluant for TLC). Two products had been formed; the major was more polar (R_f 0.15) and was 5α-cholestan-3β-ol (**12**). The α isomer had an R_f value of 0.22 and comprised ~10% of the mixture. The α and β isomers of [^{3}H]5α-cholestan-3β-ol were separated by column chromatography using 2–3 mL of silica gel and eluted with a total of 160 mL of solvent (1% ethyl acetate-/hexane). The yield of the β isomer was 60%.

[3-^{3}H]5α-Cholest-7-en-3β-ol (**1**) was prepared by using **1** purified from *Stichopus californicus* by reverse-phase HPLC as described earlier. Reverse-phase HPLC using methanol as the mobile phase resulted in separation of a sterol fraction containing 5α-cholest-7-en-3β-ol (**1**), *trans*-24-ethylidene-5α-cholest-7-en-3β-ol (**5**) and its cis isomer (**4**). This fraction was then injected on a reverse-phase HPLC using acetonitrile/methanol/ethyl acetate (22:8:6) as the mobile phase. Under these conditions, it was possible to separate **1** from **4** and **5**, which coelute in this solvent system. The 5α-cholest-7-en-3β-ol fraction was purified by reverse-phase HPLC (eluant methanol) until GC analysis indicated that the compound was pure. A 3-mg sample of the purified compound was taken up in 1 mL of methylene chloride, and 10 mg of pyridinium chlorochromate was added. The suspension was stirred overnight, after which time TLC analysis (20% ethyl acetate/hexane) indicated completion of the reaction. The product was purified by passage through a short silica gel column (1:1 hexane/ether). It was then treated as described above with 10-mCi sodium borotritide. However, this time 20% of the α isomer was formed. After separation of the two isomers by column chromatography and reverse-phase HPLC, the yield of [3-^{3}H]-labeled 5α-cholest-7-en-3β-ol was 50%.

Cholesterol labeled at C-3 was synthesized by Christopher Silva[38b] in our laboratory in a similar manner to that described above for **1** and **12**. Commercial cholesterol (Aldrich) was first separated from possible contaminants such as 5α-cholestanol and 5α-cholest-7-en-3β-ol by reverse-phase HPLC. GC analysis indicated the presence of only one compound. [24-^{3}H]-Labeled cycloartenol and lanosterol were also synthesized by Christopher Silva following an analogous scheme to that detailed below for [24-^{3}H]-labeled **21** and **26**, the $\Delta^{9(11)}$ and Δ^7 isomers of lanosterol. [3-^{3}H]-Labeled 24-methylenecholesterol was synthesized by Russell Kerr,[38c] and [3-^{3}H]-labeled isofucosterol was synthesized by George Doss.[38d]

[24-^{3}H]-Labeled parkeol (**21**) was synthesized from 24-methylenecycloartenol acetate in five steps. In the first step, 400 mg of 24-methylenecycloartenyl acetate (Tsuno Rice Fine Chemicals Co., Ltd., Wakayama, Japan) was dissolved in 70 mL of methylene chloride, and 1 mL of pyridine was added to the solution in a 100-mL round-bottomed flask. The flask was cooled in a bath containing dry ice/acetone, and ozone was bubbled in until the solution turned blue. The solution was then purged with nitrogen, 1 mL of dimethyl sulfide was added, and the solution was allowed to come to room temperature. A column was run with 2:1 hexane/methylene chloride (around 300 mL), and the eluant was then changed to 1:1 hexane/methylene chloride. The relevant fractions were evaporated to dryness, and 214 mg (53%) of 24-oxocycloartenol acetate was obtained.[39] This was taken up in 5 mL of 10% trifluoroacetic acid in benzene and left to stir for 5 days, following a method developed by José Giner in our laboratory.[40] After this time, the yellow solution was neutralized with saturated sodium bicarbonate solution and extracted with 100 mL of ethyl acetate. The product was a mixture of 83.5% 3β-acetoxylanost-9(11)-en-24-one, 17.5% 3β-acetoxylanost-8-en-24-one, and 9% 3β-acetoxylanost-7-en-24-one. We did not detect any cucurbitane isomers although these were described by Shimizu et al., who treated cycloartenol with hydrochloric acid in 2-propanol.[22c] The Δ^8 isomer has been isolated and characterized.[41] The Δ^7 isomer was first separated from the Δ^8 and $\Delta^{9(11)}$ isomers by normal-phase HPLC using 2% ethyl acetate/hexane as the mobile phase. Subsequent separation of the Δ^8 and $\Delta^{9(11)}$ isomers was achieved on reverse-phase HPLC with 97% methanol/water containing 30 mM silver nitrate as the mobile phase. However, this method did not give base-line separation, and thus many reinjections were necessary before the $\Delta^{9(11)}$ fraction was pure as judged by GC. The fractions from the HPLC separation were concentrated to dryness, extracted with ether, and then passed through a short silica column to remove any silver nitrate. It was later found that these isomers could be more easily separated by argentic TLC. This method is described later.

3β-Acetoxylanost-9(11)-en-24-one: ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.217 (m, 9-H), 4.477 (m, 3α-H), 2.051 (s, 3β-OAc), 1.098 and 1.080 (2 × 3 H, 26- and 27-H), 1.060 (3 H, s, 30-H), 0.084 (3 H, s, 19-H), 0.868 (3 H, d, J = 6.4 Hz, 21-H), 0.860 (3 H, s, 31-H), 0.732 (3 H, s, 32-H), 0.631 (3 H, s, 18-H); MS m/z (relative intensity) 484.3910 ($C_{32}H_{52}O_3$, 10.18), 470.3724 (10.57), 469.3675 (29.94), 424.367 (14.68), 410.3500 (25.83), 409.3472 (100.0), 255.2133 (10.18), 227.1817 (10.57), 203.1747 (11.94), 201.161 (10.96), 189.1627

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(15.46), 187.1477 (16.24), 175.1490 (18.79), 173.1343 (13.31), 161.1329 (16.63), 159.1167 (16.83), 149.1315 (12.52), 147.1153 (14.87), 145.0977 (15.07), 136.1228 (12.33), 135.1155 (22.50), 133.1019 (21.14), 127.1123 (32.49), 123.1178 (14.09), 121.1016 (27.20), 119.0869 (22.11), 109.1006 (16.44), 108.0936 (11.74), 107.0860 (19.77), 105.0705 (13.89), 95.0885 (28.77), 94.0807 (20.94), 93.0725 (14.29), 81.0689 (16.83), 71.0493 (38.36), 69.0709 (14.09), 57.0687 (12.331), 55.0563 (11.15), 43.0451 (26.42) $[\alpha]^{25}_D$ +80° (*c* 0.6, $CHCl_3$); mp 185–186 °C.

A 3-mg sample of purified 3β-acetoxylanost-9(11)-en-24-one was dissolved in 1 mL of 2-propanol, and 10 mCi of $NaBT_4$ was added. The vial was sealed with Parafilm, and the solution was left to stir for 48 h. TLC (20% ethyl acetate/hexane) indicated 80% conversion to the two isomeric 24-hydroxy isomers. The solvent was evaporated and dilute HCl added. The aqueous solution was extracted with ca. 25 mL of ether, dried with K_2CO_3, and then passed through a short silica column. The solvent was evaporated and any water present was removed with an ethanol/toluene azeotrope. Pyridine (1 mL) was then added followed by three drops of phosphorus oxychloride. After 2 h no starting material remained (TLC, 20% ethyl acetate/hexane). The reaction was quenched by addition of water, and the product was extracted with ether. The ether layer was dried and the product, 3β-acetoxylanosta-9(11),24-diene, was purified by passage through a short column. In the final step, this compound was treated with 10 mg of lithium aluminum hydride in 1 mL of THF. After 30 min the suspension was passed through a silica column and washed with ether. [24-^{3}H]-Labeled parkeol (**21**) was purified by column chromatography using 1% ethyl acetate/hexane as eluant, yielding 1 mg of [24-^{3}H]-**21**, a 33% yield.

[24-^{3}H]-Labeled lanosta-7,24-dien-3β-ol was synthesized in a similar way to [24-^{3}H]parkeol. 3β-Acetoxylanost-7-en-24-one was obtained by TFA/benzene isomerization of 3β-acetoxylanost-8-en-24-one, which was prepared by MCPBA epoxidation of lanosteryl acetate followed by boron trifluoride etherate treatment.[41] A 45-mg sample of 3β-acetoxylanost-7-en-24-one was taken up in 1 mL of 10% trifluoroacetic acid in benzene and left to stir for 3 days. After this time, the isomer ratio was approximately 60% Δ^8 isomer to 40% Δ^7 isomer. The solution was washed with saturated sodium bicarbonate and extracted with ether as described above for the cycloartane isomerization. The Δ^8 and Δ^7 isomers were separated by using normal-phase HPLC and 2% ethyl acetate/hexane. The retention time of 3β-acetoxylanost-8-en-24-one was 30 min. The Δ^7 isomer had a retention time of 36 min. Several reinjections were necessary before 3β-acetoxylanost-7-en-24-one was pure as judged by GC analysis.

3β-Acetoxylanost-7-en-24-one: ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.196 (m, 7-H), 4.513 (m, 3α-H), 2.051 (s, 3β-OAc), 1.097 and 1.079 (2 × 3 H, 30- and 32-H), 0.966 and 0.958 (2 × 3 H, 30- and 32-H), 0.884 (3 H, s, 19-H), 0.875 (3 H, d, J = 6.8 Hz, 21-H), 0.867 (3 H, s, 31-H), 0.628 (3 H, s, 18-H); MS *m/z* (relative intensity) 484.3916 ($C_{32}H_{52}O_3$, 7.60), 470.3712 (31.25), 469.3664 (31.25), 424.3636 (14.70), 410.3435 (30.91), 409.3406 (100.0), 381.3136 (10.81), 270.2357 (14.02), 255.2106 (17.57), 227.1802 (11.49), 213.1663 (12.50), 203.1797 (11.66), 201.1646 (10.81), 189.1646 (14.02), 187.1477 (23.14), 175.1486 (14.70), 173.1334 (19.93), 161.1355 (13.51), 159.1187 (13.85), 149.1343 (14.70), 147.1199 (16.89), 145.1060 (13.18), 135.1196 (34.29), 134.1121 (11.82), 133.1033 (22.13), 127.1123 (26.29), 123.1178 (13.18), 122.1090 (13.01), 121.1020 (19.59), 119.0865 (17.74), 109.1018 (18.92), 107.0860 (24.32), 105.0708 (16.39), 95.0873 (22.97), 93.0709 (13.51), 81.0687 (16.05), 71.0495 (48.99), 69.0707 (17.57), 55.0560 (14.53), 43.0454 (36.99), 43.0083 (26.01) $[\alpha]^{25}_D$ +22° (*c* 0.15, $CHCl_3$); mp 159–160 °C.

A 3-mg specimen of pure 3β-acetoxylanost-7-en-24-one was [^{3}H]-labeled by treatment in 1 mL of 2-propanol with 10-mCi sodium borotritide. Dehydration with $POCl_3$/pyridine afforded 3β-acetoxylanosta-7,24-diene. This was followed by deacetylation as described above for the $\Delta^{9(11)}$ isomer. Column chromatography using 1% ethyl acetate/hexane yielded lanosta-7,24-dien-3β-ol in 45% yield.

All the radiolabeling experiments described above were first run with cold material. The purity of each product was ascertained by GC analysis; if necessary, it was purified by reverse-phase HPLC. Each product from the radiolabeling experiment was then purified in exactly the same manner. In this way the purity of all the precursors used in the incorporation experiments was ensured.

Synthesis of Other Compounds. Parkeol (**21**) was synthesized as described earlier for the labeled compound. Lanost-9(11)-en-3β-ol (**20**) was isolated from the sea cucumber *Stichopus californicus* by column chromatography. Normal-phase HPLC as described earlier was followed by injection on reverse-phase HPLC using methanol as the mobile phase. Lanosterol (**24**) and 24,25-dihydrolanosterol (**25**) were purified from a commercial source (Sigma) by reverse-phase HPLC using methanol as the mobile phase. Cycloartenol was separated from 24-methylenecholesterol by reverse-phase HPLC from a commercial sample (Oryzanol, Tsuno Rice Fine Chemicals Co. Ltd., Wakayama, Japan). 24,25-Dihydrocycloartenol was prepared by hydrogenation of cycloartenol in ethyl acetate over platinum oxide for 2 h. The preparation of lanosta-7,24-dien-3β-ol was as described earlier for the labeled compound. Lanost-7-en-3β-ol was prepared by isomerization of 3β-acetoxylanost-8-ene in 10% TFA/benzene for 3 days and separation of the Δ^8 and Δ^7 isomers using reverse-phase HPLC with methanol as the mobile phase.

Separation of $\Delta^{9(11)}$ Isomers from Δ^8 and Δ^7 Isomers Using Argentic TLC. Parkeol (**21**) acetate was separated from lanosteryl acetate and 3β-acetoxylanosta-7,24-diene on argentic TLC as described by Raederstorff and Rohmer.[42] The TLC plates (14.5 × 20 cm) were prepared by dipping in a saturated solution of silver nitrate in methanol. The plates were dried at 100 °C for 10 min and the process was repeated two more times. A 5-mg sample of a mixture of isomers was loaded onto the plate followed by elution with a 7:3 solution of hexane/toluene. The plate was dried and then eluted two more times. The bands were visualized by spraying with berberine chloride and examination under UV light. Parkeol (**21**) acetate had an R_f of 0.2 after three consecutive elutions. Lanosteryl acetate, which coeluted with 3β-acetoxylanosta-7,24-diene, had an R_f of 0.4. The plates were eluted three times. Similarly, the double-bond isomers with a reduced $\Delta^{24,25}$ bond could be separated; 3β-acetoxylanost-9(11)-ene had an R_f of 0.5 and its Δ^8 isomer an R_f of 0.6.

The separation of 3β-acetoxylanost-9(11)-24-one from its Δ^8 isomer using reverse-phase HPLC was described above. Better separation was possible with argentic TLC using as eluant a solution of 5:3 hexane/toluene.

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