Minor and Trace Sterols in Marine Invertebrates. 27.[1] Isolation, Structure Elucidation, and Partial Synthesis of 25-Methylxestosterol, a New Sterol Arising from Quadruple Biomethylation in the Side Chain

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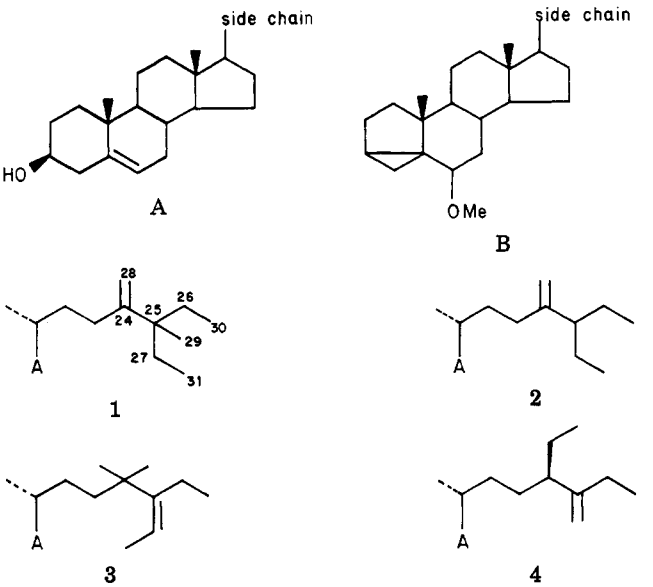
A novel C_{31} sterol, 25-methylxestosterol, resulting from quadruple biomethylation in the side chain has been isolated as a trace constituent of the sterol fraction from a Caribbean sponge (*Xestospongia* sp.). Its structure (1, 24-methylene-25,26,27-trimethylcholesterol) has been elucidated by spectroscopic methods and confirmed by partial synthesis. A biosynthetic route leading to 1 is proposed that is consistent with the hypothesis of stepwise biomethylations and with earlier discoveries of "extended" side chains among marine sterols.

Sterols from the marine environment differ from their terrestrial counterparts mainly in the side chain. While the latter rarely contain more than 10 carbon atoms,[3] frequent discoveries of new C_{11} side chains have been reported from marine sterols during the last decade.[4] Such compounds are believed to be biosynthesized from common short-chain sterols through stepwise biomethylations with *S*-adenosylmethionine.[5] The possibility that C_{12} sterol side chains might also exist was raised by the reported presence of trace quantities of unknown sterols with C_{12} side chains in a tunicate;[6] furthermore, on the basis of a mass spectrum, the structure of another C_{12} side chain was proposed for a sterol from a sponge.[7] This has prompted us to focus our search for new sterols upon compounds with long gas chromatographic retention times and has led to the publication of the first two fully characterized C_{12} side chain sterols from our laboratory.[8] We now report the discovery of 25-methylxestosterol (1)—a third member of this unusual class of sterols arising from quadruple biomethylation of the side chain.



Results and Discussion

Xestosterol (2) has been reported as the main sterol (71%) in a Caribbean sponge of the genus *Xestospongia*.[9] Capillary GC of the mother liquor from this sponge indicated the presence of trace quantities of two sterols with relative retention times (rel t_R) of 2.38 (cholesterol = 1.00) and 2.56, respectively. Separation on reverse-phase high-performance liquid chromatography (LC) yielded two trace sterols with $M^+ = 440$. One of them (rel $t_R = 2.38$) was identified as xestospongesterol (3), whose structure has been fully demonstrated.[8] The mass spectrum of the other showed typical peaks[10] (*m/z* 213, 231, 253, 271) of a Δ^5-3β-hydroxy sterol. A base peak at *m/z* 314 due to a McLafferty rearrangement is characteristic of a $\Delta^{24(28)}$ double bond.[11] That this double bond was present as a 24-methylene group was deduced from the chemical shift of two singlets at δ 4.952 and 5.104 of the 360-MHz spectrum in C_6D_6. The methyl region showed the usual singlets at δ 0.654 (3 H) and 0.944 (3 H) associated with the C-18 and C-19 methyl groups and a doublet at δ 1.017 (3 H, J = 6.5 Hz) due to the C-21 methyl group. In addition, there was noted a singlet at 0.994 (3 H) and triplet at δ 0.812 (6 H, J = 7.5 Hz), indicating the presence of a methyl group and two ethyl groups attached to a quaternary carbon in the side chain. On the basis of these MS and NMR interpretations, the structure of 24-methylene-25,26,27-trimethylcholesterol (1) (25-methylxestosterol) can be proposed for this new sterol which was also isolated from the Indo-pacific sponge *Strongylophora durissima*, which contains predominantly strongylosterol (4).[12]

To confirm this structure assignment, we synthesized 1 by the route outlined in Scheme I. (20*S*)-20-Iodomethyl-6β-methoxy-3α,5-cyclo-5α-pregnane (6) could be prepared in good yield from stigmasterol (5) by well-known procedures.[13] The synthesis of the other component,

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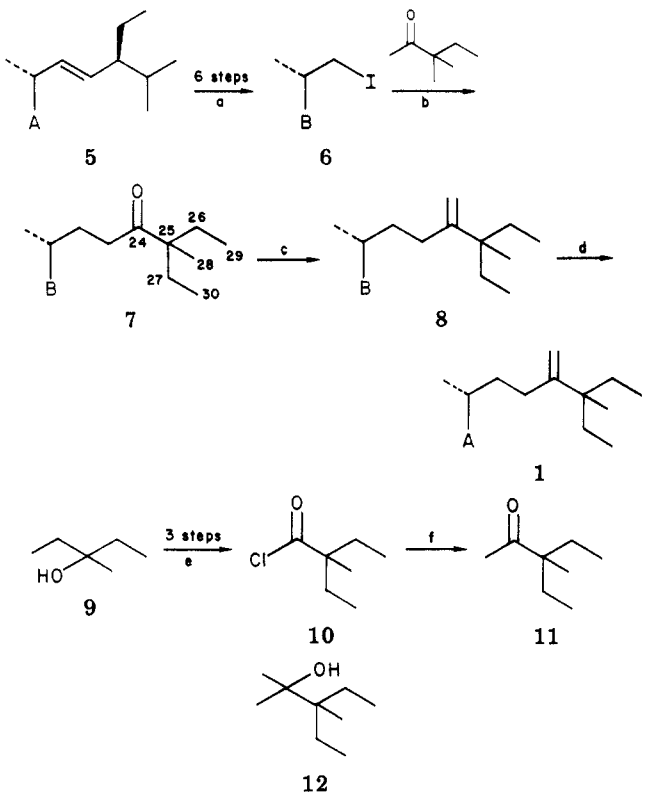
Table I. ^{13}C NMR Chemical Shifts ($CDCl_3$) of Side-Chain Carbons in 25-Methylxestosterol (1)

carbon atom	δ	carbon atom	δ
20	36.03	26	32.04
21	18.89	27	32.04
22	35.26	28	109.21
23	26.87	29	21.86
24	154.33	30	8.58
25	42.96	31	8.58

Scheme I[a]



[a] (a) reference 13; (b) *n*-BuLi, *i*-Pr_2NH; (c) $Ph_3PCH_3^+Br^-$, *n*-BuLi, THF; (d) TsOH, H_2O in *p*-dioxane; (e) ref 15; (f) MeMgI.

3-ethyl-3-methyl-2-pentanone (**11**),[14] was accomplished by a slight modification of a literature procedure,[15] starting from 3-methyl-3-pentanol (**9**). In the last step (**10** → **11**) it is essential that the Grignard reagent be added to an excess of the acid chloride (**10**) rather than the opposite procedure proposed in the literature[15] which leads predominantly to the tertiary carbinol **12**. Formation of the enolate of **11** with lithium diisopropylamide and subsequent reaction with the iodide **6** gave the ketone **7**. This procedure offers an attractive alternative to aldol and Wittig reactions often employed for preparations of sterols with modified side chains.[16] A sluggish Wittig reaction of the ketone **7** with methyltriphenylphosphonium bromide in THF followed by deprotection of the i-ether grouping of **8** yielded the desired product **1**. Coinjection on capillary GC of the synthetic product with natural material gave only one peak, their mass spectra were virtually superimposable, and their 360-MHz ^1H NMR spectra were identical in $CDCl_3$ and C_6D_6.

Since 25-methylxestosterol (**1**) was present in trace amounts, it is very unlikely that it plays a membrane role in these sponges.[17] Instead, the importance of the discovery is to be found in connection with the biosynthesis of **1**.

Experimental studies of the biosynthesis of extended side chains in trace sterols of sponges are connected with major technical problems of which laboratory culture and successful incorporation of labeled precursors are illustrative. For the time being, an indirect method has to be used instead. Employing only well-known biosynthetic steps, one considers schemes that account for the sterols found. Ideally, all the sterols found should be accounted for, and all products and intermediates proposed in the scheme should be present in the sterol mixture. Missing links will be the targets of further analysis and new sterols encountered later should also fit into the scheme.

Scheme II contains all the sterols with a long side chain found in *Xestospongia* species as well as in other sponges. In involves only four well-known[5] bioconversions: (a) one-step biomethylation with *S*-adenosylmethionine (SAM), (b) proton elimination from a carbon adjacent to the resulting carbonium ion, (c) 1,2-migration of a tertiary hydrogen to a tertiary carbonium center followed by proton loss, (d) double bond migration, presumably via a biochemical hydrogenation–dehydrogenation sequence.

Scheme II thus can account for all known sponge sterols (boxed in Scheme II) with extended side chains. The goal to discover all intermediates has not yet been fully achieved. Compounds with a $\Delta^{24(25)}$ double bond are very reactive,[18] so the intermediates **20** and **25** are probably short lived. The absence of **19** as well as further alkylated products derived from it make it a less likely candidate to appear in sterol analysis. A systematic synthesis of such highly alkylated desmosterol analogues is now under way in our laboratory with the aim of determining their physical characteristics so as to facilitate their eventual discovery in nature.

The possibility of enzymatically induced methyl migrations has been suggested by other authors[19,20] for the biosynthesis of side chains in terrestrial tetracyclic triterpenes and cannot be ruled out for marine organisms. If such processes do indeed occur—at present they are purely speculative—then they must also be included in possible biosynthetic pathways.

Experimental Section

General Methods. Analytical GC was carried out on a Hewlett-Packard 402A chromatograph equipped with a flame-ionization detector (FID) and a U-shaped glass column containing 3% OV-17 on GCQ (carrier gas, He; oven temperature, 260 °C) or a Carlo Erba capillary GC (Fractovap 4160) equipped with a FID and flexible capillary column containing SE-54 (carrier gas, H_2; oven temperature, 260 °C).

Preparative high-performance liquid chromatography was performed on two columns: (A) Whatman Partisil M9 10/50 ODS-2 and (B) Whatman Partisil M9 ODS-3 (50 cm × 1 cm), using a Waters Associates pump and dual-cell refractometer detector. Absolute methanol was used as eluant.

Combined GC–MS was performed on a Varian MAT 44 spectrometer or on a Ribermag R-10-10B (capillary GC) and high-resolution MS on a Varian MAT 711 double-focusing

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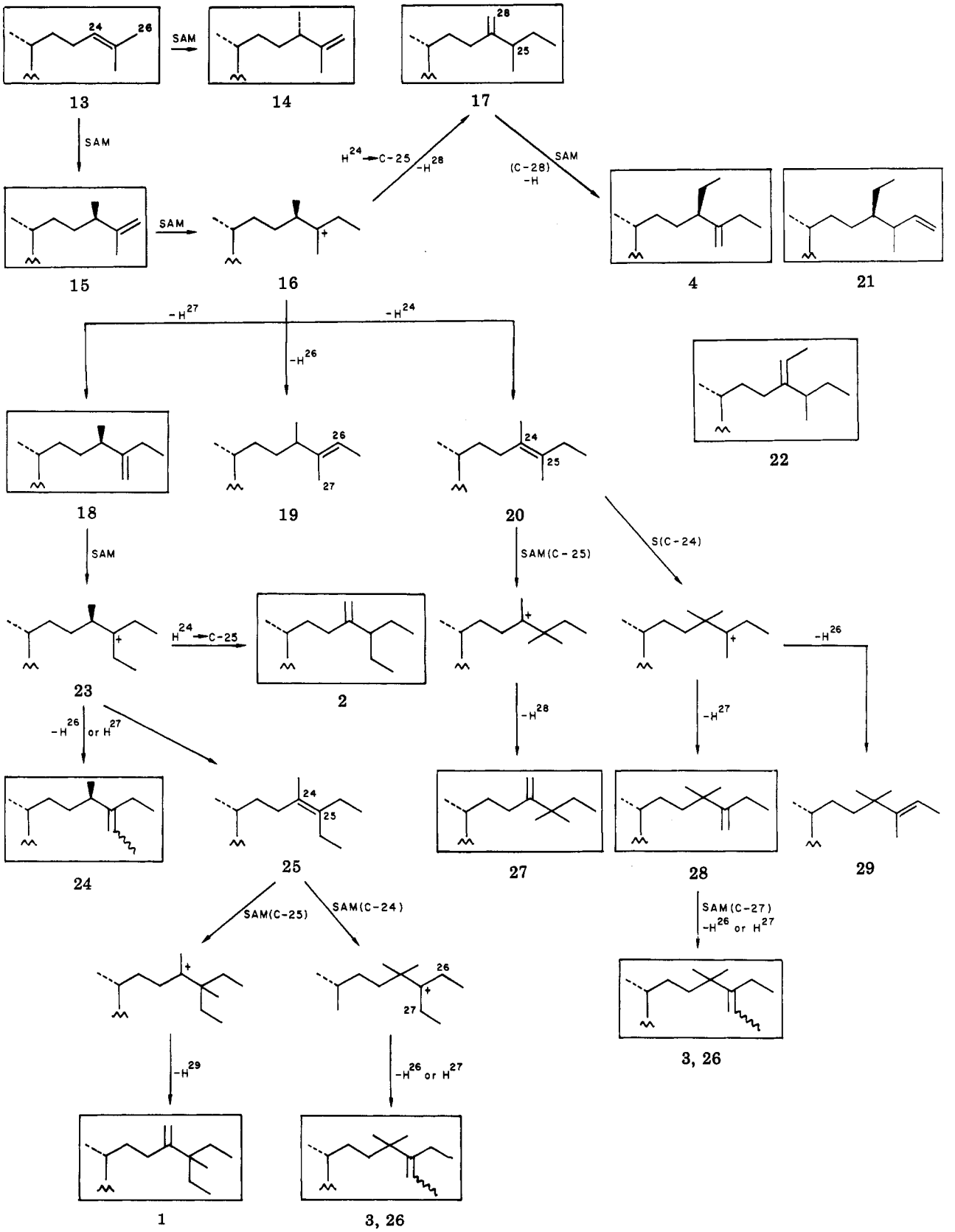
Scheme II



spectrometer.

^{1}H NMR spectra were recorded on a Bruker HXS-360 (360 MHz) spectrometer and ^{13}C NMR spectra on a Varian XL-200 (200 MHz) spectrometer. Chemical shifts are in parts per million relative to Me_4Si.

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.

Isolation of Natural 25-Methylxestosterol. The free sterols from the mother liquor of xestosterol[9] were applied on LC column A, and 19 fractions were collected and analyzed by capillary GC–MS. Fractions 15 and 19 contained sterols with $M^+ = 440$. Reinjection of fraction 19 on column B yielded pure 25-methylxestosterol (1).

25,26,27-Trimethyl-6β-methoxy-3α,5-cyclocholestan-24-one (7). A 2.4 M solution of *n*-butyllithium in hexane (1.75 mL, 4.2 mmol) was added at 0 °C under nitrogen gas to diisopropylamine (404 mg, 4.0 mmol) in anhydrous THF (10 mL). The solution was stirred at 0 °C for 15 min and cooled to −78 °C. A solution of 3-ethyl-3-methyl-2-pentanone (11)[14] (487 mg, 3.8 mmol) in THF (3 mL) was added dropwise during 5 min, and the solution kept at −78 °C for 90 min. The iodide 6[13] (456 mg, 1.0 mmol) in THF (3 mL) was added and the cooling bath was removed. The reaction mixture was heated to reflux (1 h) and left at room temperature overnight. Workup with water and ether, washing of the organic phase with HCl and sodium bicarbonate solution, drying over magnesium sulfate, and evaporation yielded a crude product, which was subjected to column chromatography on silica gel (hexane–ether, 10:1) to give the desired product 7 (142 mg, 31%) and recovered starting material 6 (288 mg, 50%): ^{1}H NMR (360 MHz) δ 0.710 (3, s, C-18 Me), 0.757 (6, t, J = 7.6, C-29/C-30 Me), 0.905 (3, d, J = 6.6, C-21 Me), 1.018 (3, s, C-19 Me), 1.042 (3, s, C-28 Me), 3.321 (3, s, C-6 OMe); mass spectrum, m/z 456 (M^+, $C_{31}H_{52}O_2$).

25,26,27-Trimethylergosta-5,24(28)-dien-3β-ol (25-Methylxestosterol, 1). Methyltriphenylphosphonium bromide (639 mg, 1.79 mmol) was suspended in anhydrous THF (5 mL) under an atmosphere of nitrogen. A solution of 2.4 M *n*-butyllithium in hexane (0.75 mL, 1.80 mmol) was added dropwise. The suspended material dissolved and gave a yellow solution, which was heated to 50 °C (1 h). The ketone 7 (125 mg, 0.274 mmol) in THF (3 mL) was added and the solution was brought to reflux. The reaction was very slow, as checked by TLC. After 6 days the product to starting material ratio was 3:1, and the reaction was stopped. Workup with water and ether as in the previous experiment yielded a yellow oil. Silica gel chromatography (hexane–ether, 9:1) separated the starting material from the product 8. The crude product (86 mg) was dissolved in *p*-dioxane (15 mL) and water (3 mL) and *p*-toluenesulfonic acid (3 mg) was added. The solution was heated under reflux for 30 min (starting material absent by TLC), poured into water, and extracted with ether. The ether was washed with sodium bicarbonate solution and water, dried, and evaporated to give a crude product, which was purified on reverse-phase LC with methanol as eluant to give a white, crystalline solid (41 mg, 34%): mp 143 °C (from MeOH); $[\alpha]^{20.0}_D$ −27 (*c* 0.76, $CHCl_3$); ^{1}H NMR (360 MHz) (C_6D_6) δ 0.653 (3, s, C-18 Me), 0.812 (6,t, J = 7.4 Hz, C-30/C-31 Me), 0.944 (3, s, C-19 Me), 0.994 (3, s, C-29 Me), 1.015 (3, d, J = 6.6 Hz, C-21 Me), 3.4 (1, m, C-3 H), 4.953 (1, s, C-28 H), 5.106 (1, s, C-28 H), 5.35 (1, br, C-6 H); mass spectrum, m/z (relative intensity) 440.4054 (M^+, $C_{31}H_{52}O$, 8; calcd 440.4018), 425 (2), 422 (4), 314 (100), 300 (8), 299 (11), 296 (9), 281 (12), 272 (9), 271 (13), 231 (4), 228 (9), 213 (7).

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Two Bicyclic C_{15} Enynes from the Sea Hare *Aplysia oculifera*[1]

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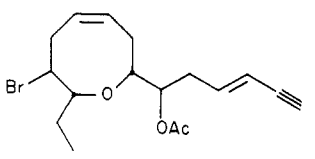
The structures of two geometric isomers (*E*)- and (*Z*)-ocellenyne, isolated from the sea hare *Aplysia oculifera*, were elucidated by chemical degradation and spectral analysis.

Sea hares are gregarious, herbivorous mollusks of the order *Anaspidea* (subclass Opisthobranchia, class Gastropoda). Their global distribution, abundance, intertidal or shallow water habitat, and the large size of some species have made aplysids attractive chemical targets. No comprehensive review of secondary metabolites of sea hares has appeared in print. This is, at least in part, due to the varied dietary origin as well as diverse biogenesis of these compounds. A preferred food of some sea hares is the red algal genus *Laurencia*, renowned for its synthetic capability, which has engendered numerous terpenoid[2a] and nonterpenoid[2b] compounds, many halogenated. One group of nonterpenoid metabolites possesses an unbranched C_{15} backbone with a conjugated enyne terminus. The first representative of this class of compounds, the oxocin derivative laurencin (1), was described in 1965 by Irie et al.[3]

From the sea hare *Aplysia oculifera* we report isolation and structure determination of two geometrically isomeric enynes, (*E*)- and (*Z*)-ocellenyne (**2**,**3**),[4] which possess a novel 2,5-dioxabicyclo[2.2.1]heptane system.

A. oculifera (Adams and Reeve, 1850) were collected on a reef flat near Pupukea, Oahu. We observed that the sea hares were feeding on an unidentified species of *Laurencia*. Extraction of the excised digestive glands from 25 animals and solvent partitioning yielded a lipid fraction, which after chromatographic purification afforded (*E*)-ocellenyne (**2**, 30 mg) as a colorless oil and (*Z*)-ocellenyne (**3**, 14 mg) as a colorless solid. Mass spectral analysis showed their composition to be $C_{15}H_{20}Br_2O_2$.



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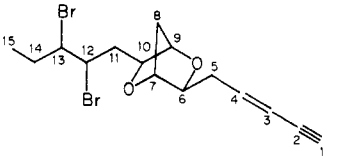

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