Enzymic Cyclization of 2,3-Dihydrosqualene and Squalene 2,3-Epoxide by Squalene Cyclases: from Pentacyclic to Tetracyclic Triterpenes

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Cell-free systems from the protozoon *Tetrahymena pyriformis* and the bacterium *Alicyclobacillus acidocaldarius* normally convert squalene into pentacyclic triterpenes of the gammacerane and hopane series. 2,3-Dihydrosqualene, a substrate analogue lacking one of the terminal double bonds of squalene and therefore making it impossible to form pentacyclic products, was converted unexpectedly into tetracyclic triterpenes, *i.e.* euph-7-ene by the *T. pyriformis* enzyme and a 1:1 mixture of (20*R*)-dammar-13(17)-ene and (20*R*)-dammar-12-ene by the system from *A. acidocaldarius*. Formation of a pentacyclic framework with six-membered D-ring might thus only depend on the assistance of the terminal double bond to the cyclization process, its lack of participation leading, probably spontaneously, to the formation of the thermodynamically favoured tetracyclic skeleton with a five-membered D-ring.

The cell-free system from *T. pyriformis* was further, for the first time, directly shown to induce cyclization of (3*S*)-[3-^{3}H]squalene epoxide into gammacerane-3β,21α-diol, and the (3*R*)-enantiomer into gammacerane-3α,21α-diol. The (3*R*)-enantiomer also afforded a novel monocyclic product with a 2,3,4-trimethylcyclohexanone structure. The enzymic cyclization of squalene epoxide is apparently exclusively initiated by an oxirane ring-opening, and not by a proton attack on the remaining terminal double bond of the molecule.

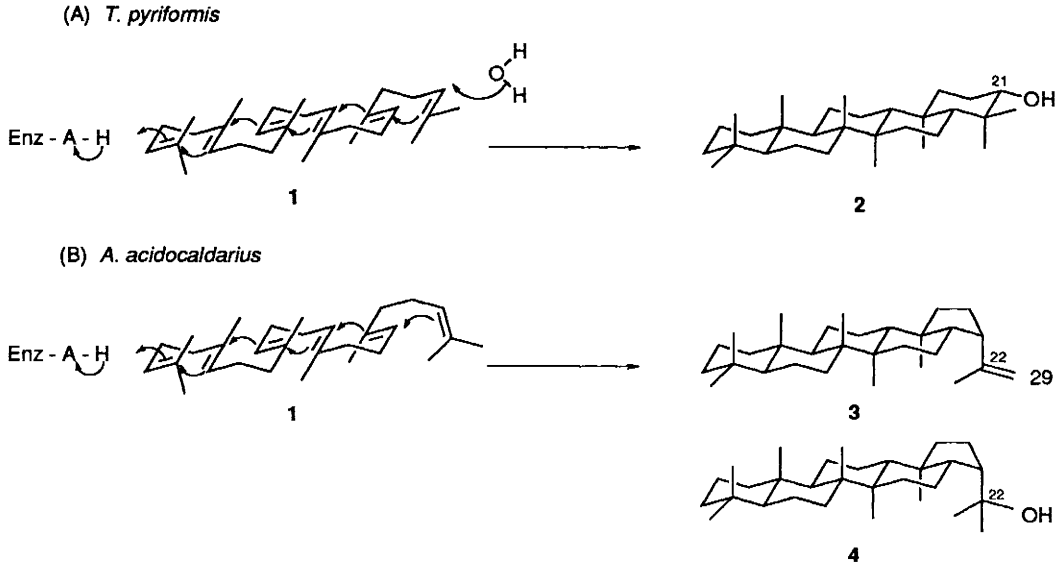
Protozoa of the genus *Tetrahymena* produce a gammacerane triterpene, tetrahymanol **2**, which is regarded as a sterol surrogate in this organism.[1] The biosynthesis of tetrahymanol has been well studied, and it was established that the formation of tetrahymanol does not proceed *via* the cyclization of squalene epoxide, but *via* a non-oxidative enzyme-catalysed cyclization of squalene **1** as for bacterial hopanoids (Scheme 1).[1-3] The cyclization of squalene, folded in its all-pre-chair conformation, is initiated by proton attack on a terminal double bond and is followed by addition of water at the resulting gammaceranyl C-21 cationic centre without carbon-skeletal rearrangement.[1-3] The squalene cyclase of *Tetrahymena* has been considered to be rather primitive because of both the simplicity of the reaction and its low substrate selectivity.[1,3] It was shown that *T. pyriformis* squalene cyclase can also cyclize racemic (*RS*)-squalene 2,3-epoxide to a mixture of gammaceranediols (see Scheme 4 below).[4] Furthermore, C_{25} and C_{30} regular polyprenyl methyl ethers were converted into a series of polycyclic products.[5]

In a preliminary communication, we have reported that a cell-free extract from *T. pyriformis* efficiently converted 2,3-dihydrosqualene **5**, a substrate lacking one of the terminal double bonds of squalene, therefore making it impossible to form pentacyclic products, into euph-7-ene **6** with an unexpected tetracyclic skeleton (Scheme 2).[6] In this paper, we further describe the result of the incubation with a cell-free system from the thermoacidophilic bacterium *Alicyclobacillus* (formerly *Bacillus*) *acidocaldarius* which normally catalyses the cyclization of squalene into pentacyclic triterpenes of the hopane series, hop-22(29)-ene **3** and hopan-22-ol **4**, in a constant molar ratio of 5:1.[7,8] In the case of hopanoid formation, the hopanyl C-22 carbocation with a five-membered E-ring is formed instead of the gammaceranyl cation, and this is then followed by either 29-H proton elimination or addition of water at the cationic centre, yielding hop-22(29)-ene or hopan-22-ol, respectively (Scheme 1B). The *A. acidocaldarius* cyclase has been reported also to accept (*E*,*E*)-homofarnesol, homogeraniol and bishomofarnesyl (1,5,9-trimethyldeca-4,8-dienyl) ether as substrates.[8] Recently, the squalene cyclase of *A. acidocaldarius* has been purified,[7,9] cloned, expressed and sequenced[10] by Poralla's group in Tübingen. Purification of tetrahymanol cyclase from *T. thermophila* has been also reported.[11]

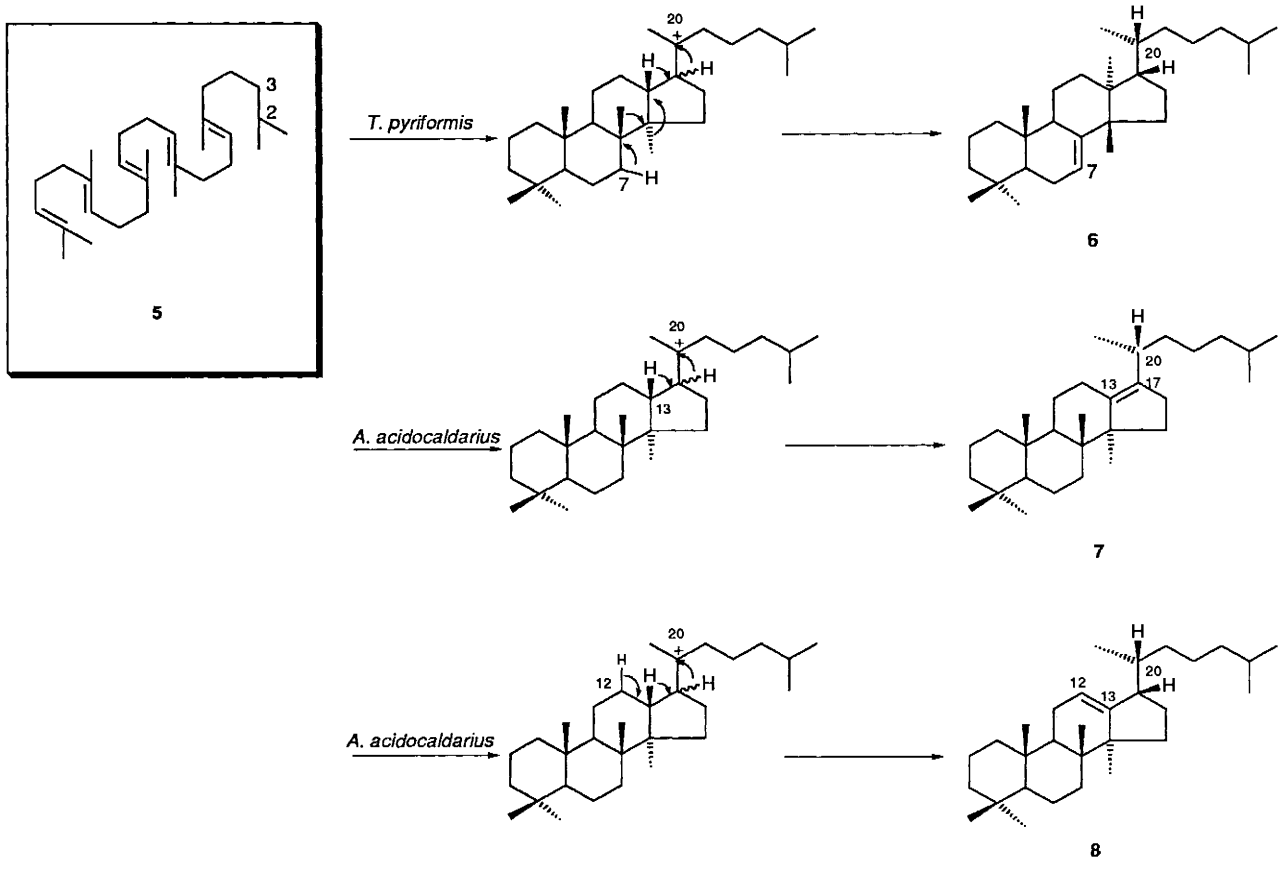
As described above, *T. pyriformis* squalene cyclase cyclized racemic (*RS*)-squalene 2,3-epoxide to a mixture of gammacerane-3α,21α-diol **15** and gammacerane-3β,21α-diol **16** (see Scheme 4). One question remained, however, unanswered. Why is the cyclization of squalene epoxide always initiated from an oxirane ring-opening reaction, and not by a proton attack on the terminal double bond of the molecule? As 2,3-dihydrosqualene **5** is efficiently transformed into euph-7-ene **6**,[6] it might therefore be expected that squalene epoxide would also be converted into other cyclization products such as 24,25-epoxyeuph-7-ene. In order to investigate this possibility, we also examined the cyclization of squalene epoxide by a cell-free system from *T. pyriformis*.

Results and discussion

Enzymic Cyclization of Dihydrosqualene.—Identification of euph-7-ene from a cell-free system of T. pyriformis. Incubation of 2,3-dihydrosqualene **5** with a cell-free system from *T. pyriformis* led to the isolation, by TLC in high yield, of one single fraction which was less polar than the starting material. Under the standard assay conditions (50 μmol dm^{-3} substrate concentration, for 4 h at 30 °C), the conversion rate was 20%, while that of squalene into tetrahymanol was 50%. The cyclization product could not be detected in a control experiment performed with a boiled enzymic preparation and could be directly identified by comparison of its spectroscopic data with those of synthetic reference materials, making it unnecessary to have recourse to radiochemical identification methods. The NMR and mass spectra of the product were characteristic of those of tetracyclic triterpenic hydrocarbons and showed good accord with those of eupha-7,24-diene, which has been isolated from a fern,[12] except for the signals due to the terminal double bond. In the ^{1}H NMR spectrum, the methyl signals for the C-4α, C-4β, C-10β, C-13α, C-14β and C-20*R*



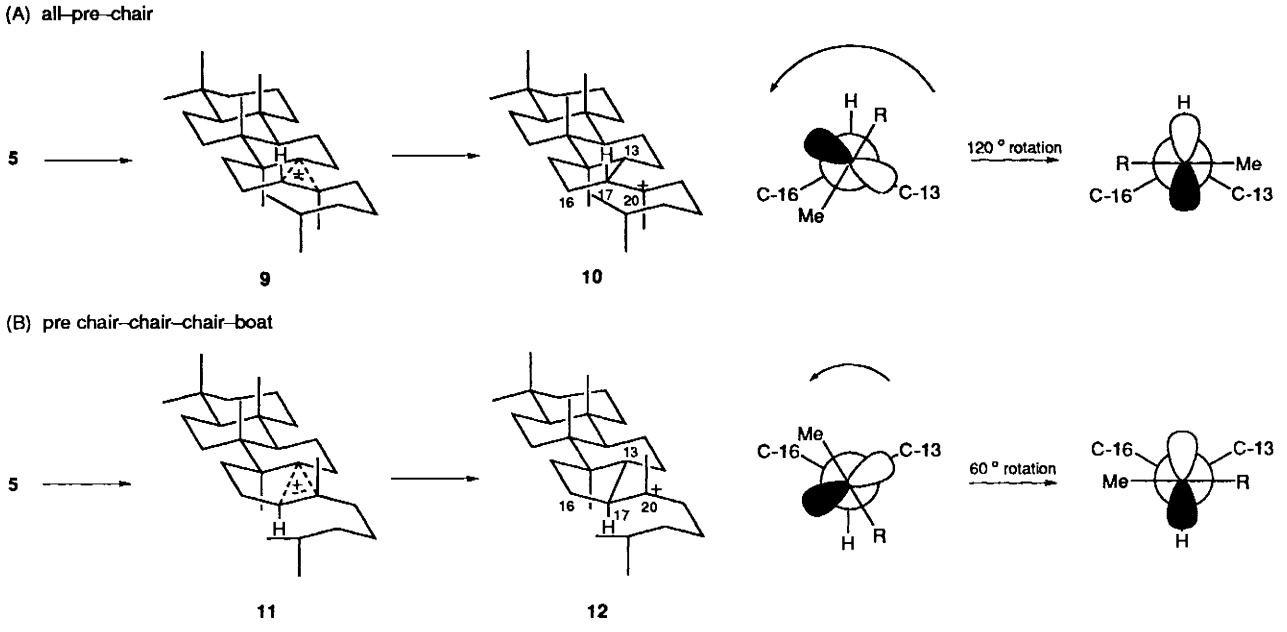
Scheme 1 (A) Cyclization of squalene **1** into tetrahymanol **2** by the squalene cyclase of *T. pyriformis*. (B) Cyclization of squalene **1** into hop-22(29)-ene **3** and hopan-22-ol **4** by the squalene cyclase of *A. acidocaldarius*.



Scheme 2 Cyclization of 2,3-dihydrosqualene **5** by the squalene cyclase of *T. pyriformis* and *A. acidocaldarius*

methyl groups and the olefinic proton signal for 7-H were almost identical with those of eupha-7,24-diene. In the GLC–MS spectrum, the base peak was observed at *m/z* 397, which corresponds to $M^+ - CH_3$. Confirmation of the structure, and the stereochemistry of C-20, was finally obtained by direct comparison (GLC, GLC–MS, ^1H and ^{13}C NMR) with the chemically synthesized euph-7-ene **6** and its (20*S*)-isomer, tirucall-7-ene, respectively obtained from butyrospermol (eupha-7,24-dien-3β-ol)[13] and masticadienoic acid (3-oxo-tirucalla-7,24-dien-25-oic acid).[14] The two isomers could be unambiguously differentiated. They were readily separable by GLC, and this was verified by co-injection giving baseline separation of the two signals. In the ^1H NMR spectrum recorded at 300 K, the methyl protons of the 20*R*-Me group of euph-7-ene appeared at δ 0.835 (*J* 6.8 Hz), while those of the 20*S*-Me group of tirucall-7-ene appeared at δ 0.865 (*J* 6.7 Hz). The cyclization product was thus shown to be euph-7-ene **6**, which has not yet been isolated.

Identification of the dammarenes from a cell-free system of A. acidocaldarius. Incubation of 2,3-dihydro[13-^3H]squalene



Scheme 3 Proposed mechanism for the cyclization of 2,3-dihydrosqualene **5** to the tetracyclic products with C-20*R* stereochemistry

with a cell-free system from *A. acidocaldarius* led to the isolation of two fractions which were less polar than the starting material. Under the standard assay conditions (50 μmol dm^{-3} substrate, at 60 °C for 15 h), the conversion rate was 2%, while that of squalene to hop-22(29)-ene was 30%. A large-scale incubation yielded enough material for spectroscopic analysis.

Spectrosopic data of the more polar product on $AgNO_3$ TLC suggested the structure of (20*R*)-dammar-13(17)-ene **7** and were in good accord with those of (20*R*)-dammara-13(17),24-diene, which has been isolated from a fern,[12] except for the signals due to the terminal double bond. In the ^1H NMR spectrum, the methyl signals for the C-4α, C-4β, C-8β, C-10β and C-14α Me groups were almost identical with those of (20*R*)-dammara-13(17),24-diene. In the GLC–MS spectrum, the base peak at *m/z* 191 corresponding to ring C cleavage is frequently observed for several tetracyclic and pentacyclic triterpenoids with quaternary C-8 and C-14 carbon atoms.[15] Confirmation of the structure including the stereochemistry of C-20 was finally obtained by direct comparison (GLC–MS, ^1H NMR) with synthetic (20*R*)-dammar-13(17)-ene and (20*S*)-dammar-13(17)-ene which were respectively obtained by treatment of euph-7-ene **6** and of tirucall-7-ene with 20% BF_3–diethyl ether in diethyl ether as reported for the conversion of eupha-7,24-diene into (20*R*)-dammara-13(17),24-diene.[12] The two isomers could also be unambiguously differentiated by GLC and ^1H NMR. In the ^1H NMR spectrum at 300 K, the methyl signal of the 20*R*-Me group of (20*R*)-dammar-13(17)-ene appeared at δ 0.910 (*J* 6.9 Hz), while those of the 20*S*-Me group of (20*S*)-dammar-13(17)-ene appeared at δ 0.951 (*J* 6.8 Hz).

The less polar product on $AgNO_3$ TLC was believed to be (20*R*)-dammar-12-ene **8**. In the ^1H NMR spectrum, the chemical shifts of the methyl groups attached to C-4α, C-4β, C-8β, C-10β and C-14α, and of the olefinic proton attached to C-12 resembled those found in the spectra of olean-12-ene,[16] neohop-12-ene[16] and bacchara-12,21-diene.[17] In the GLC–MS spectrum, the base peak at *m/z* 220 corresponding to a retro-Diels–Alder reaction is characteristic for triterpenes with a Δ^{12} double bond.[16,17] Further confirmation of the structure was obtained by quantitative conversion of this compound into (20*R*)-dammar-13(17)-ene **7** by treatment with BF_3–diethyl ether. The cyclization products were thus shown to be (20*R*)-dammar-13(17)-ene **7** and (20*R*)-dammar-12-ene **8**, which have not so far been isolated.

Discussion. 2,3-Dihydrosqualene was cyclized, on the one hand, into a single product, euph-7-ene **6**, by a cell-free system from *T. pyriformis*, and on the other hand into (20*R*)-dammar-13(17)-ene **7** and (20*R*)-dammar-12-ene **8** by an enzymic preparation from *A. acidocaldarius* (Scheme 2). Other cyclization products such as olefins or alcohols were not detected and, if formed, can only be present in much smaller amounts. Since these compounds were neither formed in the inactivated cell-free system, nor detected in these micro-organisms, their enzymic origin seems certain.

For both cyclases two features have to be mentioned. First, the cyclization produces only transposed dammarenes with a five-membered D-ring. Second, all cyclization products have only the 20*R* configuration, suggesting that the stereochemistry of the rearrangement reactions must be strictly controlled by the enzymes. Cyclization of 2,3-dihydrosqualene occurs most likely at the same squalene-binding active site, the geometry of which is already adapted for such reactions. Considering the structures of the hopanoids and tetrahymanol with a six-membered D-ring and the small structural differences between squalene, the normal substrate, and its analogue 2,3-dihydrosqualene, cyclization of the latter analogue was expected to occur in the same conformation of the polyene, *i.e.* an all-pre-chair conformation (Scheme 3, Pathway A), and to generate a secondary tetracyclic cation **9** with a six-membered D-ring. As formation of ring E was not possible due to the absence of the terminal double bond, the cyclization process yields spontaneously the most stable tertiary 17-isodammaranyl cation **10**. In this case, in order to achieve the 20*R* stereochemistry, a 120° rotation about the C-17–C-20 bond is required prior to proton migration from C-17 to C-20.

An alternative pathway would also lead to the same cyclization products. A different folding of the precursor in a pre-chair–chair–chair–boat conformation would yield another tetracyclic cation **11** with a six-membered D-ring and/or to the dammaranyl tertiary cation **12** (Scheme 3, Pathway B). Here, the C-20*R* configuration of the product can be attained *via* a least-motion pathway involving only a small (< 60°) rotation about the C-17–C-20 axis. Further, a 1,3-diaxial interaction between the two methyl groups in pre-ring D is released and the following rearrangement reaction can be explained in an all-antiparallel manner. This second hypothetic pathway is probably the less likely one: indeed, folding of the substrate in

a pre-chair–chair–chair–boat conformation differs from the all-pre-chair conformation required for the normal squalene cyclization. At this stage the two routes cannot be differentiated one from another, and the differences between them resemble those proposed for the formation of lanosterol from a protostane intermediate with either a 17α-side-chain [18,19] or with a 17β side-chain.[20]

The final olefinic reaction products are subsequently obtained by a backbone rearrangement and hydride shifts followed by elimination of proton to yield either euph-7-ene **6** in the case of the *T. pyriformis* cyclase, or (20*R*)-dammar-13(17)-ene **7** and (20*R*)-dammar-12-ene **8** in the case of the *A. acidocaldarius* enzyme, all of them with a 20*R* configuration (Scheme 2). This suggests that the conformation of the cationic intermediates **10** or **12** is strictly controlled by the enzyme. The formation of different cyclization products by the two cyclases might reflect differences in the geometry of their active sites. In chemical isomerization experiments, both euph-7-ene **6** and (20*R*)-dammar-12-ene **8** were quantitatively converted into (20*R*)-dammar-13(17)-ene **7** by treatment with BF_3–diethyl ether. Further, treatment of (20*S*)-dammaran-20-ol with $HClO_4$ in HCO_2H–$CHCl_3$ yielded the mixture of the two diastereoisomers (20*R*)- and (20*S*)-dammar-13(17)-ene as a major product (I. Abe and M. Rohmer, unpublished result). Formation of (20*R*)-dammar-13(17)-ene by the *A. acidocaldarius* cyclase therefore seems to proceed rather spontaneously from the tertiary cationic intermediate. On the other hand, cyclization into (20*R*)-dammar-12-ene and euph-7-ene, which are not the thermodynamic products, are supposed to require enzymic assistance for the elimination of, respectively, the 12α-H or 7α-H proton. Similar reactions are known from biogenetic pathways leading to several triterpene series. For example, eupha-7,24-diene and (20*R*)-dammara-13(17),24-diene have been isolated from *Polypodium* species.[12] Tetrahymanol has been reported from another fern (*Oleandra wallichii*[21]), and hydrocarbons derived from the C-22 hopanyl or moretanyl cation are widespread in the Pteridophyta. Furthermore, fern-7-ene derives from a similar backbone rearrangement as that implied in the formation of euph-7-ene.[22] The often simultaneous occurrence of several types of triterpenic hydrocarbons in ferns suggests a possible close relationship between these cyclases leading to the diverse polycyclic frameworks.

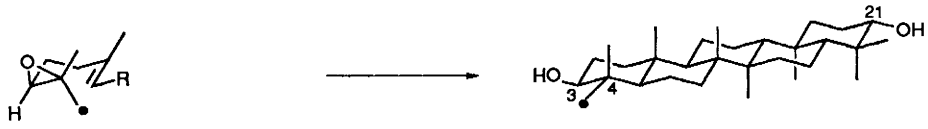
According to Ruzicka's biogenetic isoprene rule,[18] a squalene derivative is converted into various triterpenic skeletons by different enzymes which might differ one from another only by small variations of a single catalytic mechanism. Such variations might be obtained by slight modifications of the structure of the active site of the enzyme, as proposed in Ourisson's phylogenetic isoprene rule for molecular evolution of triterpene cyclases.[1,23] Indeed, recent progress in molecular biological studies of several squalene and oxidosqualene cyclases provides support for this hypothesis.[3] The versatility of the above described squalene cyclases demonstrates a further possibility that the geometry of the active site of the 'primitive' squalene cyclases has already been 'prepared' for other cyclization products. Preferential formation of a five-membered instead of a six-membered D-ring is apparently only obtained by the lack of participation of the π-system of the terminal double bond at the end of the cyclization process. The 'primitive' *T. pyriformis* enzyme catalyses the simple cyclization of squalene into the pentacyclic gammacerane skeleton without any rearrangement and with addition of water to the final cationic intermediate, but in the case of dihydrosqualene it proved to be capable of accomplishing a more complex reaction involving a transposition as well as a proton elimination. The cyclization and the subsequent rearrangement leading to the tetracyclic skeletons would appear to be a spontaneous sequence of events dictated primarily by a folding conformation of the polyene in which the terminal double bond is sufficiently distant from the pre-ring D. 2,3-Dihydrosqualene therefore seems to be an excellent substrate for the investigation of the potential ability for the synthesis of tetracyclic triterpenes by a cyclase normally catalysing the formation of pentacyclic frameworks.

Enzymic Cyclization of Squalene Epoxide by a Cell-free System of T. pyriformis.—*Identification of the cyclization products.* As described before,[4] the racemic (*RS*)-[3-^{3}H]-squalene 2,3-epoxide **13** and **14** was converted in substantial amount into two radioactive fractions on TLC with CH_2Cl_2 as developing solvent: one possessing the polarity of diols, which was further separated into gammacerane-3α,21α-diol **15** and gammacerane-3β,21α-diol **16** by TLC, and the other one possessing that of squalene epoxide. These two fractions were the only detectable radioactive bands on the plates. In particular, no radioactivity was detected in the tetrahymanol or sterol regions. The squalene epoxide-containing fraction could be further separated into two distinct radioactive compounds by TLC (5% EtOAc–cyclohexane): unchanged squalene epoxide (verified by ^{1}H NMR analysis and TLC on $AgNO_3$-impregnated silica gel) and a more polar, unknown product. Under the standard assay conditions (50 μmol dm^{-3} substrate, at 30 °C for 4 h), the conversion rates into the unknown product, gammacerane-3α,21α- and -3β,21α-diol were respectively 9, 41 and 32%, while that of squalene into tetrahymanol was 45% (Table 1). Since these products could not be detected in a control experiment performed with a boiled enzymic preparation, they could only arise from an enzymic reaction.

For identification of the cyclization products, a large-scale incubation was carried out and afforded enough pure material for spectroscopic analysis. Except for the above mentioned three compounds, no other products could be detected. The spectroscopic data (^{1}H NMR, ^{13}C NMR and GLC–MS) of the gammaceranediols as well as of the recovered unchanged squalene epoxide were identical with those reported in the literature[4] and confirmed their structures. The IR spectrum of the novel product showed a strong band at 1715 cm^{-1} compatible with the presence of a cyclohexanone moiety. On the ^{1}H NMR spectrum, four vinylic protons and three aliphatic methyl groups [two secondary methyl groups at δ 1.10 (d, *J* 7 Hz), δ 0.99 (d, *J* 7 Hz), and one tertiary methyl group at δ 0.84 (s)] indicated that the compound is a monocyclic triterpene. Further, one of the three protons in the α-position of the carbonyl carbon atom appeared at δ 2.47 as a quartet (*J* 7 Hz), indicating solely a coupling with the protons of the methyl group at δ 0.99 (d, *J* 7 Hz) and suggesting that this carbon atom is located between a quaternary carbon atom and the carbonyl group. On the GLC–MS spectrum, the fragment at *m/z* 139 corresponded to the trimethylcyclohexanone ring. From these data, the compound was suggested to be a monocyclic compound with a 2,3,4-trimethylcyclohexanone moiety **17**. The results of the ^{1}H–^{1}H COSY spectrum and of the ^{1}H–^{13}C long-range COSY experiments were consistent with the structure. In the ^{1}H–^{1}H COSY spectrum, one of the α-carbonyl protons (2-H) was coupled only to 24-H_3, and the methyl signal of the C-25 Me group to a proton at δ 1.9 (4-H). The other α-carbonyl protons (6-H) were coupled to proton signals at δ 1.68 (5-H_a) and δ 1.99 (5-H_b). Further, in order to confirm the location of the side-chain of the molecule, ^{1}H–^{13}C long-range COSY experiments were performed. The study of the proton non-decoupled spectrum led us to assign a value of 5 Hz to the ^2J and ^3J coupling constants between ^{1}H and ^{13}C, which was taken into account in the experiment. In the spectrum, the methyl protons 24-H_3 were coupled to the carbons C-1, C-2 and C-3, and those 25-H_3 were coupled to C-3, C-4 and C-5. The methyl

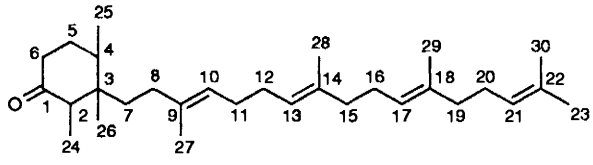
Table 1 Cyclization of (3*R*)-, (3*S*)- and (*RS*)-2,3-epoxy-2,3-dihydro-[3-^{3}H]squalene by a cell-free system from *T. pyriformis*. Specific activity of the substrates was, respectively, 6.2, 8.7 and 27.6 Ci mol^{-1}. The incubations (total volume 1 cm^3; substrate concentration 50 μmol dm^{-3}) were carried out at 30 °C for 4 h. The radioactivity was nearly quantitatively recovered after extraction and TLC separation for each substrate. Every experiment was performed in duplicate. Under the same assay conditions, the conversion rate of the squalene into tetrahymanol was 45%

	Gammacerane-3α,21α-diol **15** (%)	Gammacerane-3β,21α-diol **16** (%)	Monocyclic ketone **17** (%)	Squalene epoxide recovered (%)	Total
(3*R*)-squalene epoxide	47	< 1	15	38	100
(3*S*)-squalene epoxide	< 1	53	< 1	46	100
(*RS*)-squalene epoxide	41	32	9	20	100



14 (3*S*)-Squalene epoxide **16**

13 (3*R*)-Squalene epoxide **15**



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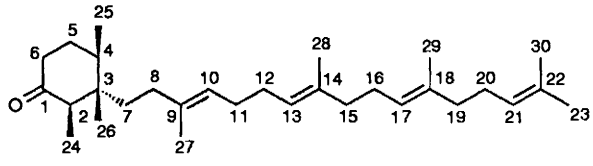

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Scheme 4 Cyclization of (3*R*)- and (3*S*)-squalene epoxide (**13**, **14**) respectively into gammacerane-3α,21α-diol **15** and monocyclic ketone **17**, and gammacerane-3β,21α-diol **16**, respectively, by the squalene cyclase of *T. pyriformis*. Monocyclic ketone **18** obtained by Lewis acid-induced cyclization of squalene epoxide. The label ● corresponds to a deuterium labelled methyl group.[4]

signal of 26-H$_3$ was coupled to C-2, C-3, C-4 and C-7 of the monocyclic ketone. These results clearly confirmed the structure **17**.

A 2,3,4-trimethylcyclohexanone structure has been assigned to synthetic compounds which were obtained, for instance, by acid-induced cyclization of (*RS*)-14,15-epoxygeranylgeranyl acetate,[24] (*RS*)-(*Z*,*Z*)-umbelliprenin epoxide,[25] and (*RS*)-squalene epoxide.[26] The naturally occurring fungal sesquiterpenoid antibiotic ascochlorin also has a (2*R*,3*S*,4*R*)-trimethylcyclohexanone ring,[27] which was established by X-ray structure analysis.[28] The synthetic compounds (actually a racemic mixture of both antipodes) were supposed to have a structure similar to that of ascochlorin with the same relative stereochemistry. In order to confirm the structure of the monocyclic product, we treated racemic (*RS*)-squalene epoxide with tin(IV) chloride in dry benzene according to van Tamelen's method.[26] One of the five major products is a monocyclic ketone. It had the same R_f-value on TLC as the biological ketone **17**, and showed almost identical GLC–MS and IR spectra. Its ^{1}H and ^{13}C NMR spectra were qualitatively similar, but the chemical shifts were, however, slightly different from those observed in the spectra of the monocyclic product obtained from the cell-free system of *T. pyriformis*. On the ^{1}H NMR spectrum, the three aliphatic methyl signals appeared at δ 0.94 (d, *J* 7 Hz), δ 0.91 (d, *J* 7 Hz) and δ 0.58 (s). The unusually high-field unsplit methyl signal is supposed to be characteristic of the (2*R*,3*S*,4*R*)-trimethylcyclohexanone structure which adopts a typical chair conformation, and the quaternary methyl group at position C-3 is fixed in an axial orientation.[29] Further, from the ^{1}H–^{1}H COSY spectrum, one of the α-carbonyl protons

at δ 2.48 (2-H) was coupled to the methyl proton signals at δ 0.94 (24-H_3) and those at δ 2.34 (6-H) to the proton signals at δ 1.6 (5-H_a) and δ 1.9 (5-H_b). The methyl signal at δ 0.91 (25-H_3) was seen to be coupled to that at δ 2.0 (4-H). These facts suggested that the monocyclic ketone obtained from the incubation mixture of *T. pyriformis* was one of the stereoisomers of the monocyclic compound **18** obtained by the Lewis acid-induced cyclization of racemic squalene oxide.

In order to obtain further information about the structure of the novel product and also to provide a direct proof of the former hypothesis that gammacerane-3α,21α-diol and gammacerane-3β,21α-diol, respectively, derive from the (3*R*)- and the (3*S*)-enantiomer of squalene epoxide.[4] Both radiolabelled enantiomers of squalene epoxide were synthesized according to the literature.[30] (*RS*)-Dihydro-[3-^{3}H]squalene-2,3-diol was first resolved into the (3*R*)-(+)- and (3*S*)-(−)-[3-^{3}H]-enantiomer by TLC of its 3β-acetoxyandrost-5-ene-17β-carboxylate ester, which were respectively converted to (3*S*)-(−)- and (3*R*)-(+)-[3-^{3}H]squalene 2,3-epoxide. The incubation results were consistent with the former hypothesis and clearly showed that the monocyclic product exclusively derived from (3*R*)-enantiomer of squalene epoxide. Under the standard assay conditions (50 μmol dm^{-3} substrate, at 30 °C for 4 h), the conversion rates of the (3*S*)-enantiomer into gammacerane-3β,21α-diol was 53%, while that of the (3*R*)-enantiomer into gammacerane-3α,21α-diol and the monocyclic product were respectively 47 and 15% (Table 1).

Discussion. Why is the cyclization of squalene epoxide, which is not a normal substrate for the squalene cyclase in *T. pyriformis*, always initiated by an oxirane ring-opening, and not by a proton attack on the terminal double bond as on squalene, the normal substrate? As the squalene cyclase efficiently transforms 2,3-dihydrosqualene, a substrate lacking one of the terminal double bonds of squalene, into euph-7-ene, it might be expected that squalene epoxide would also be transformed into other cyclization products, such as 24,25-epoxyeuph-7-ene, whose formation is initiated by a proton attack on the terminal double bond of the molecule. However, such products could not be detected, or, even if they were formed, they could be present only in much smaller amounts. Instead, a novel monocyclic product was isolated from the incubation mixture. It was thus concluded that the cyclization of squalene epoxide by the squalene cyclase of *T. pyriformis* is exclusively initiated by protonation of the oxirane ring such as that in eukaryotic squalene epoxide cyclization. The same specificity towards the oxirane ring of squalene epoxide has been observed with other bacterial squalene cyclases of *Acetobacter pasteurianus*[31] and *Methylococcus capsulatus*.[32] The active sites of these bacterial squalene cyclases appear to recognize the oxirane ring of squalene epoxide. There is probably more affinity between the basic oxygen atom of the oxirane ring and the acidic site of the squalene cyclase than between the terminal double bond and this site. Furthermore, the replacement of a double bond by an oxirane seems even to accelerate slightly the cyclization, as the consumption rate of squalene epoxide is higher than that of squalene (Table 1). This might result from the fact that squalene is a little more hydrophobic than its epoxide and thus less easily available for the active site of the cyclase in aqueous solution.

Incubation with either the (3*R*)- or the (3*S*)-enantiomer of squalene epoxide provided a direct proof that the (3*R*)-enantiomer is cyclized to gammacerane-3α,21α-diol and the monocyclic product, while the (3*S*)-enantiomer is cyclized only into gammacerane-3β,21α-diol. This confirms directly the results obtained with the racemic (*RS*)-[3-^{3}H]squalene epoxide before.[4] As shown in the previous paper by incubation of racemic stereospecifically deuteriated squalene epoxide, the (3*S*)-enantiomer of squalene epoxide has to be cyclized in an all-pre-chair conformation as for the cyclization of squalene into tetrahymanol, whereas the (3*R*)-enantiomer is cyclized in a pre-boat conformation as concerns the formation of ring A (Scheme 3, Pathway B).[4] Only these two conformations allow one to locate the oxygen atom of the oxirane in the same position with respect to the acidic site of the cyclase and to fulfil the optimal stereoelectronic requirements for a concerted *trans* antiparallel addition of the Δ^6 double bond on the oxirane. It has to be pointed out that both enantiomers are almost equally accepted as a substrate by the same squalene cyclase.

The novel monocyclic product has a 2,3,4-trimethylcyclohexanone moiety **17**, one of the stereoisomers of which is the monocyclic ketone **18** obtained by the acid-induced cyclization of racemic squalene epoxide. It derives uniquely from the (3*R*)-enantiomer of squalene epoxide. This is the first demonstration of the enzymic conversion of squalene epoxide into a monocyclic triterpene, which has never been isolated from this organism. Although we are not quite sure whether the monocyclic product is formed at the same active site of the squalene cyclase; it might be considered as a side-product from the cyclization into gammacerane-3α,21α-diol. As mentioned above, cyclization of the (3*R*)-enantiomer into the pentacyclic product requires the substrate to be folded in its pre-boat conformation as concerns the formation of ring A. However, in this 'unnatural conformation', further cyclization might probably be interrupted for some stereoelectronical reasons, and formation of the monocyclic cationic intermediate is followed by a series of hydride and methyl shifts. The reaction is supposed to be controlled by the enzyme and specifically yields the monocyclic ketone as a single product. On the other hand, the acid-induced cyclization product (racemic) as well as the 2,3,4-trimethylcyclohexanone moiety of ascochlorin is formed by an antiparallel concerted rearrangement reaction from an initially generated monocyclic cationic intermediate.[24,25] In order to establish the structure of the monocyclic product, further investigation is necessary.

Experimental

Chemicals.—2,3-Dihydrosqualene **5** was chemically synthesized according to Corey's method.[33] 2,3-Dihydro-[13-^{3}H]squalene (specific activity 6.4 Ci mol^{-1}) was a gift from Professor D. Arigoni. [11,12-^{14}C]Squalene (specific activity 0.5 Ci mol^{-1}) was obtained from the Commissariat à l'Energie Atomique (France). (*RS*)-2,3-Epoxy-2,3-dihydro-[3-^{3}H]squalene (specific activity 27.6 Ci mol^{-1}) was chemically synthesized as described before.[31] (3*R*)-2,3-Epoxy-2,3-dihydro-[3-^{3}H]squalene (specific activity 6.2 Ci mol^{-1}) and (3*S*)-2,3-epoxy-2,3-dihydro-[3-^{3}H]squalene (specific activity 8.7 Ci mol^{-1}) were synthesized according to the method of Boar and Damp.[30]

Analytical Methods.—^{1}H NMR and ^{13}C NMR spectra were recorded in C^2HCl_3 at 300 K on a Bruker ACF 250 spectrometer, using $CHCl_3$ (δ 7.260) as internal standard for ^{1}H NMR and $^{13}C^2HCl_3$ (δ 77.0) for ^{13}C NMR. *J*-Values are given in Hz. Tentative ^{13}C assignments were performed according to the literature.[34–36] The 2D ^{1}H–^{1}H COSY spectra of the monocyclic ketone **17** and **18** were recorded with the Bruker COSYPH.AUR program on an AM-400 spectrometer. The 2D long-range ^{1}H–^{13}C COSY spectrum of ketone **17** was recorded with the Bruker INVDR2LP.AUR program on the AM-400 instrument. A 5 Hz value for ^2J and ^3J coupling constants between ^{1}H and ^{13}C was selected after a study of the proton non-decoupled ^{13}C NMR spectrum of ketone **17**. GLC analysis was performed on a Carlo Erba Fractovap 4160 chromatograph equipped with a flame ionization detector fitted with a DB-1 surface-bonded fused-silica capillary column (30 m; film thickness 0.1 μm). Detector temperature was 310 °C. The

analyses were performed with a temperature program from 50 to 220 °C (20 °C min^{-1}) and from 220 to 310 °C (4 °C min^{-1}) with a hydrogen column pressure of 0.7 kg cm^{-2}. GLC–MS spectrometric analyses were performed on a LKB 9000S spectrometer under electronic impact at 70 eV. Injection, separator and source temperature were respectively 260, 270 and 290 °C; oven temperature was programmed from 200 to 300 °C (4 °C min^{-1}). Analyses were performed on the same columns as those utilized for GLC. The radioactivity on the TLC plates was recorded using a Berthold LB2832 thin-layer scanner. Radioactivity was measured on a Packard Prias PL Tricarb liquid scintillation counter. TLC plates were visualized under UV light (366 nm) after spraying with a 0.1% alcoholic solution of berberine hydrochloride. Optical solutions ($[\alpha]_D$ in units of 10^{-1} deg cm^2 g^{-1}) were measured on a Perkin–Elmer polarimeter, and CD spectra on a Roussel–Jouan polarimeter.

Incubation with a Cell-free System from T. pyriformis.—(a) 2,3-*Dihydrosqualene. Tetrahymena pyriformis* (strain L1630/1W from the Culture Collection of Algae and Protozoa, Cambridge, UK) was grown as described before.[4,5] The standard assay incubation (total volume 1 cm^3; substrate concentration 50 μmol dm^{-3}) was carried out with 2,3-dihydro-[13-^{3}H]squalene (7.1×10^5 dpm) or [11,12-^{14}C]squalene (5.6×10^4 dpm) at 30 °C for 4 h by using the cell-free system as described before. Large-scale incubation, which permitted spectroscopic identification of the reaction product, was performed using the cells (150 g, wet weight) from a 18 dm^3 culture giving, by sonication, a 290 cm^3 cell-free system. Unlabelled and labelled 2,3-dihydrosqualene (in total, 78 mg; 5.9×10^7 dpm; final concentration 650 μmol dm^{-3}) were dissolved in a 0.5% solution of Tween 80 in ethanol (5 cm^3), added to the cell-free system and incubated at 30 °C for 17 h. A control experiment was performed with a boiled preparation in order to verify that the incubation product arose from an enzymic reaction. The incubation was stopped by freezing and lyophilization. The freeze-dried preparation was refluxed with $CHCl_3$–MeOH (2:1 v/v; 2×200 cm^3). The combined extracts were evaporated to dryness, methanolysed by being refluxed for 1 h with a 6% solution of KOH in MeOH (20 cm^3), and extracted with cyclohexane after addition of two volumes of water. The unsaponifiable fraction was separated by preparative TLC (cyclohexane) to give unchanged labelled 2,3-dihydrosqualene (R_f 0.66) and a less polar labelled product (R_f 0.94), which was further purified by TLC on $AgNO_3$-impregnated silica gel (cyclohexane) as a radioactive oily compound (R_f 0.59; 26 mg; 2.0×10^7 dpm; pure by GLC).

(b) *Squalene epoxide.* The standard assay incubation (total volume 1 cm^3; substrate concentration 50 μmol dm^{-3}) was carried out with (*RS*)-2,3-epoxy-2,3-dihydro-[3-^{3}H]squalene (3.2×10^6 dpm), (3*R*)-2,3-epoxy-2,3-dihydro-[3-^{3}H]squalene (7.2×10^5 dpm) or (3*S*)-2,3-epoxy-2,3-dihydro-[3-^{3}H]-squalene (1.1×10^6 dpm) at 30 °C for 4 h. A control experiment was performed with the boiled preparation. For large-scale incubation, the cell-free system (300 cm^3) prepared from the cells (210 g) was incubated with unlabelled (*RS*)-2,3-epoxy-2,3-dihydrosqualene (100 mg) dissolved in a 0.5% solution of Tween 80 in ethanol (5 cm^3). In parallel with the preparative incubation, a small-scale incubation of (*RS*)-2,3-epoxy-2,3-dihydro-[3-^{3}H]squalene (1.1×10^6 dpm) was carried out. The incubations were shaken at 30 °C for 14 h and were stopped by freezing and lyophilization. The freeze-dried preparation was refluxed in $CHCl_3$–MeOH (2:1 v/v; 2×100 cm^3). The combined extracts were evaporated to dryness, methanolysed by being refluxed for 1 h with a 6% solution of KOH in MeOH (20 cm^3), and extracted with cyclohexane after addition of two volumes of water.

The unsaponifiable fractions were mixed in order to detect the products easily by their radioactivity, and they were first separated into two radioactive fractions by TLC (CH_2Cl_2), one possessing the polarity of diols (R_f 0.05) and the other possessing that of squalene epoxide (R_f 0.41). The diol fraction was separated by TLC (30% EtOAc–cyclohexane) into gammacerane-3α,21α-diol (35.4 mg; R_f 0.38) and gammaceranе-3β,21α-diol (19.0 mg; R_f 0.30) as described previously.[4] On the other hand, the squalene epoxide fraction was further separated into two distinct radioactive fractions by TLC (5% EtOAc–cyclohexane): unchanged squalene epoxide (16.9 mg; R_f 0.37) and a more polar, unknown product (7.9 mg; R_f 0.27) which was pure on GLC.

Incubation with a Cell-free System from A. acidocaldarius.—*A. acidocaldarius* ATCC 27009, obtained from Professor K. Poralla, was grown in sporulation medium[37] at pH 3 and 60 °C in a 10 dm^3 Biolafitte fermentation vessel. The cells from 7 dm^3 of medium (9.3 g, wet weight) were harvested during the exponential growing phase (15 h), washed with the incubation buffer (100 mmol dm^{-3} sodium citrate, pH 6.0), resuspended in this buffer (90 cm^3) and disrupted by sonication at 0 °C (200 W; 10 × 1 min burst with 2 min interval). After removal of residual cells by low-speed centrifugation (6000 *g*), the crude cell-free system was utilized for the incubation without further purification. The standard assay incubation (total volume 1 cm^3; substrate concentration 50 μmol dm^{-3}) was carried out with 2,3-dihydro[13-^{3}H]squalene (7.1×10^5 dpm) or [11,12-^{14}C]squalene (5.6×10^4 dpm) in the presence of 0.1% (w/v) Triton X-100 at 60 °C for 15 h. For large-scale assay, the cell-free system (80 cm^3) was incubated with unlabelled 2,3-dihydrosqualene (40 mg; final concentration 1.2 mmol dm^{-3}) in the presence of 0.1% (w/v) Triton X-100 at 60 °C for 72 h. A small-scale incubation (total volume 2 cm^3) was separately carried out with 2,3-dihydro[13-^{3}H]squalene (3.8×10^6 dpm) under the same conditions. A control experiment was performed at 10 °C, at which temperature this thermostable enzyme was completely inactive.[7] The incubations were stopped by freezing and lyophilization. The freeze-dried preparations were refluxed in cyclohexane (3×150 cm^3). The combined extracts were evaporated to dryness. The extracts from both incubations were mixed in order to detect the reaction products easily. They were separated by TLC (cyclohexane) into unchanged 2,3-dihydrosqualene (R_f 0.66) and a less polar fraction containing the reaction products (R_f 0.94; 3.9 mg; the two cyclization products representing ~25% of the mixture), which was further separated by TLC on $AgNO_3$-impregnated silica gel (cyclohexane) to give two radioactive oily compounds (R_f 0.19 and 0.31; 0.1 mg each; pure by GLC).

Chemical Synthesis.—Synthetic euph-7-ene **6** and its (20*S*)-isomer tirucall-7-ene were obtained as follows. Butyrospermol (eupha-7,24-dien-3β-ol) isolated from the fruits of *Maclura pomifera* (Osage oranges)[13] was first hydrogenated over palladium on carbon (10%) in EtOAc to give euph-7-en-3β-ol, which was oxidized by CrO_3–pyridine complex in dry CH_2Cl_2[38] to give the corresponding ketone. This ketone was finally converted into euph-7-ene by Wolff–Kishner reduction in diethylene glycol–butan-1-ol (2:1 v/v).[39]

For the synthesis of tirucall-7-ene, masticadienoic acid (3-oxotirucalla-7,24-dien-25-oic acid) isolated from commercial mastic gum (Roth) was converted into tirucall-7-en-3-ol according to Barton's method,[14] which was further oxidized to ketone; Wolff–Kishner reduction as described above then yielded tirucall-7-ene.

Synthetic (20*R*)-dammar-13(17)-ene **7** was obtained by treatment of euph-7-ene **6** with 20% BF_3–diethyl ether in diethyl ether at 20 °C for 24 h as reported for the conversion of eupha-7,24-diene into (20*R*)-dammara-13(17),24-diene.[12]

(20*S*)-Dammar-13(17)-ene was synthesized in the same way from tirucall-7-ene. For confirmation of the structure of (20*R*)-dammar-12-ene **8**, the isolated compound from enzymic cyclization (10 μg) was quantitatively converted by BF_3–diethyl ether treatment into (20*R*)-dammar-13(17)-ene **7**, which was identified by GLC and GLC–MS.

According to van Tamelen's method,[26] (*RS*)-2,3-epoxy-2,3-dihydrosqualene (1 g) was treated with tin(IV) chloride (0.2 mol equiv.) in dry benzene at 10 °C for 5 min. One of the five major products (205 mg, 21% yield; pure on GLC) having the same R_f-value on TLC (5% EtOAc–cyclohexane) showed similar spectroscopic data to those of the novel monocyclic product obtained from the incubation mixture of *T. pyriformis*.

Euph-7-ene **6**. GLC: t_R 17.0 min; δ_H(250 MHz) 0.748 (3 H, s, 10β-Me), 0.822 (3 H, s, 13α-Me), 0.835 (3 H, d, *J* 6.8, 20*R*-Me), 0.849 (3 H, s, 4α-Me), 0.872 (6 H, d, *J* 6.8, 2 × 25-Me), 0.886 (3 H, s, 4β-Me), 0.980 (3 H, s, 14β-Me) and 5.24 (1 H, dt, *J* 4 and 3, 7-H); δ_C (65 MHz) 146.2 (C-8), 118.0 (C-7), 53.3 (C-9), 51.4 (C-14), 51.4 and 49.1 (C-5 and -17), 43.6 (C-13), 42.5 (C-3), 39.5 and 39.1 (C-1 and -24), 36.1 (C-20), 35.3 (C-22), 35.2 (C-10), 34.0 and 33.9 (C-15 and -16), 33.2 (C-4), 33.1 (4α-Me), 28.5 (C-6), 28.1 (C-25), 27.4 (14β-Me), 24.5 and 24.4 (C-12 and -23), 22.8 and 22.7 (25-Me_2), 22.1 (13α-Me), 21.4 (4β-Me), 19.1 (C-2), 18.7 (20*R*-Me), 18.2 (C-11) and 13.1 (10β-Me); *m/z* (70 eV, GLC–MS) 412 (M^+, 11%), 397 ($M^+ - CH_3$, 100), 288 (5), 273 (9), 259 (5), 243 (5), 231 (5), 203 (5), 189 (6) and 175 (12). These data were identical for the synthetic triterpenes and for those obtained by enzymic cyclizations.

Tirucall-7-ene. GLC: t_R 17.8 min; δ_H(250 MHz) 0.748 (3 H, s, 10β-Me), 0.823 (3 H, s, 13α-Me), 0.851 (3 H, s, 4α-Me), 0.865 (3 H, d, *J* 6.7, 20*S*-Me), 0.871 (6 H, d, *J* 6.9, 25-Me_2), 0.885 (3 H, s, 4β-Me), 0.975 (3 H, s, 14β-Me) and 5.24 (1 H, dt, *J* 4 and 3, 7-H); δ_C(65 MHz) 146.2 (C-8), 118.0 (C-7), 53.1 (C-9), 51.4 (C-14), 51.3 and 49.2 (C-5 and -17), 43.5 (C-13), 42.5 (C-3), 39.6 and 39.1 (C-1 and -24), 36.4 (C-20), 36.2 (C-22), 35.2 (C-10), 34.1 and 34.0 (C-15 and -16), 33.2 (C-4), 33.1 (4α-Me), 28.3 (C-6), 28.0 (C-25), 27.4 (14β-Me), 24.4 and 24.2 (C-12 and -23), 22.9 and 22.6 (25-Me_2), 22.0 (13α-Me), 21.4 (4β-Me), 19.1 (C-2), 18.5 (20*R*-Me), 18.2 (C-11) and 13.1 (10β-Me); *m/z* (70 eV, GLC–MS) 412 (M^+, 12%), 397 ($M^+ - CH_3$, 100), 288 (4), 273 (7), 259 (4), 243 (4), 231 (4), 203 (4), 189 (5) and 175 (10).

(20R)-*Dammar*-13(17)-*ene* **7**. GLC: t_R 15.4 min; δ_H(250 MHz) 0.803 (3 H, s, 4β-Me), 0.836 (3 H, s, 8β-Me), 0.848 (3 H, s, 10β-Me), 0.855 (6 H, d, *J* 6.6, 25-Me_2), 0.863 (3 H, s, 4α-Me), 0.910 (3 H, d, *J* 6.9, 20*R*-Me) and 1.074 (3 H, s, 14α-Me); δ_C(65 MHz) 139.0 and 134.7 (C-13 and -17), 57.0 (C-5), 56.6 (C-14), 51.8 (C-9), 42.1 (C-3), 41.3 (C-8), 40.7 (C-1), 39.2 (C-24), 37.8 (C-10), 35.7 and 35.4 (C-7 and -22), 33.5 (4α-Me), 33.4 (C-4), 32.0 (C-20), 30.7 and 29.1 (C-15 and -16), 28.1 (C-25), 25.9 and 23.0 (C-12 and -23), 23.0 (14α-Me), 22.7 and 22.6 (25-Me_2), 21.8 (C-11), 21.7 (4β-Me), 20.1 (20*R*-Me), 18.8 and 18.6 (C-2 and -6), 17.2 (8β-Me) and 16.4 (10β-Me); *m/z* (70 eV, GLC–MS) 412 (M^+, 72%), 397 ($M^+ - CH_3$, 9), 299 (59), 231 (29), 220 (54), 205 (62), 191 (ring C cleavage, 100), 177 (17) and 161 (41). These data were identical for the synthetic triterpenes and for those obtained by enzymic cyclizations.

(20S)-*Dammar*-13(17)-*ene*. GLC: t_R 15.8 min; δ_H(250 MHz) 0.802 (3 H, s, 4β-Me), 0.820 (3 H, s, 8β-Me), 0.840 (6 H, d, *J* 6.6, 25-Me_2), 0.847 (3 H, s, 10β-Me), 0.861 (3 H, s, 4α-Me), 0.951 (3 H, d, *J* 6.8, 20*S*-Me) and 1.082 (3 H, s, 14α-Me); δ_C(65 MHz) 139.0 and 134.6 (C-13 and -17), 57.0 (C-5), 56.5 (C-14), 51.8 (C-9), 42.1 (C-3), 41.5 (C-8), 40.7 (C-1), 39.1 (C-24), 37.8 (C-10), 35.8 and 35.3 (C-7 and -22), 33.5 (4α-Me), 33.4 (C-4), 31.8 (C-20), 30.7 and 29.1 (C-15 and -16), 28.0 (C-25), 25.5 and 23.2 (C-12 and -23), 23.1 (14α-Me), 22.8 and 22.6 (25-Me_2), 21.9 (C-11), 21.7 (4β-Me), 20.1 (20*R*-Me), 18.8 and 18.6 (C-2 and -6), 16.9 (8β-Me) and 16.4 (10β-Me); *m/z* (70 eV, GLC–MS) 412 (M^+, 73%), 397 ($M^+ - CH_3$, 10), 299 (59), 231 (29), 220 (55), 205 (63), 191 (ring C cleavage, 100), 177 (18) and 161 (42).

(20R)-*Dammar*-12-*ene* **8**. GLC: t_R 16.8 min; δ_H(250 MHz) 0.784 (3 H, d, *J* 6.5, 20*R*-Me), 0.789 (3 H, s, 4β-Me), 0.829 (3 H, s, 4α-Me), 0.871 (6 H, d, *J* 6.5, 25-Me_2), 0.877 (3 H, s, 10β-Me), 0.937 (3 H, s, 8β-Me), 0.953 (3 H, s, 14α-Me) and 5.19 (1 H, dt, *J* 3 and 4, 12-H); *m/z* (70 eV, GLC–MS) 412 (M^+, 9%), 397 ($M^+ - CH_3$, 5), 299 (7), 220 (retro-Diels–Alder reaction induced by Δ^{12} double bond, 83%), 205 (9), 192 (33), 191 (ring C cleavage, 39), 177 (17), 161 (23) and 107 (100).

Monocyclic ketone **17** *obtained from incubation mixtures of* T. pyriformis. δ_H(250 MHz) 0.84 (3 H, s, 26-H_3), 0.99 (3 H, d, *J* 7, 24-H_3), 1.10 (3 H, d, *J* 7, 25-H_3), 1.35 (2 H, m, 7-H), 1.60 (12 H, s, 27-, 28-, 29- and 30-H_3), 1.68 (4 H, s, 23-H_3; m, 5-Ha), 1.90 (3 H, m, 4-H and 8-H), 1.99 (13 H, m, allylic H, 5-Hb), 2.34 (2 H, m, 6-H), 2.47 (1 H, q, *J* 7, 2-H) and 5.12 (4 H, m, vinylic H); δ_C(65 MHz) 209.1 (C-1), 135.6, 135.3 and 135.0 (C-9, -14 and -18), 131.2 (C-22), 124.5, 124.4, 124.4 and 124.3 (C-10, -13, -17 and -21), 50.3 (C-2), 42.6 (C-3), 39.8 and 39.8 (C-15 and -19), 37.0 (C-6), 35.5 (C-7), 34.7 (C-4), 33.3 (C-8), 29.6 (C-5), 28.4 and 28.3 (C-11 and -12), 26.9 and 26.8 (C-16 and -20), 25.7 (C-23), 21.1 (C-25), 17.7 (C-30), 16.1, 16.0 and 16.0 (C-27, -28 and -29), 14.3 (C-24) and 9.3 (C-26). This tentative ^{13}C NMR assignment was performed according to the literature;[29,40] *m/z* (70 eV, GLC–MS) 426 (M^+, 9%), 357 (6), 234 (5), 221 (9), 205 (4), 203 (7), 191 (4), 177 (5), 163 (5), 149 (15), 139 (trimethylcyclohexanone ring, 55) and 69 (100%); $\nu_{max}(CCl_4)/cm^{-1}$ 1715 (CO); $[\alpha]_D$ −1.1 (*c* 3.79 mg cm^{-3} $CHCl_3$). Circular dichroism (in 1,4-dioxane): $[\theta]$ +1140 degrees cm^2 $dmol^{-1}$ (294 nm).

Monocyclic product **18** *obtained from tin*(IV) *chloride treatment of racemic* (RS)-*squalene epoxide*. δ_H(250 MHz) 0.58 (3 H, s, 26-H_3), 0.91 (3 H, d, *J* 7, 25-H_3), 0.94 (3 H, d, *J* 7, 24-H_3), 1.40 (2 H, m, 7-H), 1.6 (13 H, s, 27-, 28-, 29- and 30-H_3; m, 5-Ha), 1.68 (3 H, s, 23-H_3), 1.9 (1 H, m, 5-Hb), 2.0 (15 H, m, allylic H, 8-H and 4-H), 2.34 (2 H, m, 6-H), 2.48 (1 H, q, *J* 7, 2-H) and 5.13 (4 H, m, vinylic H); δ_C(65 MHz) 202.3 (C-1), 135.3, 135.2 and 134.9 (C-9, -14 and -18), 131.1 (C-22), 124.6, 124.6, 124.4 and 124.3 (C-10, -13, -17 and -21), 50.6 (C-2), 43.6 (C-3), 41.6 (C-6), 39.8 and 39.8 (C-15 and -19), 36.3 (C-4), 36.2 (C-7), 32.8 (C-8), 31.1 (C-5), 28.4 and 28.3 (C-11 and -12), 26.9 and 26.8 (C-16 and -20), 25.6 (C-23), 17.6 (C-30), 16.2, 16.1 and 16.0 (C-27, -28 and -29), 15.3 (C-24), 15.1 (C-25) and 7.5 (C-26). The ^{13}C NMR assignment was performed according to the literature;[29,40] *m/z* (70 eV, GLC–MS) 426 (M^+, 7%), 357 (4), 234 (3), 221 (6), 205 (5), 203 (5), 191 (9), 177 (5), 163 (4), 149 (15), 139 (trimethylcyclohexanone ring, 68) and 69 (100); $\nu_{max}(CCl_4)/cm^{-1}$ 1715 (CO).

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