Enyzmatic Cyclization of 2,3-Dihydrosqualene into Euph-7-ene by a Cell-free System from the Protozoon *Tetrahymena pyriformis*

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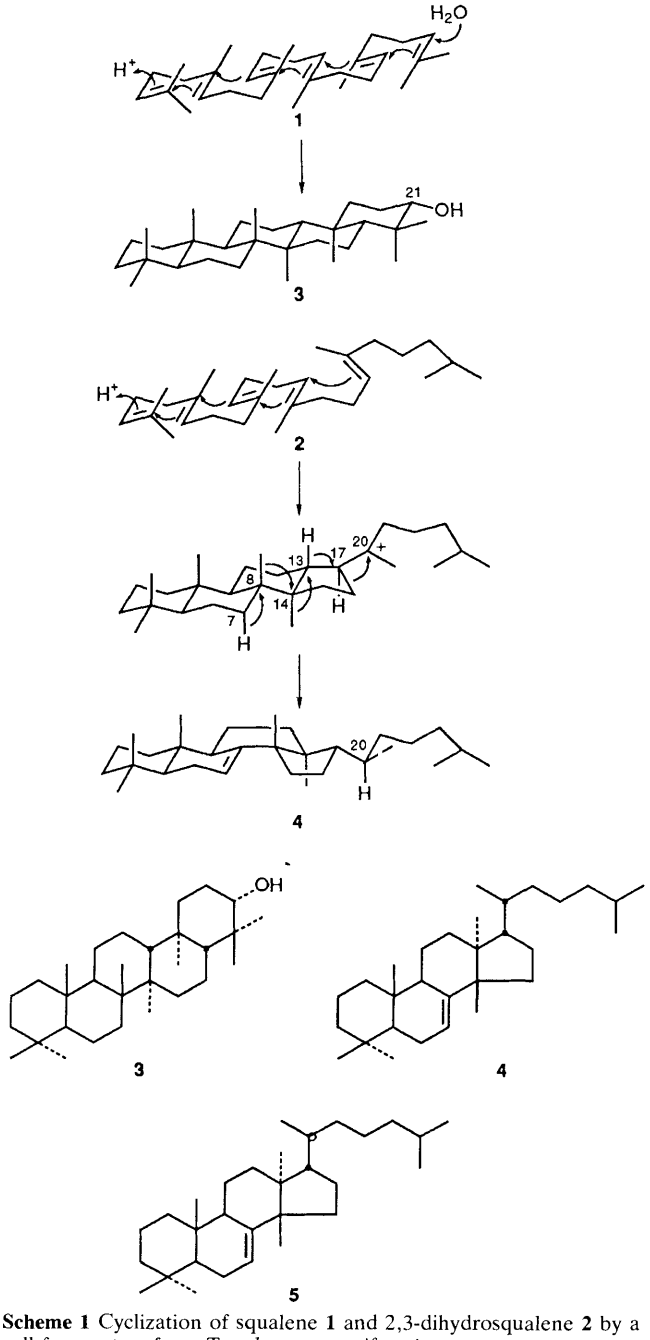
The cyclase of the protozoon *Tetrahymena pyriformis*, which normally converts squalene into the pentacyclic tetrahymanol, cyclized 2,3-dihydrosqualene into euph-7-ene with an unexpected tetracyclic skeleton and back-bone rearrangement.

The protozoon *Tetrahymena* produces a gammacerane triterpene, tetrahymanol **3**, 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.[1,2] The cyclization of squalene, folded in its all pre-chair conformation, is initiated by a proton attack on a terminal double bond and followed by addition of H_2O at the resulting C-21 cationic centre without carbon skeletal rearrangement (Scheme 1).[1,2] The squalene cyclase of *Tetrahymena* has been considered to be rather primitive because of the simplicity of the reaction[1,2] and its low substrate selectivity.[1] It was shown that the squalene cyclase of *T. pyriformis* can also cyclize (3*R*,*S*)-squalene epoxide into a mixture of two epimeric gammaceranediols.[3] Further, C_{25} and C_{30} regular polyprenol methyl ethers were converted into a series of polycyclic products.[4] Recently, squalene–tetrahymanol cyclase has been purified from *T. thermophila*.[5] In this paper, we present the result of the incubation of a cell-free extract from *T. pyriformis* with 2,3-dihydrosqualene **2**, a substrate lacking one of the terminal double-bonds of squalene, therefore making it impossible to form pentacyclic products.



Scheme 1 Cyclization of squalene **1** and 2,3-dihydrosqualene **2** by a cell-free system from *Tetrahymena pyriformis*

The cell-free system from *T. pyriformis* (strain L1630/1W from the Culture Collection of Algae and Protozoa, Cambridge, UK, 150 g, wet weight) was prepared as described before.[3] It was incubated with chemically synthesized [13-^{3}H]-2,3-dihydrosqualene (78 mg, 5.9×10^7 dpm) for 17 h at 30 °C. The incubation was stopped by freezing and lyophilized. After extraction with $CHCl_3$–MeOH (2 : 1) and final separation by TLC on $AgNO_3$ impregnated silica gel (cyclohexane), a less polar oily compound (R_f = 0.59, 26 mg, 2.0×10^7 dpm, pure by GLC) was isolated as a single product. Under 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 control experiments performed with a boiled enzymatic 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 hydrocarbons and showed good accordance with eupha-7,24-diene, which has been isolated from a fern,[6] except the signals due to the terminal double bond. Confirmation of the structure, and the stereochemistry of C-20, was finally obtained by comparison (GLC, ^{1}H and ^{13}C NMR) with euph-7-ene **4**† and its (20*S*)-isomer, tirucall-7-ene **5**, respectively obtained from butyrospermol (eupha-7,24-dien-3β-ol)[7] and masticadienoic acid (3-oxo-tirucalla-7,24-dien-25-oic acid).[8] The cyclization product was thus proved to be euph-7-ene, which has been isolated until now neither from this organism, nor from other natural sources. As described above, eupha-7,24-diene has been isolated from a fern, on the

† *Spectroscopic data* for euph-7-ene **4**: ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.761 (s, 3 H, 10β-Me), 0.835 (s, 3 H, 13α-Me), 0.846 (d, *J* 5.6 Hz, 3 H, 20*R*-Me), 0.857 (s, 3 H, 4α-Me), 0.881 (d, *J* 6.7 Hz, 6 H, 25-Me), 0.894 (s, 3 H, 4β-Me), 0.990 (s, 3 H, 14β-Me) and 5.25 (ddd, *J* 2.8, 2.8 and 4.0 Hz, 1 H, H-7). ^{13}C NMR (65 MHz, $CDCl_3$) δ 146.2, 117.9, 53.3, 51.4, 51.4, 49.1, 43.6, 42.5, 39.5, 39.1, 36.1, 35.3, 35.2, 34.0, 33.9, 33.2, 33.1, 28.5, 28.1, 27.4, 24.5, 24.4, 22.8, 22.7, 22.1, 21.4, 19.1, 18.7, 18.2 and 13.1. Mass spectrum (GLC–MS, electronic impact 70 eV) *m/z* 412 (M^+, 11%), 397 (M^+ − Me, 100%), 288 (5%), 273 (9%), 259 (5%), 243 (5%), 231 (5%), 203 (5%), 189 (6%) and 175 (12%).

other hand, tetrahymanol has also been found in another fern,[9] which suggests that squalene–tetrahymanol cyclase and squalene-eupha-7,24-diene cyclase might have a common origin.

It was quite unexpected that the tetrahymanol cyclase could also catalyse the cyclization into euph-7-ene, whose structure is apparently different from tetrahymanol, in such a good yield. It might be presumed that the reaction with 2,3-dihydrosqualene occurs at the same active site of the tetrahymanol cyclase, where the geometry had been already prepared for it. The cyclization of 2,3-dihydrosqualene is also thought to proceed in its all pre-chair conformation. The proton initiated cyclization first produces the dammaranyl C-20 cation, and the subsequent back-bone rearrangement (H-17α→20α, H-13β→17β, Me-14α→13α, Me-8β→14β) with elimination of H-7α proton gives the euphene framework (Scheme 1). Here the formation of ring D in the cyclization into euph-7-ene involves a thermodynamically favoured 'all-Markownikow' process with formation of a tertiary carbocation. The cyclization into tetrahymanol requires, however, an 'anti-Markownikow' process with a secondary cation. Once the folding of the precursor is imposed by the geometry of the cyclase active site determining the stereochemistry of the cyclization product, this suggests that the formation of a five- or six-membered D-ring in a triterpenic skeleton might be only regulated by stereoelectronic condition induced by the terminal double-bond of the substrate.

The versatility of the presumed primitive *Tetrahymena* cyclase is of great interest from the view-point of molecular evolution. Indeed, the cyclization and subsequent rearrangement leading to the lanosterol or cycloartenol skeletons, the precursors of the universal sterol nucleus in eukaryotes, would appear to be a spontaneous sequence of events dictated primarily by stereoelectronic effects. In this case a conformation of squalene oxide putting away the terminal double-bond from pre-ring D would automatically lead to the tetracyclic nucleus. Only the knowledge of the three-dimensional structures of cyclase active sites would help to prove or disprove the former hypothesis. In the meantime it is interesting to investigate whether other bacterial squalene–hopanoid cyclase can cyclize 2,3-dihydrosqualene into similar products, which is now under study.

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