121.0, 121.3, 121.6, 126.9, 128.4, 134.7, 136.2, 140.7, 148.1, 155.1; IR (KBr): $\tilde{\nu}$ = 3336, 2965, 1589, 1571, 1441, 1390, 1358, 739 cm^{-1}; MS: *m*/*z*: 280, 223, 196.

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Occurrence of Cationic Intermediates and Deficient Control during the Enzymatic Cyclization of Squalene to Hopanoids**

Catherine Pale-Grosdemange,* Corinna Feil, Michel Rohmer,* and Karl Poralla

Triterpenes belong to a group of natural products for which the theme of enzymic polyene cyclization is rich in variations. Such cyclizations—including the formation of all cyclic isoprenoids, regardless of the series to which they belong—produce the most diverse array of natural products.[1, 2] Concerning only triterpenes, about 100 different skeletons are found in nature.[3] Owing to genetic techniques and new purification procedures, highly purified squalene-hop-22(29)-ene cyclase (SHC) is now available without any contamination by cellular lipids.[4] This cyclase, like lanosterol and cycloartenol cyclase, catalyzes the most sophisticated one-step reaction known in biochemistry.[5] For the formation of the hopane skeleton, 13 covalent bonds are broken or formed, 9 chiral centers are established, and 5 rings are produced. The reaction is thought to be sensitive to side reactions because of the postulated occurrence of reactive carbocationic intermediates.[6] Furthermore, exclusion of water from the active site is also a problem for SHC as hopan-22-ol (**3**, diplopterol) is always produced along with hop-22(29)-ene (**2**, diploptene).[6]

Several minor hydrocarbons are produced along with diploptene (**2**) upon the SHC-catalyzed cyclization of squalene (**1**). The presence of such triterpenes helped explain the intermediacy of tetra- and pentacyclic carbocations during the formation of the hopane skeleton. Upon analyzing the products of the enzymatic cyclization of squalene (**1**) by GLC, peaks for minor products (each representing 0.9 – 2 % of the area of the peak for diploptene) were observed between the peaks corresponding to squalene and diploptene. The same product distribution also appeared when testing a highly purified SHC without a His tag, which was otherwise necessary for purification on a nickel affinity column. Preliminary GC-MS results showed that all these compounds had a relative molecular mass of 410 and were isomers of squalene and diploptene.

These compounds were separated by thin-layer chromatography on silica gel impregnated with silver nitrate (argenta-

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tion TLC) and identified by GC-MS. Neohop-13(18)-ene (**4**) and eupha-7,24-diene (**8**) were obtained as pure compounds. Their ^{1}H NMR and mass spectra were identical with those reported in the literature.[7–9] The structure of **8** was finally confirmed by direct comparison (GC, GC-MS, ^{1}H NMR) with **8** synthesized from butyrospermol. Neohop-13(18)-ene (**4**) was directly identified by coinjection with a reference sample during the GC experiment and then GC-MS. The two isomeric hydrocarbons **6** and **7** could not be separated from one another. ^{1}H NMR and mass spectra of **6** were identical with those reported in the literature for dammara-13(17),24-diene isolated from *Polypodium fauriei*, whose structure was confirmed by comparison with the hydrogenated cyclization product of 2,3-dihydrosqualene.[10] Hydrocarbon **7** was identified as 17-isodammara-12,24-diene by comparison of the ^{1}H NMR spectrum with that of the 24,25-dihydro derivative 17-isodammar-12-ene, which was previously obtained by enzymatic cyclization of 2,3-dihydrosqualene with a cell-free system from *Alicyclobacillus acidocaldarius*.[11] Catalytic hydrogenation of the mixture of **6** and **7** selectively reduced the double bond of the side chain, yielding a mixture of 17-isodammar-12-ene and dammar-13(17)-ene; these were identified by comparison with reference compounds (GC-MS, ^{1}H NMR).[11] Chemical shifts of the methyl groups from the tetracyclic moiety of **6** were nearly identical with those of the 24,25-dihydro compound. The unsaturated side chain with a Δ^{24} double bond was characterized by the two broad singlets of the methyl groups from this double bond at $\delta = 1.606$ and 1.643, the broad triplet from vinylic proton H24 at $\delta = 5.10$ ($J = 6.0$ Hz), and the C21 methyl doublet at $\delta = 0.789$ ($J = 7.0$ Hz). For compound **7**, the presence of a Δ^{12} double bond was corroborated by the signal of the olefinic proton H12 appearing as a double doublet at $\delta = 5.20$ ($J = 3.2$ and 5.6 Hz) and by the intense signal at m/z 218 arising from the retro-Diels–Alder product, which is characteristic for Δ^{12} double bonds in a triterpenic skeleton. These last two features were previously observed in the ^{1}H NMR and mass spectra of dammar-12-ene.[10]

Hydrocarbons **5** and **9** were also obtained as a mixture. ^{1}H NMR and GC-MS spectra of the minor component **9** were identical with those reported for dammara-20(21),24-diene.[8] The mass spectrum of **5** was nearly identical with that of **9**,[8] but the chemical shifts of the five methyl group singlets differed significantly.[8] The signals from the side-chain protons were similar, however, and indicated the presence of two double bonds in the side chain of **9**. An isopropylidene group corresponding to a Δ^{24} double bond was shown by the two broad singlets of the methyl groups on a double bond at $\delta =$ 1.618 and 1.684, and by the broad triplet of the vinylic proton H24 at $\delta = 5.11$ ($J = 6.7$ Hz). The second double bond was characterized by two olefinic proton singlets at $\delta = 4.87$ and 4.91 (in contrast to an olefinic methylene group) and corresponded to a $\Delta^{20(21)}$ double bond. Hydrocarbon **5** was therefore tentatively identified as 17-isodammara-20(21),24-diene. Its structure is in accordance with the hypothetical biogenetic scheme which can be proposed for the formation of the triterpenoids from *A. acidocaldarius* (Scheme 1). A 17-isodammarane derivative resulting from trapping of a cationic intermediate was found as a cyclization product of (3*S*)-29-methylidene-2,3-oxidosqualene by SHC.[12] Since compounds from the 17-isodammarane series were never found as natural products, definitive identification of **5** requires the synthesis of a reference compound.

Six minor products (**4**–**9**), all resulting from squalene cyclization, were formed in addition to diploptene (**2**) and diplopterol (**3**) from the purified SHC from *A. acidocaldarius* (Scheme 1). These hydrocarbons were not artifacts, resulting from disfunctioning of the enzyme induced by its removal from its natural membrane environment, because they were detected in trace amounts in the hydrocarbon fraction of *A. acidocaldarius*.

The structures of the minor compounds can be deduced from those of carbocationic intermediates possibly involved in the formation of the hopane skeleton (Scheme 1). The pentacyclic neohopa-13(18)-ene (**4**) results from rearrangement induced by the cationic center at C22 of the hopane intermediate **10**. All tetracyclic hydrocarbons arise from cationic 17-isodammarane (**11**) or dammarane (**12**) intermediates. On the one hand, cation **11** results from an all-pre-chair conformation of squalene, which is most likely for the formation of the hopane skeleton.[10] Intervention of a 17-isodammarane intermediate is further supported by the structure of the cyclization product of (3*S*)-29-methylidene-2,3-oxidosqualene. Cyclization of this nonnatural substrate of SHC yielded a 17-isodammarane derivative resulting from trapping of the isodammarane C20 cationic intermediate **11**.[12] Proton elimination yields 17-isodammara-20(21),24-diene (**5**). On the other hand, the 7-isodammarane C20 cation **12** may result either from direct squalene cyclization in a pre-chair-chair-chair-boat conformation[10] or from the 17-isodammarane cation **11** by 1,2-hydride shifts and rotation of the side chain, yielding finally after proton elimination **9** (Scheme 1). The three remaining tetracyclic hydrocarbons **6**, **7**, and **8** resulted from the rearrangement of cation **13** by 1,2-hydride shifts (in the case of **8** by a 1,2-methyl shift) followed by proton elimination.

Formation of tetracyclic triterpenes with a five-membered ring D by SHC may be interpreted in the same way as that of ring C in lanosterol biosynthesis.[13, 14] Cyclization of artificial substrate analogues such as 20-oxa-2,3-oxidosqualene or of a truncated C_{20} oxidosqualene analogue by the yeast oxidosqualene cyclase afforded compounds with a five-membered ring C. Their formation is facilitated since it occurs by a Markovnikov process, whereas the expected six-membered ring C would result from an anti-Markovnikov addition. A cationic intermediate with a five-membered ring C was therefore postulated as a regular intermediate in the formation of lanosterol, which results from expansion to the six-membered ring. Computational investigations were in accordance with the likeliness of such a process, but also predicted that the equilibrium between the tertiary cation (Markovnikov) and the nearly isoenergetic secondary cation (anti-Markovnikov) can be readily shifted by selective placement of a nucleophilic group. The involvement of nucleophiles from the protein were proposed,[15] but a double bound from the substrate could play the same role. A similar process could be imagined for the formation of ring D in the cyclization of squalene into diploptene. In this case, however, for unknown reasons the C20 cationic intermediate (**11** and/or **12**) escapes ring expansion and yields the tetracyclic triterpenes **5**–**9**.

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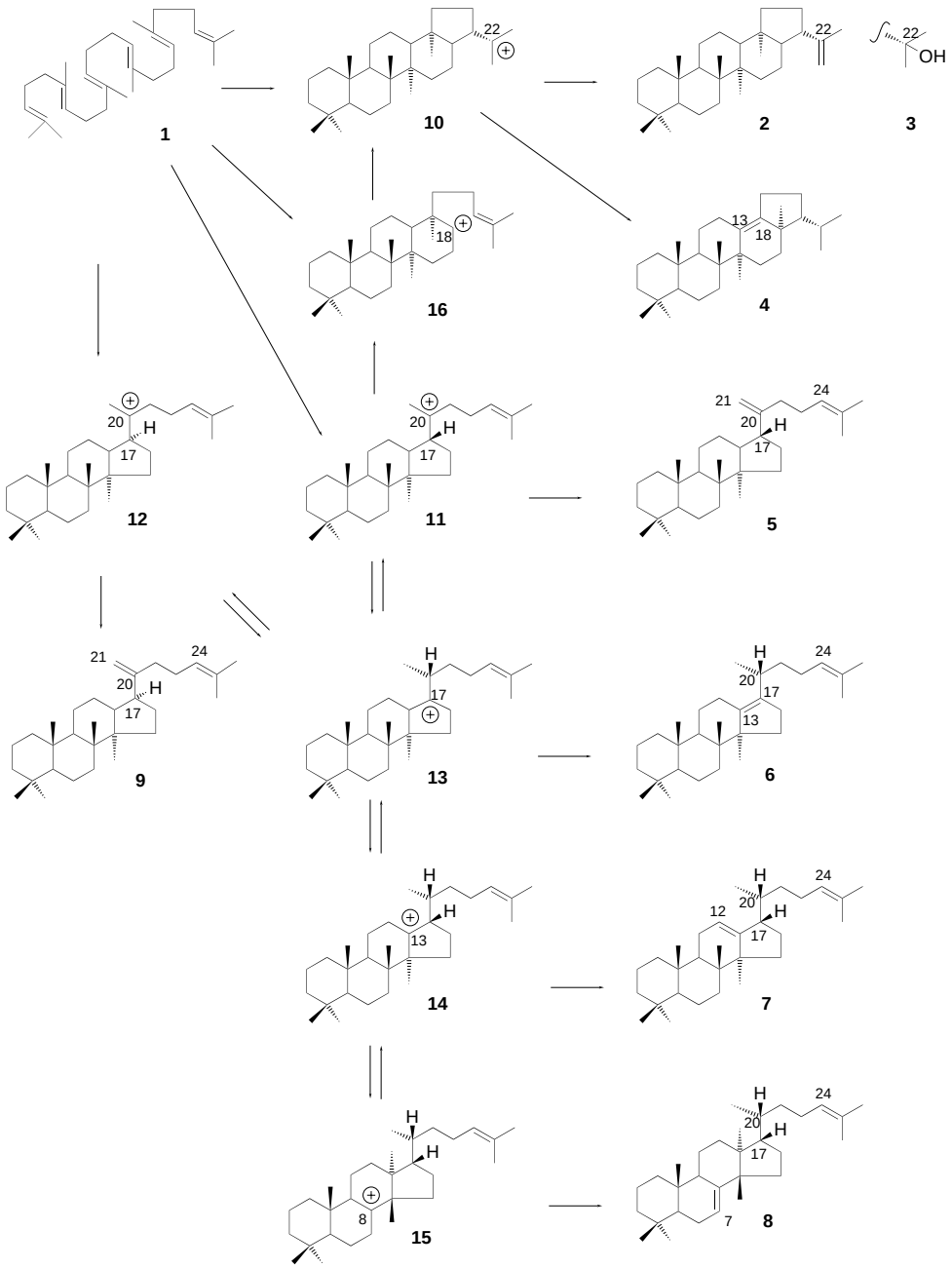

Scheme 1. Hypothetical biogenetic scheme for the formation of triterpenic skeletons by sqalene/hopene cyclase of *Alicyclobacillus acidocaldarius*.

Another mode of formation of the five-membered ring D is possible. Cyclization of 2,3-dihydrosqualene by squalene cyclases from *Tetrahymena pyriformis* or from *A. acidocaldarius* yielded tetracyclic triterpenes with a five-membered ring D.[11] Their carbon skeletons were identical with those of some of the minor compounds described here. This finding suggests that the terminal double bond of squalene may stabilize the secondary C18 cation **16** and is thus required for the formation of a six-membered ring D.[15] In its absence only the Markovnikov addition occurred, yielding triterpenes derived from dammarane or 17-isodammarane tertiary carbocations. If positioning of the terminal double bond of squalene with respect to the cationic site is not exact, formation of intermediate **16** may be hampered. This results in the production of the most stable tertiary cationic intermediate **11** or **12**, incomplete cyclization (as in the absence of the double bond), and finally formation of triterpenes of the dammarane and euphane series which represent the signature of the error made by the SHC. Since these alternative cyclization products were isolated from normal bacterial cells, these results suggest that the carbocation intermediates formed during the SHC reaction are sufficiently long-lived to allow alternative cyclization pathways to be followed.

Such a versatility and lack of specificity of SHC was previously demonstrated for the formation of ring A. All tested SHC cyclize squalene, their normal substrate, as well as the two enantiomers of oxidosqualene.[16–19] Cyclization of the 3*S* enantiomer requires a prechair conformation for the formation of ring A, that of the 3*R* enantiomer a pre-boat conformation.[18] Thus, such cyclizations with different foldings of the substrate are most probably linked to at least small changes in the geometry of the active site and suggests some conformational flexibility of the protein.

Although the X-ray structure of SHC is known,[20] it is not yet possible to localize precisely squalene in the active site and to understand the interactions inside the catalytic cavity. Side products have never been observed for the formation of lanosterol. The oxidosqualene cyclase leading to this sterol precursor seems efficiently controlled and has allowed its utilization for the biosynthesis of essential metabolites of higher organisms. In contrast, cyclizations leading to mono- and sesquiterpenes in plants afford side products in significant yields.[21, 22] Many triterpene cyclases might be supposed to show a similar lack of catalytic specificity, yielding the numerous triterpenic skeletons issued from secondary metabolism in plants. One has just to remember all mono-,[23] bi-,[24] tri-,[25] tetra-,[7, 8] and pentacyclic[26] triterpenic hydrocarbons found in ferns and, based on the absence of an oxygen functionality at C3, most probably

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derived from squalene cyclization as in the case of bacterial hopanoids.

Experimental Section

A His-tag (6 His residues) was ligated to the N terminus of the SHC cloned from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*. This altered enzyme was produced in *Escherichia coli* and purified to homogeneity by complexation chromatography in the last step.[4]

The NMR spectra were recorded on a Bruker WP400 spectrometer in $CDCl_3$ at 300 K; $CHCl_3$ ($\delta = 7.260$) was used as internal standard. The GC-MS measurements were made on a Finnigan TSQ700 spectrometer. Chromatographic conditions were as those previously described.[11] Triterpenes were extracted from the enzymatic reaction mixture with hexane/propan-2-ol (3/2). This crude fraction (229 mg) was separated by flash chromatography (CH_2Cl_2/hexane (1/1) followed by CH_2Cl_2) into a hydrocarbon fraction (179 mg) and diplopterol (31 mg).[27] A further flash chromatography (cyclohexane) yielded a fraction containing all polycyclic triterpenes (112 mg) and unchanged squalene (60 mg). The polycyclic triterpenes were separated by argentation TLC (cyclohexane/toluene (9/1), two migrations),[28] yielding (in order of increasing polarity) a fraction containing neohop-13(18)-ene (**4**, Scheme 1) and apolar hydrocarbons; a mixture of dammara-12,24-diene (**7**), dammara-13(17),24-diene (**6**), and eupha-7,24-diene (**8**); diploptene (**2**); and finally a mixture of dammara-20(21),24-diene (**9**), a hydrocarbon tentatively identified as 17-isodammara-20(21),24-diene (**5**), and some residual **2**. These fractions were further purified by another argentation TLC. Pure **4** ($R_f = 0.70$, 0.3% yield from the crude reaction mixture) was obtained with cyclohexane as eluent. The mixture of **6**, **7**, and **8** yielded with cyclohexane/toluene (9/1) as eluent a mixture of **6** and **7** in a 54:46 ratio which could not be separated ($R_f = 0.41$, 0.9% yield) and **8** ($R_f = 0.48$, 0.2% yield) The most polar fraction was separated with cyclohexane/toluene (60/40) as eluent into **2** ($R_f = 0.64$) and an inseparable mixture of **5** and **9** ($R_f = 0.25$, 0.6% yield) in a 82:18 ratio. Only spectroscopic data concerning the two new hydrocarbons **5** and **7** are extensively described here.

5 (tentative structure assignment): ^{1}H NMR: $\delta = 0.803$ (s, CH_3), 0.833 (s, CH_3), 0.845 (s, CH_3), 0.902 (s, CH_3), 0.942 (s, CH_3), 1.618 and 1.684 (2s, 2 × 25-CH_3), 4.87 and 4.91 (2s, 2 × 21-H), 5.11 (br t, $J = 6.7$ Hz, 24-H); GC-MS: m/z (%) = 410 (18, [M^+]), 395 (3, [$M^+ - CH_3$]), 367 (3), 341 (2), 299 (5), 231 (15), 218 (11), 203 (11), 191 (100, cleavage of ring C), 189 (27, cleavage of ring C), 109 (24).

7: ^{1}H NMR: $\delta = 0.789$ (d, $J = 7.0$ Hz, 20-CH_3), 0.821 (s, 4α-CH_3), 0.867 (s, 10β-CH_3), 0.927 (s, 8β-CH_3), 0.946 (s, 14α-CH_3), 1.606 and 1.643 (2s, 2 × 25-CH_3), 5.10 (br t, $J = 6$ Hz, 24-H), 5.20 (dd, $J = 3$ and 4 Hz, 12-H); GC-MS: m/z (%) = 410 (100, [M^+]), 395 (24, [$M^+ - CH_3$]), 367 (2), 341 (9, allylic cleavage between C22 and C23), 326 (11), 297 (36 [M^+ – side chain – 2H), 284 (21), 218 (32, retro-Diels–Alder reaction induced by the Δ^{12} double bond), 191 (82, cleavage of ring C), 147 (62), 134 (78), 109 (51), 107 (46), 105 (30).

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Crystal Engineering of Organometallic Compounds through Cooperative Strong and Weak Hydrogen Bonds: A Simple Route to Mixed-Metal Systems**

Dario Braga,* Lucia Maini, and Fabrizia Grepioni*

Crystal engineering of organometallic compounds is an emerging field of research.[1] Much of the excitement arises from the idea of being able to combine the plethora of characteristics provided by functional groups of organic molecules[2] with the coordination geometry, variable ionic charges, and valence and spin states typical of organometallic complexes to obtain novel crystalline materials.[3] An intelligent choice of the building blocks may yield materials with magnetic, conducting, superconducting, and nonlinear optical properties.

We now report our success in synthesizing new organometallic crystalline materials by allowing a (rather unconventional) polycarboxylic acid—namely, the neutral organome-

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