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Unnatural natural triterpenes produced by altering isoleucine into alanine at position 261 in hopene synthase and the importance of having the appropriate bulk size at this position for directing the stereochemical destiny during the polycyclization cascade†

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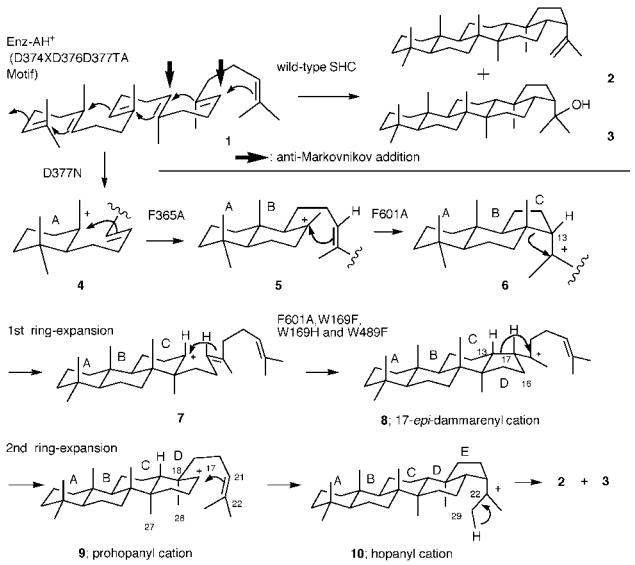
Incubation of squalene with the site-directed mutant of Ile261Ala of squalene-hopene cyclase from *Alicyclobacillus acidocaldarius* afforded multiple triterpenes consisting of tri-, tetra- and penta-cyclic skeletons, together with a final product of hopene, among which the previously unknown 6/6/6/6-fused tetracyclic skeletal compounds, denoted pro-hopene A and B, are included.

Squalene-hopene cyclase (SHC) catalyses the cyclization reaction of squalene **1** into pentacyclic triterpenes, hopene **2** and hopanol **3**, under a fine regio- and stereo-chemical specificity. The reaction has been believed to proceed through all *pre*-chair conformation,[1] but recent studies on the site-directed mutagenesis have indicated that the reaction proceeds *via* two expansion steps from the 5-membered C- and D-ring intermediates, which had been previously formed under Markovnikov closure, into the corresponding 6-membered rings (anti-Markovnikov adduct), as shown in Scheme 1.[2a,b] The ring-expansion processes have been further supported by trapping experiments of the 6/6/5-fused tricyclic intermediate **6** and the 6/6/6/5-fused tetracyclic intermediate **8** (Markovnikov adducts) using the substrate analogues having a highly nucleophilic hydroxyl group.[2a,b] The stereochemistry of 13H in **6** and 17H in **8** have been established to be of β-configuration; no enzymic products having the α-configurations have been obtained in the trapping experiments. Recently, we and Poralla's group have reported the isolation of the enzymatic products derived from the carbocation intermediates of mono- **4**,[3] bi- **5**,[4a,c] tri- **6**,[2b,4c] and tetra-cyclic **8**;[2a,b,4b,c] these discrete cationic intermediates were produced by a single amino acid replacement of D377N (D377C), F365A, Y420A, F601A, W169F, W169 H or W489F in *A. acidocaldarius* SHC (Scheme 1). The abortive cyclization products formed by each point mutation have revealed not only the catalytic function of given amino acids but also the cyclization mechanism for **1**; the cyclization is triggered by the DXDDTA motif;[3] D377 stabilises monocyclic cation **4** probably *via* the carboxylate anion; and both F365[4a] and F601[2b] stabilise the transient carbocations at each cyclization stage **5** → **8** possibly *via* a cation–π interaction. The aromatic residues of W169, W489 and Y420 are likely to play a significant role for folding the substrate **1**.[2a,4c] In a series of the site-directed mutagenesis experiments, we have focused on Ile261 as a next experiment, which is highly conserved among all the known SHCs and oxidosqualene cyclases. The mutated SHCs of I261A produced the unknown 6/6/6/6-fused tetracyclic **14** and **15** ('unnatural' natural products) together with the known **11**, **12**, **13** and **16**.[5] The stereochemistry at 13- and 17-positions of premature products **11** and **12** was of α-configuration and in contrast to those of true intermediates **6** and **8**. We report here, that the appropriate bulk size and shape at position 261 has a critical role in directing the steric control for constructing the hopane skeleton.



Scheme 1

† Electronic supplementary information (ESI) available: EIMS and NMR assignments. See http://www.rsc.org/suppdata/cc/b0/b000711k/

With the cell-free homogenates (150 ml), prepared from a 3 L culture of *E. coli* clone encoding I261A SHC, 100 mg of **1** was incubated for 15 h at optimum catalytic conditions (pH 6 and 55 °C). The GC analysis (30 m DB1, capillary column) showed six new enzymic products other than **2** in a hexane-extract of the reaction mixture.

Column chromatography over SiO_2 eluting with hexane afforded five fractions in the following order: **16**, **2** + **13**, **12** + **14** + **15**, **11** and the recovered **1**. The complete separation of each product was achieved by reverse-phase HPLC (C18) with THF–H_2O (6 : 4). The yields of each product, estimated by GC, were as follows: 4.0, 19.0, 4.1, 4.1, 3.7, 3.2, 48.6 and 3.1 mg for **11**, **12**, **13**, **14**, **15**, **16**, **2** and **3**, respectively, and 0.6 mg for the recovered **1**. No other products were observed in a detectable amount.

Structures of all the isolated compounds were determined as shown in Fig. 1 by EIMS and NMR spectra (H–H COSY 45, HOHAHA, NOESY, DEPT, HMQC and HMBC).† The EIMS, ^1H and ^{13}C NMR spectra of **11** were identical to those of the authentic (17*E*)-(13αH)-malabarica-14(27),17,21-triene,[2b] the stereochemistry of H13 being further confirmed to be of α-configuration by a strong NOE between H9 and H13. The epimer of **11**, (13βH)-malabaricatriene **11′**[2b] with a longer retention time (rt) on the GC than **11**, was never detected in the reaction mixture. The fragmentation pattern of **12** in the EIMS spectrum was superimposable to that of 17-*epi*-dammara-20(21),24-diene **12′**,[2a] but the rt of **12** on the GC is shorter than that of **12′**, and the NOE between 30-Me and H17 was found, thus proving the α-orientation of H17 in **12**. The double bond of **13** was $\Delta^{13,17}$ by HMBC correlation both from 30-Me to C13

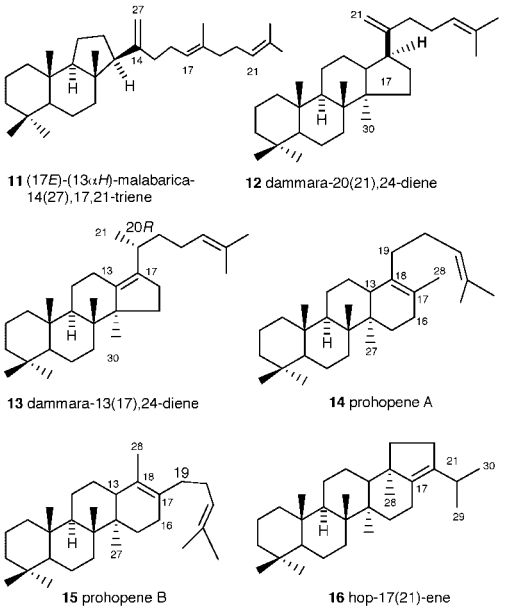
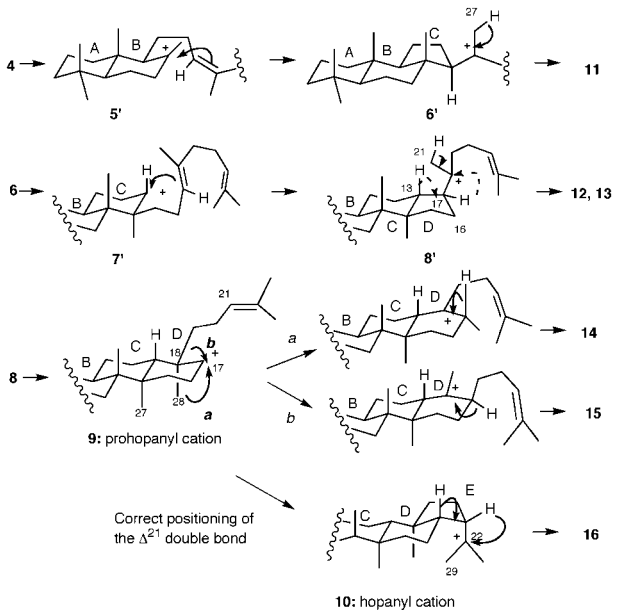


Fig. 1 Structures of the enzymatic products by the mutated I261A SHC.

and from 21-Me for C17. The proton chemical shift of 21-Me was 0.919 ppm (*J* 6.7 Hz) in $CDCl_3$, which allowed the assignment of 20*R*-stereochemistry.[6] The EIMS spectrum of **14** was essentially the same as that of **15**. The 6/6/6/6-fused tetracyclic skeletons of **14** and **15** were revealed by NMR analyses. An HMBC correlation between 28-Me and C13 was observed for **15**, but not for **14**. A strong NOE was observed between H16 and H19 for **15**, while no NOE was seen for **14**. Product **16** differed from **2** only in the double bond position, which was determined by the HMBC cross peaks of 28-Me/C17, 29-Me/C21 and 30-Me/C2l.

Tricyclic **11** could be produced *via* **6′** and two tetracyclics **12** and **13**, *via* **8′** (Scheme 2). Proton elimination from 21-Me of **8′** could give **12**, but a hydride shift to C18 of **8′** followed by deprotonation of the 13H could afford **13**. Formation of tri- and tetra-cyclic skeletons **11**–**13** may have occurred owing to the local change, especially near the site(s) responsible for the C/D-ring formation; the mutated SHC replaced by a smaller bulk size of Ala could not perfectly fit with **1**.[7] Compounds **11**–**13** were accumulated, while the corresponding epimers **11′** and **12′** from **6** and **8** were not detected. This suggests that **6′** and **8′** cannot undergo further cyclizations, whereas the intermediates **6** and **8**, which had been produced by this mutated SHC, could undergo



Scheme 2

subsequent cyclizations to yield **2**. Therefore, it is likely that the (13βH)- and (17βH)-configurations in 6/6/5- and 6/6/6/5-ring systems are required for the completion of two ring-expansion processes to give **9**. This hypothesis agrees well with the previous results[2a,b] that only **6** and **8** were trapped when the squalene analogues having a hydroxy group were incubated with the native SHC, but that **6′** and **8′** were not. The secondary cation **9** has been assumed as an intermediate in hopene biosynthesis, but no experimental evidence has been given. Formation of **14** and **15** gave definitive evidence for the involvement of intermediate **9**. The 1,2-shift of 28-Me to the C17 cation could give **14** (path *a* in Scheme 2), whereas rearrangement of the isoprenoid side chain to the cation could afford **15** (path *b*). Triterpenes **14** and **15** have never been reported before. We propose to denote the prohopane skeleton for **9**, prohopene A for **14** and prohopene B for **15**. It is noteworthy that **8′** is the biosynthetic intermediate of some plant triterpenes.[1] A looser binding near the C/D-ring could also perturb the correct positioning of the Δ^{21} double bond in **9**, thus leading to the accumulation of **14** and **15**; the appropriate arrangement of the double bond could allow a further cyclization to give **10** through the nucleophilic attack of the double bond toward cation **9**. Product **16** could be formed perhaps due to the erroneous positioning of the deprotonation site for introducing the $\Delta^{22,29}$ double bond of **2**, which may have also occurred concomitantly with the local change near the C/D-rings.

With respect to the mutated I261V SHC, no abortive cyclization product was found and the kinetic data were almost the same as that of the wild-type SHC,[7] but the mutant I261A gave a looser affinity and a slower velocity for hopene biosynthesis.[7] Site-directed mutagenesis not only helps to understand the fundamental issue of the reaction mechanism (molecular recognition and catalytic function), but also generates the previously unknown 'unnatural' natural products,[8a] as represented by **14** and **15**. The rational genetic engineering of the active and/or recognition sites is a promising tool for the creation of novel natural products.[4a,8b]

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Notes and references

1 I. Abe, M. Rohmer and G. D. Prestwich, *Chem. Rev.*, 1993, **93**, 2189.
2 (*a*) T. Sato, T. Abe and T. Hoshino, *Chem. Commun.*, 1998, 2617; (*b*) T. Hoshino, M. Kouda, T. Abe and S. Ohashi, *Biosci. Biotechnol. Biochem.*, 1999, **63**, 2038.
3 T. Sato and T. Hoshino, *Biosci. Biotechnol. Biochem.*, 1999, **63**, 2189.
4 (*a*) T. Hoshino and T. Sato, *Chem. Commun.*, 1999, 2205; (*b*) T. Merkofer, C. Pale-Grosdemange, M. Rohmer and K. Poralla, *Tetrahedron Lett.*, 1999, **40**, 2121; (*c*) C. Pale-Grosdemange, T. Merkofer, M. Rohmer and K. Poralla, *Tetrahedron Lett.*, 1999, **40**, 6009.
5 For **11**; K. Masuda, K. Shiojima and H. Ageta, *Chem. Pharm. Bull.*, 1989, **34**, 1140; for **12**; H. Yamashita, K. Masuda, T. Kobayashi, H. Ageta and K. Shiojima, *Phytochemistry*, 1998, **49**, 2461; for **13**; Y. Arai, K. Masuda and H. Ageta, *Chem. Pharm. Bull.*, 1982, **30**, 4219; for **16**, H. Ageta, K. Shiojima and Y. Arai, *Chem. Pharm. Bull.*, 1987, **35**, 2705.
6 I. Abe and M. Rohmer, *J. Chem. Soc., Perkin Trans 1*, 1994, 783.
7 Optimum catalysis was found at 55 °C for the mutants I261A, but at 60 °C for the wild-type and I261V. Kinetic values of K_m and V_{max} were determined at 55 °C and pH 6.0 from Lineweaver–Burk plots as follows; K_m: 25.8, 34.7 and 178 μM; V_{max}: 3.87, 3.57 and 0.43 nmol min^{-1} μg^{-1}, respectively, for the wild-type, I261V, and I261A. The I261G mutant, having the smallest size, also produced **11**–**13** in twice the quantity than I261A, despite the cyclase activity being significantly decreased (the amount of **2** was 17% of that of I261A), which further supports the importance of a bulk size at 261 position for the stereoselective cyclization reactions.
8 (*a*) Examples of 'unnatural' natural products by altering the active sites of sesquiterpenes or polyketide synthases. D. E. Cane and Q. Xue, *J. Am. Chem. Soc.*, 1996, **118**, 1563; R. McDaniel, A. Thamchaipenet, C. Gustafsson, H. Fu, M. Betlach, M. Betlach and G. Ashley, *Proc. Natl. Acad. Sci., USA*, 1999, **96**, 1846; (*b*) C. A. Roessner and A. I. Scott, *Chem. Biol.*, 1996, **3**, 325.

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