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# Functional analysis of Phe605, a conserved aromatic amino acid in squalene–hopene cyclases†

**Tsutomu Hoshino,* Masanori Kouda, Takamasa Abe and Tsutomu Sato**

*Department of Applied Biological Chemistry, Faculty of Agriculture and Graduate School of Science and Technology Niigata University, Ikarashi, Niigata 950-2181, Japan*

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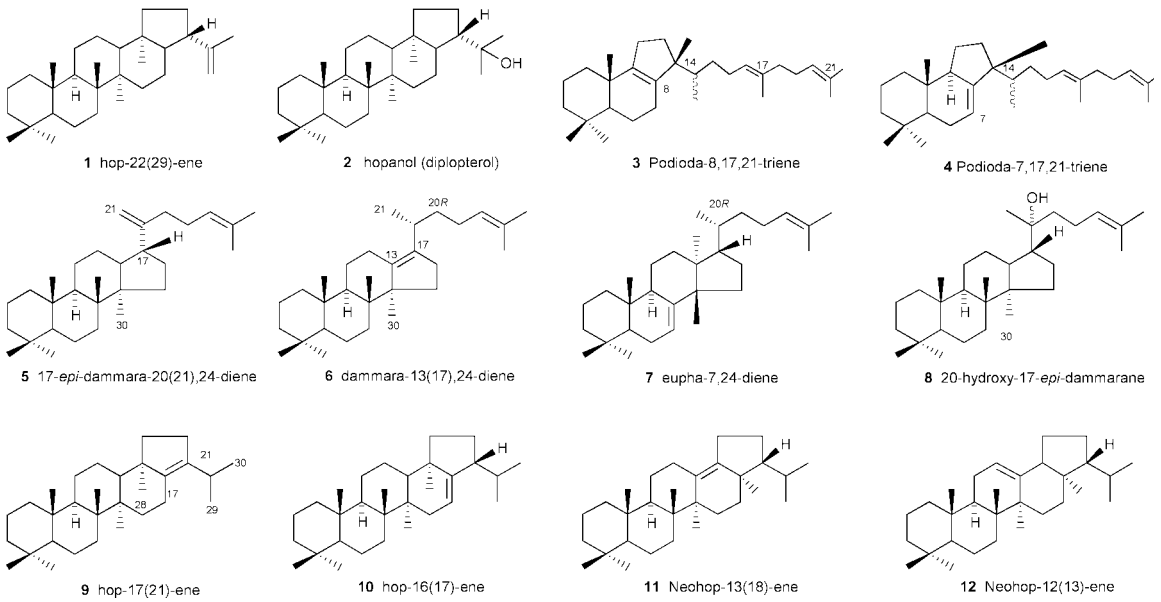
**Incubation of squalene with the site-directed mutant F605A of squalene–hopene cyclase from *Alicyclobacillus acidocaldarius* yielded many triterpenes consisting of the 6/6/5-fused tri-, 6/6/6/5-fused tetra-, and 6/6/6/6/5-fused pentacyclic skeletons, the function of F605 being assignable for facilitating the ring expansion and for stabilizing the hopanyl C22-cation, possibly *via* cation-π interactions.**

Squalene–hopene cyclase (SHC) converts acyclic squalene into pentacyclic triterpenes, hopene **1** and hopanol **2**, with regio- and stereochemical specificity.[1] In the past few years, there have been remarkable advances in addressing both the cyclization mechanism and the catalytic sites by site-directed mutagenesis experiments.[2] The cyclization cascade is triggered by proton attack at the terminal double bond, which is supplied by D376 and/or D374.[2a] The carbocation intermediates of the initially cyclized A-ring and the bicyclic A/B-fused ring, progressively formed during the polycyclization reaction, are stabilized by the carboxylate anion of D377[2a] and by the π-electrons of F365[2b] *via* a cation-π interaction, respectively. The 6-membered C-ring (anti-Markovnikov adduct) is formed as a result of a ring expansion process from the 5-membered C-ring that has been previously formed under Markovnikov closure.[2c] This expansion may be facilitated by π-electrons of F601. The point mutation experiments targeted for W 169 and W 489 allowed us to propose the involvement of the ring expansion from the 5- to 6-membered D-ring.[2d] The bulk size at the 261 position is critical in directing the stereochemical destiny for the formation of the hopane skeleton.[2f] The question still remained unanswered as to how the last 5-membered E-ring is constructed. Phe605 is strictly conserved in all the known SHCs, but not in oxidosqualene cyclases. We describe here the enzymic products obtained by the mutant F605A and the functional analysis of Phe605.

With the cell-free homogenates from a 8L-culture of the transformed *E. coli* encoding F605A SHC, 400 mg of squalene was incubated for 14 h at optimum catalytic conditions (pH 6.0 and 50 °C). Ten new products were homogeneously purified with $SiO_2$ column chromatography (5% $AgNO_3$) eluting with hexane. The yields of **1**–**12** were estimated by GC to be 141, 133, 9.9, 6.6, 23.3, 11.5, 9.3, 20.4, 7.4, 0.5, 12.4, 12.6 mg, respectively, and to be 1.2 mg for the recovered squalene.

Scheme 1 shows the structures of all the isolated products, which were determined by the detailed analyses of EIMS and 2D NMR spectra. Products **3** and **4** (**3**:**4** = 1.5:1) had a 6/6/5-fused tricyclic skeleton. All the NMR data and the specific rotation of **3** and **4** were indistinguishable from those of the known podioda-8,17,21-triene and -7,17,21-triene,[3] respectively, but the stereochemistry at the 14-position has remained undetermined. These two products were formed from the common biosynthetic intermediate **13** (Scheme 2). The successive rearrangement reactions of 13βH to C14 and of the 26-Me to C13 on **13** gave the C8-cation (podiodatrienyl cation **13′**). The deprotonation of H9 or H7 afforded **3** or **4**, respectively. The cyclization reaction proceeds through intermediates **13**–**17**, as shown in Scheme 2. A ring expansion of **13** to form **14** and a further cyclization could produce **15**. Products **5**–**8** were produced *via* **15** by deprotonation reactions at different positions, rearrangement reactions of hydride and methyl, or nucleophilic attack of a water molecule. Intermediate

† Electronic supplementary information (ESI) available: EIMS spectra and NMR assignments of products **3**–**12**. See http://www.rsc.org/suppdata/cc/b0/b004129g/




**Scheme 1** Structures of the enzymic products obtained by the mutant F605A.

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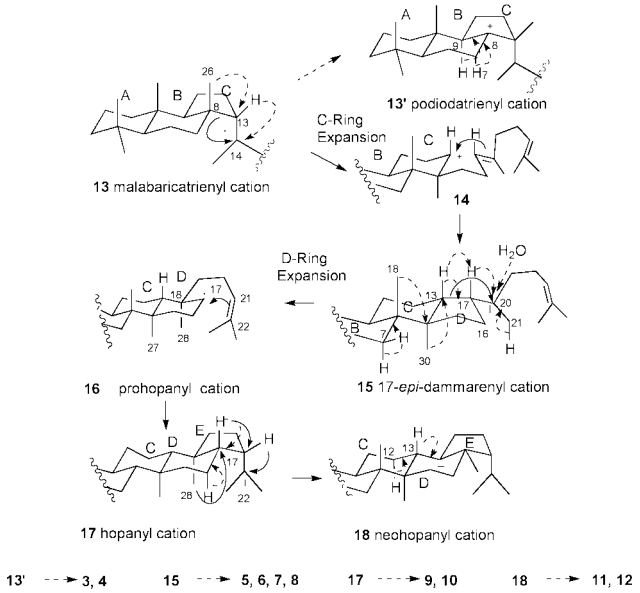


**Scheme 2**

**15** underwent ring expansion and a further cyclization to give **16**,[2f] and **17**, respectively. **9**[2f] and **10** were produced *via* **17**. The migration of 28-Me into C17 in **17** gave **18**, which was subjected to deprotonation reactions to yield **11** and **12**. The product ratio of **2**:**1** was significantly increased by this mutation (0.9:1), compared with that of the native SHC (0.2:1). This fact strongly suggests that the perturbation occurred around the E-ring, which allowed the easy access of a water molecule to the enzyme cavity and also led to the incorrect positioning of the deprotonation site for introducing the $\Delta^{22:29}$ double bond in **1**. Therefore, a larger amount of **2** and **8** could be accumulated together with the pentacyclic **9**–**12** having different double bond positions due to the improper deprotonation reactions. The significant perturbation around the E-ring may have had a greater influence on the earlier reaction steps in forming tri- and tetracyclic ring systems to afford **3**–**8**. Many different triterpene skeletons have been found from various point mutants;[2] but the high production of podiodatrienes (**3**,**4**) and neohopanes (**11**,**12**) has not previously been reported.

To identify the function of F605, we constructed the mutants of F605Y, F605W and F605H. If F605 stabilises a carbocation intermediate through a cation–π interaction, the higher π-electron density gives the faster reaction rate.[2b] Fig. 1 shows the specific activities for the formation of **1** or of both **1** and **2** against incubation temperatures. At lower temperatures, F605Y and F605W had faster reactions than the wild-type,[4] suggesting that a cation–π interaction may be involved in the cyclization cascade.[2b] The F605Y had a bell-shape, while the F605W had a steady state in the range 45–55 °C. The cyclase activities of the mutants were somewhat sensitive to the incubation temperatures. These features may have occurred due to the decreased binding (the largest $K_m$ of F605W[5]) and/or to the occurrence of the geometrical change around the active site region by elevating the temperature, as previously discussed for the F365Y and F365W SHCs.[2b] The mutants F605Y and F605W afforded a larger amount of **2** relative to **1** than the wild-type (Fig. 1);[4] the bulky substituents make the active site region (around the E-ring) less compact, thus the water molecule has easier access to the cavity resulting in the production of a larger amount of **2**. However, the quantities of **3**–**12** produced by the Tyr and Trp mutants were negligible, strongly suggesting that the perturbation was lower compared to that of the Ala mutant and that a cation–π interaction was still effective in the active site (Fig. 1). F605H afforded **5** in a significantly higher yield (14%) than the wild-type (1%), despite the total amount of **1** and **2** being decreased (84% of the native SHC); the His moiety would have abstracted the proton from 21-Me of **15** owing to the basic function. Thus, F605 can be situated near to 21-Me in **15**. The more favourable placement of F605 on the C17 cation (the secondary cation) in **16**, though near to the C20-cation in **15** (the tertiary cation), may facilitate the ring expansion reaction to give the energetically unfavourable **16**, as is predicted from computational studies.[6]

Tetracyclic **5**–**8** were accumulated with F605A, but never with F605Y and F605W, which definitively suggests that the π-electrons of Phe605 have a crucial role for the D-ring expansion process from **15** to **16**. A nucleophilic attack of the $\Delta^{21}$ double bond to the C17 cation in **16** yields the E-ring and the last C22 cation of **17**. The E-ring formation reaction would be further accelerated if the C22 cation could be stabilized *via* a cation–π interaction. Phe605 is not conserved in oxidosqualene cyclases. For lanosterol and cycloartenol biosyntheses, the polycyclization is quenched at the tetracyclic stage without a D-ring expansion.[1] For the syntheses of pentacyclic lupeol and β-amyrin, the ring expansion is also involved to give a common intermediate of the baccharenyl cation, but the position of the carbocation is different from that of the **16** cation.[1,2d,f] The different cyclization mechanism between eukaryotic and prokaryotic cyclases further support the proposed function of Phe605 which is conserved only in SHCs.

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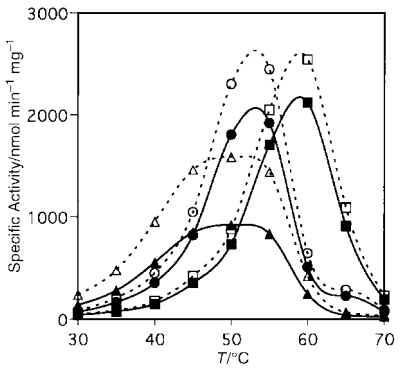


**Fig. 1** Specific activities for the formation of **1** (closed symbols, solid lines) or of the both of **1** and **2** (open symbols, dotted lines) against incubation temperatures are given for the wild-type SHC (■,□), the mutants of F365Y (●,○) and F365W (▲,△). One mg of squalene was incubated with 5 μ of the homogeneously purified SHCs for 60 min at pH 6.0. The ratio of **1** to **2** can also be estimated from the difference between the solid and the dotted lines.

## Notes and references

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4 On prolonged incubation for 20 h at 45 °C and pH 6.0 with equal amounts of squalene and the enzyme, the total amounts of **1** and **2** produced were almost equal for the three different SHCs. However, the product ratio of **1** to **2** was significantly altered as follows: 5.0 for the native SHC; 3.65 for the F605Y; 1.37 for the F605W (Fig. 1).
5 Kinetic data for the total amount of **1** and **2**, produced by incubating squalene at 35 or 45 °C (in parentheses) and for 60 min, were as follows: $k_{cat}$s; 0.25 (1.48), 0.18, 0.92 (4.52) and 1.67 (9.11) $s^{-1}$, and $K_m$s; 17.8 (18.2), 47.9, 21.1 (69.6) and 21.1 (109) μM, respectively, for the wild-type, F605A, F605Y and F605W. The more bulky substituents gave the larger $K_m$ (looser binding) with elevating incubation temperatures, which may result in the profile of steady state in the range of 45–55 °C (Fig. 1), despite π-electron density being enhanced.[2b]
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