# Mutational Studies on Triterpene Synthases: Engineering Lupeol Synthase into β-Amyrin Synthase

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**Abstract:** Site-directed mutagenesis was carried out on two triterpene synthases, β-amyrin (PNY) and lupeol (OEW) synthases, to identify the amino acid residues responsible for their product specificity. In addition to sequence comparison among known oxidosqualene cyclases, our previous chimeric studies suggested that $^{258}$MWCYCR$^{263}$ sequence of β-amyrin synthase PNY ($^{255}$MLCYCR$^{260}$ sequence of lupeol synthase OEW) would participate in product differentiation. To test this hypothesis, Trp259 (M**W**CYCR of PNY) was mutated to Leu (PNY W259L mutant). Functional expression in yeast and product analysis revealed that this mutant produced lupeol as a major product together with β-amyrin in 2:1 ratio. Some other minor products including butyrospermol were also produced. On the other hand, Leu256 (M**L**CYCR of OEW) was mutated to Trp (OEW L256W mutant). This mutant produced exclusively β-amyrin with only minor amount of lupeol, demonstrating that a single mutation had engineered lupeol synthase into β-amyrin synthase. Therefore, Trp259 of β-amyrin synthase was identified to be the residue controlling β-amyrin formation presumably through stabilization of oleanyl cation, while lack of this effect by Leu residue may terminate the reaction at lupenyl cation stage. In further mutation studies, Tyr residue (MWC**Y**CR in PNY and MLC**Y**CR in OEW) conserved in all of the OSCs producing pentacyclic triterpenes was mutated into His which is found in all of those producing tetracyclic carbon skeletons to investigate the role of this Tyr261 of PNY. PNY Y261H mutant produced dammara-18,21-dien-3β-ol (as a 3:5 mixture of *E*/*Z* isomer at $\Delta^{18}$) together with a minor amount of dammara-18(28),21-dien-3β-ol, demonstrating that Tyr261 of β-amyrin synthase plays an important role in producing pentacyclic triterpenes presumably by stabilizing one of the cation intermediates generated after dammarenyl cation.

## Introduction

Triterpenes are one of the most abundant natural products mainly found in plants and exhibit huge structural diversity. More than 90 different carbon skeletons are known, and further oxidative modifications and glycosidations generate even more diverstiy.[1] Among these triterpenoids, especially as glycosides, saponins, are medicinally important compounds. Triterpene alcohols themselves, even without further structural modification, are also known to exhibit important biological activities, such as anticarcinogenic and antiinflammatory acitivities.[2] Therefore, these triterpenoids are regarded as an important chemical pool for new drug development in the future. In higher plants, one plant species usually contains several different skeletal types of triterpenes; however, the spectrum of triterpenes present differs in one species to another. For example, *Panax ginseng* contains mainly dammarane-type triterpenes,[3] whereas *Alisma orientalis* contains mainly protostane type triterpenes.[4] It is not well understood whether any particular triterpene has significant physiological role in producing plant, yet it would be an important issue for plants to specifically control the structure of triterpenes being produced to maintain the spectrum of these compounds.

All of these triterpenes are biosynthesized from a common precursor, 2,3-oxidosqualene. The enzyme triterpene synthases are responsible for the formation of these diverse triterpene skeletons.[5] These fascinating enzymes catalyze cyclization of acyclic substrate into various tetra- and pentacyclic carbon frameworks, generating multiple stereogenic centers in a single reaction. It is thus tempting to speculate that subtle difference in the active site structure may alter the product specificities exhibited by these enzymes. To elucidate the mechanism of product specificities exhibited by triterpene synthases, molecular cloning of several cDNAs encoding these enzymes were carried out. Up to now, eight different triterpene synthase cDNAs have been cloned from plant sources. These are β-amyrin synthases from *P. ginseng* (PNY and PNY2),[6,7] *Pisum sativum* (PSY),[8]

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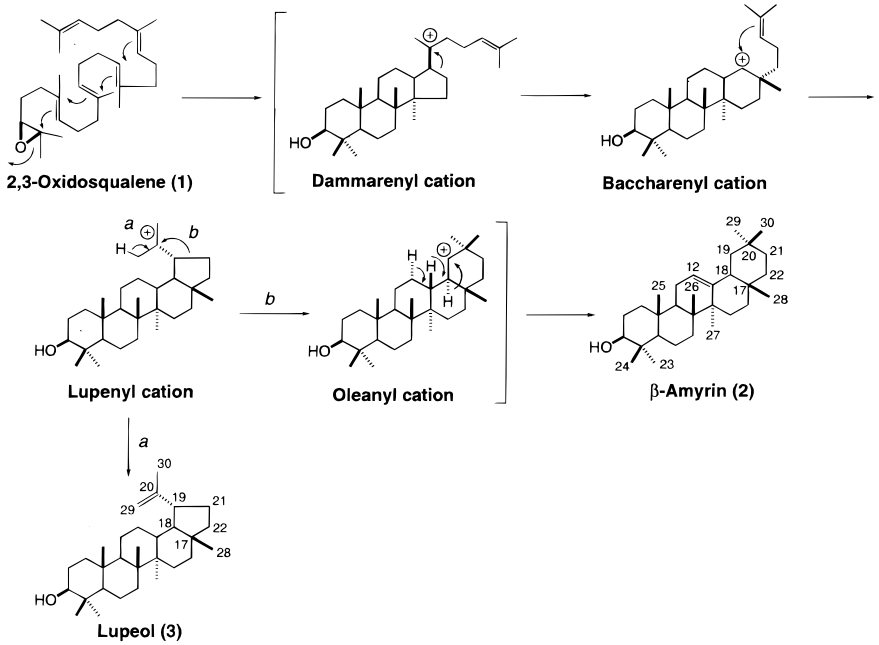
**Scheme 1.** Cyclization of 2,3-Oxidosqualene into $\beta$-Amyrin and Lupeol




and *Glycyrrhiza glabra* (GgbAS1),[9] lupeol synthases from *Olea europaea* (OEW) and *Taraxacum officinale* (TRW),[10] another type of lupeol synthase from *Arabidopsis thaliana* (LUP1),[11] and α-amyrin producing mixed amyrin synthase from *P. sativum* (PSM).[8] Among these, a mixed amyrin synthase which produces both α- and $\beta$-amyrin should be noted here. The presence of this multifunctional triterpene synthase in nature, raised a question as to whether there actually exist corresponding number of product specific triterpene synthases in higher plants. In addition, [1,2-$^{13}C_2$] acetate feeding experiments demonstrated that in the reaction of lupeol synthases OEW and TRW, the final deprotonation takes place specifically from the methyl group derived from C-6 of mevalonate[12] while in that of LUP1, the final deprotonation occurs on both methyl groups of lupenyl cation in equal amount, indicating the lack of control in this enzyme.[13]

Considerable efforts have been directed toward the understanding of cyclization mechanism of oxidosqualene cyclases (OSCs) and the active site residues probed using substrate analogues or site-directed mutagenesis.[14] However, these studies were carried out only on lanosterol synthases, and none of studies have been focused on the context of product specificities, until very recent report on cycloartenol synthase from *A. thaliana*, where a single mutation of Ile to Val has resulted in loss of product specificity.[15] We have initiated our study toward understanding the mechanism of product differentiation exhibited by $\beta$-amyrin and lupeol synthases, which produce two of the major triterpenes found in nature. As shown in Scheme 1, the mechanism of both $\beta$-amyrin and lupeol formation is identical up to lupenyl cation stage, through tetracyclic dammarenyl and baccharenyl cations. Proton loss from one of the *gem*-dimethyl groups will produce lupeol, while ring expansion to oleanyl cation followed by 1,2-hydride shifts and proton loss from C-12 will produce $\beta$-amyrin. Any factor from the enzyme proteins that controls the product specificity should be acting at the stage discriminating between proton loss and ring expansion.

Our recent domain swapping studies on $\beta$-amyrin synthase (PNY) and lupeol synthase (LUP1) have shown that the region B, the second quarter from the N terminus, is most important for $\beta$-amyrin formation, and further that only 80 amino acid long sequence located within this region has great influence on the product distribution between $\beta$-amyrin and lupeol.[13] Interestingly, all of these chimeric enzymes were multifunctional, producing mainly $\beta$-amyrin and lupeol together with some other minor triterpenes. However, it remained to be answered which of amino acid residues within this region are responsible for such product distribution.

X-ray crystal structure of a related enzyme, squalene−hopene cyclase (SHC) from bacteria, has been reported, and several amino acid residues within the active site were shown to play an important role in catalysis.[16] However, the overall amino acid sequence identity beetween SHC and OSCs is only about 20%. From the nature of the cyclization reaction, it is obvious that only a slight difference in the active site of the enzyme should alter the product outcome, and therefore, it is impossible to speculate the important residues on triterpene synthases governing the product specificity only from the X-ray crystal structure of SHC.

We have set out a series of site-directed mutagenesis studies on two triterpene synthases, namely, $\beta$-amyrin synthase (PNY) and two types of lupeol synthase (OEW and LUP1), to see if

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**Figure 1.** Alignment of amino acid sequences around MWCYCR sequence of $\beta$-amyrin synthases. *S. cerevisiae* lanosterol synthase (ERG7), $\beta$-amyrin synthases of *P. ginseng* (PNY and PNY2), *P. sativum* (PSY), and *G. glabra* (GgbAS1), and lupeol synthases of *O. europaea* (OEW), *T. officinale* (TRW), and *A. thaliana* (LUP1) are shown. Identical amino acid residues are boxed. The arrow indicates the beginning of the important 80 amino acid long sequence within region B, shown in ref 13. MWCYCR motif (in $\beta$-amyrin synthase) is double-underlined. The number indicates the position of amino acid residues in each full length sequence.

any particular residues govern the product outcome. The present contribution describes identification of the residues which play a crucial role in product determination.

## Results and Discussion

**Mutagenesis on $\beta$-Amyrin Synthase PNY.** From our previous chimeric studies, it was shown that region B, the second quarter of the polypeptide from the N terminus, is important for $\beta$-amyrin formation.[13] Especially, swapping of the 80 amino acid long sequence between Cys260 and Trp340 within this region of $\beta$-amyrin synthase into the corresponding LUP1 sequence drastically altered the product ratio between lupeol and $\beta$-amyrin. The finding by Corey et al. that peptide WWVHTR (231−236) of lanosterol synthase from *Saccharomyces cerevisiae* was affinity labeled with a substrate analogue, 20-oxa-2,3-oxidosqualene, suggested that this residue might be located close to C-20 protosteryl cation during lanosterol formation.[14c] The corresponding sequence of triterpene synthases are located at the N-terminal end of the important 80 amino acid long sequence inside region B. In all of the four $\beta$-amyrin synthases, this sequence is MWCYCR, while in two of the authentic lupeol synthases, this is MLCYCR (Figure 1). In addition, *A. thaliana* lupeol synthase (LUP1) possesses ILCYSR sequence. If we assume the same orientation of substrate binding relative to enzyme proteins as in lanosterol synthase, and a similar overall structure of the active sites, these residues might be located near E-ring of lupenyl and/or oleanyl cation during cyclization reaction, and therefore, might play an important role in product differentiation during lupeol and $\beta$-amyrin formation. Furthermore, comparison of the important 80 amino acid sequences of all the $\beta$-amyrin and lupeol synthases cloned so far, revealed a clear difference between the two enzymes at this residue. Thus, Trp of M**W**CYCR sequence is conserved among $\beta$-amyrin synthases, while Leu of M**L**CYCR/I**L**CYSR sequences is conserved among lupeol synthases. Therefore, our first mutation study was targeted to Trp259 of $\beta$-amyrin synthase (PNY) and Leu256 of lupeol synthase (OEW).

Site-directed mutagenesis was carried out by PCR method. First PCR between N-terminal and anti-sense mutation primers with native full length cDNA as a template gave 800 bp fragment, which was used as a "sense primer" in the second PCR with C-terminal primer to give 2.3 kb fragment corresponding to the full length cDNA. The resulting clone was completely sequenced to confirm only the desired mutation had taken place, and subcloned into yeast expression vector pYES2 (Invitrogen) under the control of *GAL1* promoter. Since the in vivo expression system using yeast mutant GIL77 which lacks lanosterol synthase activity, was successfully applied in analyzing the function of introduced clones, we employed this system in the present study. The fatal mutation in *erg7* lanosterol synthase gene abolishes the production of background lanosterol by the host yeast,[17] and the cyclization products of expressed clone accumulate within the cells.

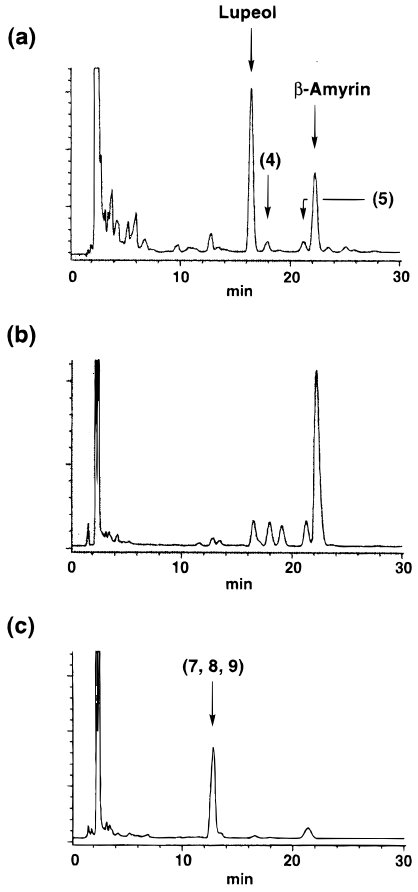


**Figure 2.** HPLC profiles of the extract from (a) PNY W259L mutant, (b) OEW L256W mutant, and (c) PNY Y261H mutant. HPLC conditions are in Experimental Section. Arrows indicate peaks of corresponding triterpenes. Horizontal line indicates the retention time in minutes.

Trp259 of PNY $\beta$-amyrin synthase was mutated to Leu to give PNY W259L mutant (MLCYCR). The transformed yeast was cultured, expression of recombinant mutant enzyme was induced with galactose, and the cyclization products were extracted and purified as described previously.[6] The results revealed the production of significant amount of lupeol together with $\beta$-amyrin as shown in the HPLC chart (Figure 2(a), Table 1). Indeed lupeol was the major product of this mutant. In addition to these two products, small amount of other products were also observed. Careful analysis of the product mixture by $^1$H NMR revealed two of the minor products to be eupha-7,21-dien-3$\beta$-ol (butyrospermol, **4**) and olean-18-en-3$\beta$-ol (germanicol, **5**), as characteristic olefinic proton signals of H-21 and H-7 of butyrospermol at $\delta$ 5.093 and $\delta$ 5.251,[18] respectively, and of H-19 of germanicol at $\delta$ 4.851 were observed.[19] Co-

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**Table 1.** Product Ratios of Each Mutant[a]

| | $\beta$-amyrin | lupeol | **4** | **5** | **7/8/9** |
|---|---|---|---|---|---|
| PNY W259L | 30.3 | 54.6 | 3.6 | 3.4 | |
| PNY MW258IL | 40.5 | 53.4 | 3.6 | 2.5 | |
| PNY C262S | 100 | | | | |
| PNY Y261H | | 2.4 | | 13.6 | 84.0 |
| OEW L256W | 74.8 | 6.9 | 9.9 | 8.4 | |
| OEW L256F | 9.8 | 69.7 | 17.9 | | |
| OEW L256Y | 1.6 | 54.8 | 22.7 | | 18.7 |
| OEW L256H | 3.8 | 69.5 | 10.0 | | 13.5 |
| OEW L256A | <1 | 68.2 | 20.2 | | 7.5 |
| OEW Y258H | | 43.6 | 6.2 | | 42.4 |
| LUP1 L255W[b] | 14.0 | 55.0 | 16.0 | | |

[a] Numbers are shown in percentage of total triterpene monoalcohol products estimated from the peak area on HPLC. [b] This mutant also produced 3$\beta$,20-dihydroxylupane (**6**). See text.

injection of the authentic samples identified a peak at 18.0 min to be butyrospermol and one at 21.2 min to be germanicol. The other minor products are still unknown. Chimera 1 of our previous work, in which N-terminal half is PNY and C-terminal half is LUP1, also produced butyrospermol (peak at 17 min on the Figure 2 HPLC chart of ref 13) as a minor product. The total amount of triterpenes produced by this mutant decreased to about one-third of the native enzyme, presumably due to a partial loss of catalytic efficiency caused by the mutation in the active site. W259L mutation on $\beta$-amyrin synthase caused notable production of lupeol, indicating that aliphatic leucine residue might play an important role in lupeol formation. Conversely, aromatic tryptophan might be an important residue for $\beta$-amyrin formation. In addition, identification of minor products provided some insights on the mechanism of product diversity. Production of butyrospermol indicates that the cyclization was derailed to some extent at tetracyclic dammarenyl cation stage, and successive methyl and hydride shifts and proton loss at C-7 took place (Scheme 2). The presence of germanicol, on the other hand, indicates that the proton loss from C-18 took place at oleanyl cation stage to form C-18, 19 double bond. The exact reason for these side reactions is not clear, however, the change in active site conformation by substitution of amino acid residue might have resulted in aberrant termination of the cyclization reaction.

Whether the lupeol formed by this mutant shows methyl group scrambling during proton loss at lupenyl cation stage as has been demonstrated for LUP1 lupeol synthase,[13] was examined by feeding [1,2-$^{13}C_2$] acetate. $^{13}$C NMR analysis showed accompanying doublets at both C-29 ($\delta$ 109.3, d, $J$ = 72.5 Hz) and C-30 ($\delta$ 19.3, d, $J$ = 42.7 Hz) indicating that scrambling had taken place. On the other hand, $\beta$-amyrin formed by the same mutant exhibited an accompanying doublet at C-29 ($\delta$ 33.3, d, $J$ = 35.1 Hz) and a singlet at C-30 ($\delta$ 23.7, s) indicating that both methyl groups were strictly discriminated. Therefore, during the formation of lupeol by this mutant, deprotonation took place from both methyl groups and was not controlled by the enzyme. This lack of control at the lupenyl cation stage suggests that the ring expansion to oleanyl cation did not occur and nonspecific deprotonation took place presumably by the solvent water molecule within the active site of the enzyme. The scrambling of methyl groups was previously observed for *A. thaliana* lupeol synthase (LUP1) but not for *O. europaea* and *T. officinale* lupeol synthases (OEW, TRW).[12,13] The present results also suggest that original function of LUP1 might not be lupeol synthase but a synthase of other triterpenes located downstream of oleanyl cation stage and this clone might have lost the ability to form oleanyl cation from lupenyl cation during the course of evolution. In any case, scrambling of methyl groups indicates the aberrant termination of the reaction at the lupenyl cation stage. It is noteworthy that the mutant still retains the ability to produce $\beta$-amyrin, with strict control of two methyl groups during the ring expansion process.

Since *A. thaliana* LUP1 possesses $^{254}$ILCYSR$^{259}$ sequence at this position (Figure 1), we also prepared other mutants on $\beta$-amyrin synthase in order to examine whether other amino acid residues also have influence on lupeol formation. Both Met258 and Trp259 of $\beta$-amyrin synthase were mutated to Ile and Leu to give PNY MW258IL mutant (ILCYCR). The result was the same with PNY W259L mutant as lupeol was formed as the major product together with $\beta$-amyrin and two other minor products, namely butyrospermol and germanicol (Table 1). Therefore, the product specificity is exclusively governed by Leu255, and not by Ile254 of LUP1. In addition, Cys262 of $\beta$-amyrin synthase was mutated to Ser to give PNY C262S mutant (MWCYSR). In this case, the result was completely the same with the native $\beta$-amyrin synthase and no production of lupeol was detected (Table 1). These results further confirm that the product specificity is governed only by a limited number of amino acids.

**Mutagenesis on Lupeol Synthase OEW.** With the results of $\beta$-amyrin synthase mutants, we then examined the opposite mutations on lupeol synthase OEW. Leu256 of OEW was mutated to Trp to give OEW L256W mutant (MWCYCR). The result is shown in Figure 2(b). Surprisingly, this time, $\beta$-amyrin was formed predominantly over some minor products including lupeol. This result is really astonishing in that by a single amino acid substitution, lupeol synthase was engineered into $\beta$-amyrin synthase. Although this mutant still produces lupeol, the majority of the product is $\beta$-amyrin sharing about 75% of the total products (Table 1). The total amount of products was nearly the same as native OEW, and therefore, the mutation did not affect the catalytic efficiency. The minor products included again butyrospermol and germanicol as determined by $^1$H and $^{13}$C NMR analysis. Feeding experiment of [1,2-$^{13}C_2$] acetate was carried out in order to see whether the terminal *gem*-dimethyl groups were derived from the same carbons as with the native $\beta$-amyrin synthase. The result showed that C-29 ($\delta$ 33.3, d, $J$ = 35.1 Hz) appeared with an accompanying doublet while C-30 ($\delta$ 23.7, s) appeared as a singlet, indicating that C-29 derived from C-6 of mevalonate while C-30 from C-2 of mevalonate, which is completely identical with the native $\beta$-amyrin synthase.[13] Therefore, OEW L256W mutant strictly controls clockwise rotation of isopropyl group of lupenyl cation during the ring expansion process. This is quite surprising as it seems that lupeol synthase had already prepared the active site so as to produce $\beta$-amyrin in a completely identical mechanism with the native $\beta$-amyrin synthase, although the amino acid sequence identity between these two enzymes is only 63.7%. From our previous phylogenetic analysis on triterpene synthases, these clones form distinct branches and are located quite far apart, suggesting that the two enzymes have diverged early in the evolution and evolved independently afterward.[10] However, the fact that a single mutation on each clone restores the catalytic activity to produce the opposite ones implies that their evolutionary course is quite common and both possess the hidden pontential to form each product. Since the formation of $\beta$-amyrin requires additional steps to the formation of lupeol, this mutation could be considered as an addition of a new function into the existing lupeol synthase.

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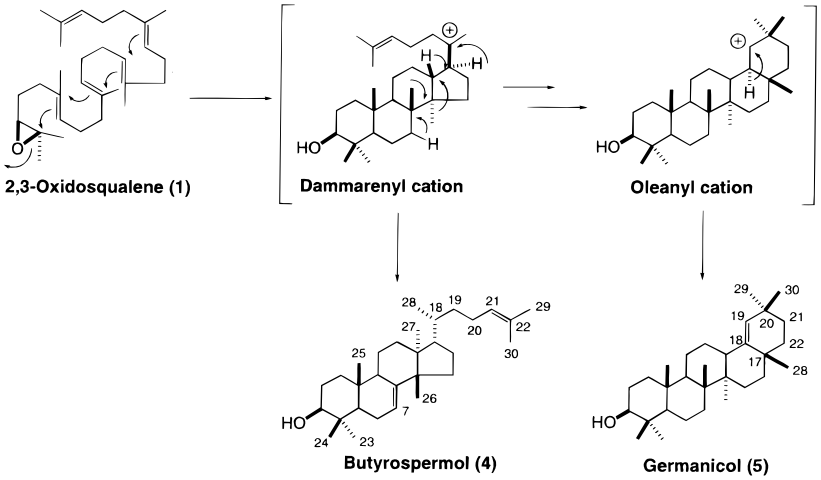
**Scheme 2.** Formation of Butyrospermol and Germanicol by PNY W259L Mutant




As it became obvious that the Trp residue on MWCYCR sequence plays a crucial role in $\beta$-amyrin formation, we have generated several mutants of OEW in the same position in order to investigate the function of this residue. The original Leu was replaced by Phe, Tyr, His, and Ala. Phe and Tyr mutants were tested to see whether the $\pi$-electrons of aromatic ring might be important for $\beta$-amyrin formation. Expression of OEW L256F mutant (MFCYCR) has shown that still lupeol was produced as a major product, however, minor amount of $\beta$-amyrin was formed (14% of lupeol) together with butyrospermol (Table 1). On the other hand, OEW L256Y mutant (MYCYCR) also gave lupeol as a major product, together with butyrospermol and unknown compound (retention time 13 min, see below) in small amount. $\beta$-Amyrin was also observed, however, the amount was only 1.6% of the total products (Table 1). The overall triterpene production of this mutant was reduced to about 50% of the native enzyme suggesting that the substitution to Tyr residue has caused significant loss in catalytic efficiency. OEW L256H mutant (MHCYCR) exhibited almost identical product pattern with the native enzyme except that an unknown compound at 13 min was produced in significant amount (13.5% of the total products) together with $\beta$-amyrin (3.8% of the total products) and butyrospermol (Table 1). OEW L256A mutant (MACYCR) also gave lupeol as a major product with butyrospermol and an unknown compound at 13 min as a minor prouducts, the product pattern being similar to L256Y mutant. $\beta$-Amyrin production was less than 1% of the total products (Table 1). These results indicated that the amino acid residues other than Trp have little influence on $\beta$-amyrin formation, and lupeol was still produced as a major product. However, in the case of Leu to Phe mutant, minor amount of $\beta$-amyrin was formed (14% of lupeol), suggesting that the aromatic ring of Phe has crucial effect to produce $\beta$-amyrin, although this effect is not absolute as is the case with Tyr. Besides lupeol, butyrospermol and unknown compound at 13 min were formed in significant amount by these mutants. The formation of butyrospermol indicates the aberrant termination of the cyclization reaction presumably due to altered conformation of the active site. The unknown compound will be discussed in the following section.

To examine whether Trp residue at this position also promotes the formation of $\beta$-amyrin in LUP1, LUP1 L255W mutant (IWCYSR) was prepared. As reported previously, LUP1 produces $\beta$-amyrin in its native form.[11] The result showed enhanced production of $\beta$-amyrin compared with the native enzyme (25% of lupeol produced compared with 10% in native one). However, still lupeol was produced in major amount

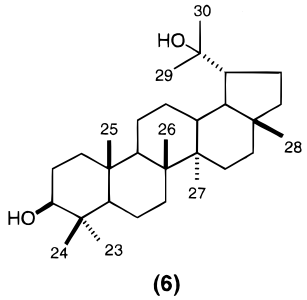



**Figure 3.**

together with some other minor products (Table 1). Therefore, in LUP1, the substitution of Leu to Trp did not cause drastic change in catalytic function as in OEW. Presumably, the active site of LUP1 has already evolved so as to produce both lupeol and $\beta$-amyrin with some other minor triterpenes.

During extraction and purification of cyclization products of LUP1 L255W mutant, we noticed a more polar spot (Rf: 0.15, benzene:acetone=19:1) on TLC than the spot corresponding to lupeol and $\beta$-amyrin (Rf: 0.4) which is apparently derived from expressed protein. The identical spot was also observed in native LUP1 products. Isolation, characterization by $^1$H, $^{13}$C NMR, HMBC and HMQC analysis, and comparison with literature data revealed it to be 3$\beta$,20-dihydroxylupane (**6**) (Figure 3).[20] The formation of **6** would be best understood by the addition of water to lupenyl cation. This is the first report on the production of any triterpene which is formed by addition of water to the final carbocation intermediate by recombinant triterpene synthases. Previously, (20*S*)-dammarenediol synthase activity was detected in the microsomal preparation of the hairy root of *P. ginseng*, which is another rare example of the formation of triterpene by addition of water.[21] The amount of **6** produced is about the same as total triterpene monoalcohol produced, and did not differ between L255W mutant and the native clone. This suggests that in LUP1 reaction, the final quenching of lupenyl cation is accomplished by both deprotonation from the methyl group and addition of water in almost equal ratio. The formation of both compounds is reminiscent of SHC which produces both hopene and diplopterol. Together with the scrambling of methyl groups during lupeol formation, this would imply that, in LUP1 reaction, the final deprotonation

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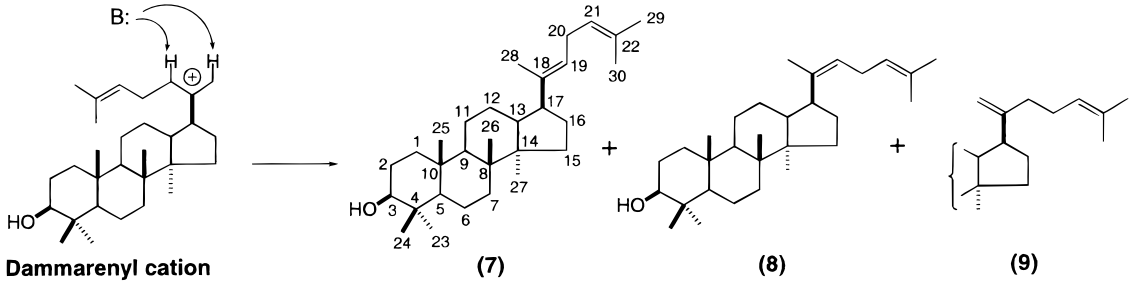
(21) Kushiro, T.; Ohno, Y.; Shibuya, M.; Ebizuka, Y. *Biol. Pharm. Bull.* **1997**, *20*, 292−294.

**Scheme 3.** Formation of **7**, **8**, and **9** by PNY Y261H Mutant




is mediated by water molecule and not by specific amino acid residue within the active site of the enzyme as suggested in the case of SHC reaction.[16] 3$\beta$,20-Dihydroxylupane and its derivatives are known in plants such as *Rhus taitensis* and *Relhania calycina*.[20,22] It would be interesting to know whether LUP1 type triterpene synthases are responsible for the production of these triterpenes.

**Mutation of Tyr Residue to His of M(W/L)CYCR Sequence on $\beta$-Amyrin and Lupeol Synthases.** Since Trp and Leu residues of this motif have significant effect on lupeol and $\beta$-amyrin formation, we then turned our attention to Tyr261 of PNY $\beta$-amyrin synthase, which is also present in lupeol synthases. Amino acid sequence comparison between all the known OSCs have revealed that pentacyclic triterpene synthases such as $\beta$-amyrin, lupeol, and α-, $\beta$-mixed amyrin synthases all have Tyr residue at this position in common. On the other hand, in all lanosterol and cycloartenol synthases, which produce tetracyclic carbon skeletons, His residue is conserved in this position. Therefore, we have assumed that this Tyr residue might play an important role in producing pentacyclic tritepenes. PNY Y261H (MWCHCR) and OEW Y258H (MLCHCR) mutants were prepared and expressed in yeast. PNY Y261H mutant, produced no $\beta$-amyrin and instead, an unknown peak appeared at 13 min on HPLC (Figure 2(c)). The peak was separated and analyzed by $^1$H and $^{13}$C NMR, DEPT-135, HMBC, and HMQC. The results revealed the presence of three dammarane type triterpene products in this peak. On $^1$H NMR spectrum, characteristic signals around $\delta$ 2.73 (m) were observed which could be assignable to methylene protons adjacent to two olefins. Five singlet methyl signals appeared at $\delta$ 0.775, 0.844, 0.902, 0.963, 0.979, four vinylic methyl signals at $\delta$ 1.540, 1.608, 1.615, and 1.680, and an oxymethine proton at $\delta$ 3.203 (dd $J$ = 11.3, 4.9 Hz). From the integration of each proton signals, it was obviously ~5:3 mixture of two major products. The olefinic region showed the major peaks at $\delta$ 5.048, 5.085, and 5.152, and a minor *exo*-methylene protons at $\delta$ 4.703 and 4.738. Further analysis of $^{13}$C NMR signals and comparison with the data of known dammarene type triterpenes,[23] suggested these major products to be a mixture of dammara-18(*E*),21-dien-3$\beta$-ol (**7**) and its 18(*Z*) isomer **8**. To confirm each structure, authentic **7** was prepared from hydroxydammarenone I, isolated from commercial dammar resin,[24] through $POCl_3$/pyridine mediated dehydration, followed by separation of olefinic isomers on 20% $AgNO_3$-impregnated silica gel, and $LiAlH_4$ reduction. 18(*E*) Isomer **7** was obtained as a major product with minor amount of (*Z*) isomer **8** ( ~6:1). Comparison of their $^1$H, and $^{13}$C NMR spectra unambiguously established the assignment of signals for **7** and **8** (see Experimental section). The other minor product was established to be dammara-18(28),21-dien-3$\beta$-ol (**9**), an olefinic isomer of **7** and **8** (Scheme 3).[25] The ratio between these three products were estimated to be **7**:**8**:**9** = 3:5:0.5. The presence of (*Z*) isomer as a major product is in contrast with a chemically prepared sample from hydroxydammarenone I through dehydration of C18 hydroxyl group, which gave (*E*) isomer as a major product. This would indicate that the side chain moiety of dammarenyl cation is held in such a way in the active site of the enzyme so as to form 18(*Z*) stereochemistry, which is chemically less favorable. In addition to these dammaradienols, minor amount of lupeol and germanicol were also produced (Table 1).

OEW Y258H mutant, on the other hand, gave lupeol as a major product, however, significant amount of dammaradienols were formed together with some other minor products (Table 1).

The formation of these dammaradienols by the Tyr to His mutants of PNY and OEW strongly suggest that this Tyr residue play a crucial role in the formation of pentacyclic triterpenes. Dammaradienols **7**, **8**, and **9** were apparently formed from intermediate dammarenyl cation through deprotonation on C-19 and C-28, respectively (Scheme 3). Therefore, in these mutants, the cyclization reaction did not proceed further than dammarenyl cation stage. The absence of euphane type triterpenes such as butyrospermol, which had been observed in many of the mutants described above, indicates that none of methyl and hydride shifts could occur and dammarenyl cation is immediately quenched by deprotonation from the adjacent carbons, which could be attributed to the replaced basic His residue. This would indicate that the His residue, thus original Tyr residue in native triterpene synthases, is located close to C-19 of dammarenyl cation. In addition, the formation of 18(*Z*) isomer **8** as a major product suggests that the side chain moiety is fixed to some extent, in a conformation that probably would have led to lupenyl cation, and is not freely rotating. As far as we know, dammara-18,21-dien-3$\beta$-ols (**7**, **8**) have not been reported from any natural sources, and therefore, considered to be "unnatural" natural products, although several dammarenoids having C-18, 19 double bond, such as Cordialin A from *Cordia verbenacea*,[26] and viburnudienone $H_1$ and $H_2$ from *Viburnum dilatatum*,[27] have been reported. It is not known whether these dammarenoids are directly biosynthesized from **7**, **8** by oxidative modification, or rather derived from more common dammarenoids such as dammarenediol or **9** through stereospecfic dehydration of C-18 hydroxyl group, or isomerization of *exo* to *endo*-olefin, respectively.

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(23) Yamashita, H.; Masuda, K.; Kobayashi, T.; Ageta, H.; Shiojima, K. *Phytochemistry* **1998**, *49*, 2461−2466.
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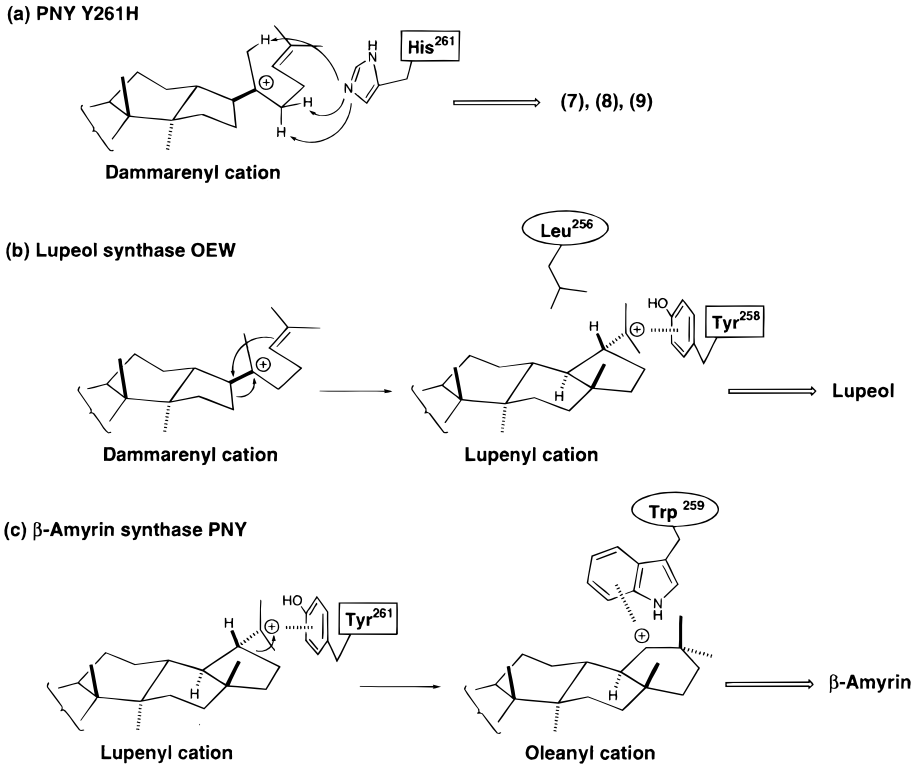
(27) Machida, K.; Kikuchi, M. *Chem. Pharm. Bull.* **1999**, *47*, 692−694.

**Scheme 4.** Proposed Role of Critical Amino Acid Residues during the Formation of Each Triterpene[a]




[a] Residues at the identical positions in the sequences are boxed or circled.

## Conclusions

The present results described above have pointed out that MWCYCR sequence of $\beta$-amyrin synthase (MLCYCR sequence for lupeol synthase), which is located within the 80 amino acid sequence in region B from the previous work,[13] plays the major role in product determination. Especially, the Trp residue is crucial for $\beta$-amyrin formation. As seen in PNY W259L mutant, substitution of this Trp residue into Leu greatly changed the product pattern where lupeol was formed as a major product. The formation of butyrospermol and germanicol indicated the aberrant termination of the reaction presumably due to the change of acitive site conformation. On the other hand, OEW L256W mutant gave exclusively $\beta$-amyrin while still maintaining the same overall production level of triterpenes as in the native OEW. Lupeol was formed in only tiny amount that corresponded to the level of butyrospermol and germanicol formation. From the [1,2-$^{13}C_2$] acetate feeding experiment, *gem*-dimethyl groups of lupenyl cation were strictly discriminated by this mutant enzyme during the formation of $\beta$-amyrin. The results were tremendous in that the single mutation on lupeol synthase had changed its function to $\beta$-amyrin synthase, and that OEW had already prepared the active site in such a way as to produce $\beta$-amyrin. At present, the role of this Trp residue is only speculative, however, we suggest its role as a stabilization of secondary oleanyl cation through cation-$\pi$ interaction during the ring expansion process (Scheme 4). As OEW L256F and L256Y mutants produced $\beta$-amyrin only in small amount, not only this electrostatic effect but also bulkiness of Trp might have generated conformational strain on the intermediate to favor the ring expansion from lupenyl cation. The reduced level of $\beta$-amyrin production by L256F and L256Y mutants might also be due to the improper orientation of these aromatic residues to stabilize the oleanyl cation. As evidenced from the [1,2-$^{13}C_2$] acetate feeding experiment, rotation of the isopropyl group prior to ring expansion should be restricted in order to discriminate two methyl groups during $\beta$-amyrin formation. In lupeol synthases, this residue is aliphatic Leu, and hence, the reaction is favored to terminate at lupenyl cation stage by deprotonation rather than to proceed to oleanyl cation to give six-membered E-ring. We are not sure whether the specific amino acid residue participates in the final deprotonation step in these triterpene synthases, however, the formation of 3$\beta$,20-dihydroxylupane in LUP1 suggests that water molecule in the active site, instead, may perform this task. Such mechanism have been proposed for the bacterial SHC catalyzed reaction, as this enzyme gives both hopene and diplopterol, and from the X-ray crystal structure, no basic residues could be found in close proximity to the methyl groups which undergoes deprotonation.[16]

The MWCYCR sequence not only contains amino acid residues responsible for determination of product specificity between lupeol and $\beta$-amyrin, but it also contains Tyr residue surely responsible for the formation of pentacyclic triterpenes. Mutation of this Tyr of PNY and OEW into His, which is conserved in all lanosterol and cycloartenol synthases, resulted in production of tetracyclic dammaradienols, mainly dammara-18,21-dien-3$\beta$-ol. The result was drastic in PNY case, as it gave exclusively these compounds and no $\beta$-amyrin was formed. Therefore, the His residue had stopped the reaction at dammarenyl cation stage. One possible role of this Tyr might be the stabilization of secondary baccharenyl cation right after the D-ring expansion from dammarenyl cation, and another is the stabilization of tertiary lupenyl cation after cation-$\pi$ E-ring closure. Since none of the baccharane type triterpene has been detected even as a minor product, and from the analogy of bacterial SHC reaction, where the terminal double bond of squalene is proposed to participate in the stabilization of secondary cation of D-ring before E-ring closure,[28] we favor the latter possibility of Tyr residue as stabilizing lupenyl cation

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(Scheme 4). Production of very minor amount of lupeol and germanicol by this mutant indicated that reaction control is somewhat leaky to produce pentacyclic skeletons. In fact, OEW Y258H mutant still retains the ability to produce lupeol. This maybe attributed to the ability of His residue to stabilize tertiary carbocation.

The structural diversity of triterpenes found in nature can be correlated to each other on the basis of cyclization mechanism. For example, dammarane, baccharane, lupane, and oleanane type triterpenes are all produced from reaction intermediates generated enroute to $\beta$-amyrin. As a result, very precise control by the enzyme should be required at each carbocationic intermediate stage in order to distinguish the specific reaction pathway from the variety of possible choices. In the present study, Tyr261 of $\beta$-amyrin synthase was shown to play a major role at dammarenyl cation stage facilitating the formation of E-ring presumably by stabilization of resulting tertiary lupenyl cation. The role of corresponding His residue in lanosterol and cycloartenol synthases is not clear, however, it is highly possible that this residue participates in cation-$\pi$ interaction with protosteryl cation as proposed by Corey et al.[14c] Trp259 of $\beta$-amyrin synthase, on the other hand, was shown to lead the reaction to $\beta$-amyrin formation. Presumably, this Trp residue stabilizes the secondary oleanyl cation right after E-ring expansion and also generates conformational strain on the intermediate. In lupeol synthase having aliphatic Leu residue at this position, the absence of this effect would terminate the reaction to produce lupeol. These results certainly opened up the possibility of generating new triterpene synthases with additional novel functions through point mutations. Such enzymes should be valuable in the future for generating diverse chemical library of triterpenes useful to search for pharmacologically important compounds. Therefore, search for other residues which govern the cyclization reaction at various intermediate stages would be necessary. As we already have cloned α-amyrin producing mixed amyrin synthase, studies toward identifying the residues responsible for α- and $\beta$-amyrin formation are now underway.

## Experimental Section

Synthesis of oligo DNA was carried out by Nihon Bioservice (Saitama, Japan). Yeast strain GIL 77 (*gal2 hem3-6 erg7 ura3-167*) was constructed by crossing GL7 (*MAT***a** *gal2 hem3-6 erg7*)[17] to INVSC2 (*MAT*α *his3-D200 ura3-167*) (Invitrogen), sporulating the resultant diploid and selecting a segregant with the desired phenotype.[29] Yeast GIL77 was maintained on YEPD medium supplemented with ergosterol (20 $\mu$g/mL) and Tween 80 (5 mg/mL) as described previously.[6] $^1$H and $^{13}$C NMR spectra were recorded on JEOL α500 spectrometer. Chloroform-*d* (99.8% atom $^2$H, ISOTEC) was used as a solvent with solvent signal $\delta$ 7.26 for $^1$H and $\delta$ 77.0 (t) for $^{13}$C as references for chemical shifts. Merck 60 $F_{254}$ (0.25 mm thickness, 20 × 20 cm) was used for silica gel TLC. All the other reagents were of reagent grade unless otherwise noted.

**Construction of Mutant Clones.** Mutant clones were constructed using PCR method. Following primers were used as an anti-sense mutation primers.

For PNY mutants:

PNY W259L: 5′-**T**ACCATCCGGCAATAACAC**A**ACATTTTAGCTGG-3′

(mutated bases in bold face, underlined is a part of silent restriction enzyme sites for *Acc*I)

PNY MW258IL: 5′-**T**ACCATCCGGCAATAACAC**A**A**A**ATTTTAGCTGG-3′

PNY C262S: 5′-CATCCGGC**T**ATAACACCACATTTTAGC-3′

PNY Y261H: 5′-**T**ACCATCCGGCAAT**G**ACACCACATTTTAGCTGG-3′

For OEW mutants:

OEW L256W: 5′-CATGTA**A**ACCAACCGACAATAACAC**C**ACATCTT-3′

(underline indicates the silent *Acc*I site)

OEW L256F: 5′-CATGTA**A**ACCAACCGACAATAACA**A**AACATCTT-3′

OEW L256Y: 5′-CATGTA**A**ACCAACCGACAATAACA**AT**ACATCTT-3′

OEW L256H: 5′-CATGTA**A**ACCAACCGACAATAACA**ATG**CATCTT-3′

OEW L256A: 5′-CATGTA**A**ACCAACCGACAATAACA**TGC**CATCTT-3′

OEW Y258H: 5′-CATGTA**A**ACCAACCGACAAT**G**ACACAACATCTT-3′

For LUP1 mutants:

LUP1 L255W: 5′-GCTATAACA**CCA**AATTTTCCC**G**GGATGTAT-3′

(underline indicates the introduced *Sma*I site)

First PCR was performed with 1 $\mu$L (1 $\mu$g) of N-terminal primer (for PNY: 5′-ATAAGGTACCATGTGGAAGCTTAAGATAGCG-3′ (*Kpn*I site underlined), for OEW: 5′-TACAAGCTTATGTGGAAGTTGAGATTGCTGAT-3′ (*Hin*dIII site underlined), and for LUP1 5′-GTACGGTACCATGTGGAAGTTGAAGATAGGA-3′ (*Kpn*I site underlined)) and 1 $\mu$L (1 $\mu$g) mutation primer with 1 $\mu$L (0.05 $\mu$g) of plasmid DNA which contains the cDNA for each clone (PNY: $pOSC_{PNY}$,[6] OEW: $pOSC_{OEW}$,[10] LUP1: $pOSC_{LUP1}$[13]) as a template. Ex Taq DNA polymerase (Takara Shuzo) was used with dNTP (0.2 mM) in a final volume of 100 $\mu$L according to the manufacturer's protocol. The reaction was carried out for 20 cycles using Gradient 40 (Stratagene) with a program (94 °C, 1 min, 58 °C, 1 min, 72 °C, 1 min, and final extension at 72 °C, 10 min). The resulting 800 bp fragment was separated on agarose gel (2%) electrophoresis and purified using a Wizard PCR Preps Kit (Promega), eluted from the column with 50 $\mu$L of water. Second PCR was carried out with 10 $\mu$L of this fragment as a sense primer, and 1 $\mu$L (1 $\mu$g) of C-terminal primer (for PNY: 5′-GATATAGCTCGAGTTAGGTGCCTAGGGACGG-3′, for OEW: 5′-TCGCTCGAGCTATGTTTGTGCATGAAGAATCCG-3′, and for LUP1: 5′-AATAAGTCTCGAGTTAATTAACGATAAACAC-3′ (*Xho*I sites underlined)) with 1 $\mu$L (0.05 $\mu$g) of plasmid DNA containing the cDNA of each clone as a template. The reaction was carried out for 20 cycles with a program (94 °C, 1 min, 58 °C, 2 min, 72 °C, 3 min, and final extension at 72 °C, 10 min). The resulting 2.3 kb band corresponding to full length cDNA fragments were digested with each restriction enzymes and ligated into yeast expression vector pYES2 (Invitrogen) digested with the same restriction enzymes. These plasmids were subcloned into *Escherichia coli* strain NovaBlue (Novagen) and purified using GFX Purification Kit (Pharmacia). For sequencing, the full length clones were subcloned into pT7Blue (Novagen) and completely sequenced on both strands using a Thermo Sequenase Cycle Sequencing Kit (Aloka).

**Functional Expression of Mutant Clones.** To analyze the function of each mutants, these plasmids were introduced into yeast strain GIL77 using the lithium acetate method[29] and plated onto synthetic complete medium without uracil (SC−U) supplemented with ergosterol (20 $\mu$g/mL), hemin (13 $\mu$g/mL), and Tween 80 (5 mg/mL) and cultured at 30 °C for selecting the desired transformants. The culture of transformant yeast, expression of protein, and isolation and purification of the products were done as described previously.[13] HPLC analysis was carried out using a SUPER-ODS column (4.6 × 200 mm) (TOSOH) with 95% $CH_3CN$ (aqueous) as a solvent (flow rate 1.0 mL/min, detection UV 202 nm) at 40 °C.

[1,2-$^{13}C_2$] Sodium acetate (90% atom $^{13}$C, MSD ISOTOPES) 85 mg was mixed with 165 mg of nonlabeled sodium acetate for incubation of yeast culture (1000 mL) during the galactose induction and resting period as described previously.[13] NMR signal assignments on lupeol and $\beta$-amyrin obtained were also carried out as previously described.[13]

**Structural Analysis of Minor Triterpenes.** Yeast GIL77 harboring PNY W259L mutant was cultured (2000 mL), expression induced with

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galactose, cells harvested and refluxed with 20% KOH/50% EtOH (aqueous), and extracted with hexane. Purification by silica gel column, with benzene as an eluent, gave triterpene products (6 mg).

**Butyrospermol (4):** $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 0.744 (3H, s, H-25), 0.804 (3H, s, H-27), 0.848 (3H, d, $J$ = 6.0 Hz, H-28), 0.854 (3H, s, H-24), 0.970 (6H, s, H-23 and H-26), 1.603 (3H, s, H-30), 1.680 (3H, s, H-29), 3.243 (1H, dd, $J$ = 11.0, 4.0 Hz, H-3), 5.093 (1H, m, H-21), 5.251 (1H, m, H-7).

$^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 13.095 (C-25), 14.716 (C-24), 17.661 (C-30), 18.138 (C-11), 18.582 (C-28), 22.069 (C-27), 23.937 (C-6), 25.352 (C-20), 25.722 (C-29), 27.309 (C-26), 27.646 (C-2), 27.696 (C-23), 28.453 (C-16), 33.816 (C-12), 33.947 (C-15), 34.934 (C-10), 35.156 (C-19), 35.782 (C-18), 37.180 (C-1), 38.957 (C-4), 43.522 (C-13), 48.910 (C-9), 50.621 (C-5), 51.221 (C-14), 53.220 (C-17), 79.254 (C-3), 117.782 (C-7), 125.128 (C-21), 130.927 (C-22), 145.889 (C-8).

**Germanicol (5):** $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 0.732 (3H, s, H-27), 0.766 (3H, s, H-24), 0.875 (3H, s, H-25), 0.935 (6H, s, H-29, 30), 0.968 (3H, s, H-23), 1.015 (3H, s, H-28), 1.073 (3H, s, H-26), 3.199 (1H, m, H-3), 4.851 (1H, s, H-19).

$^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 14.584 (C-27), 15.399 (C-24), 16.081 (C-25), 16.698 (C-26), 18.245 (C-6), 21.091 (C-11), 25.253 (C-28), 26.199 (C-12), 27.408 (C-2), 27.507 (C-15), 27.951 (C-23), 29.185 (C-30), 31.340 (C-29), 32.351 (C-20), 33.314 (C-21), 34.342 (C-17), 34.572 (C-7), 37.213 (C-10), 37.353 (C-22), 37.698 (C-16), 38.397 (C-1), 38.874 (C-4), 38.924 (C-13), 40.733 (C-8), 43.316 (C-14), 51.196 (C-9), 55.474 (C-5), 78.982 (C-3), 129.701 (C-19), 142.747 (C-18).

**Isolation of 6.** Yeast culture harboring LUP1 L255W or LUP1 (2000 mL), were cultured and harvested as described above. Silica gel column with benzene as an eluent gave triterpene monoalcohol fraction (6.4 mg). Further elution with benzene:acetone = 18:1 gave 3$\beta$,20-dihydroxylupane (**6**) (7.7 mg).

**3$\beta$,20-Dihydroxylupane (6):** $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 0.762 (3H, s, H-24), 0.808 (3H, s, H-28), 0.838 (3H, s, H-25), 0.955 (3H, s, H-27), 0.971 (3H, s, H-23), 1.057 (3H, s, H-26), 1.121 (3H, s, H-29/30), 1.224 (3H, s, H-29/30), 3.199 (1H, dd, $J$ = 11.3, 4.9 Hz, H-3).

$^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 14.831 (C-27), 15.382 (C-24), 16.147 (C-25, 26), 18.327 (C-6), 19.207 (C-28), 21.387 (C-11), 24.759 (C-29/30), 27.375 (C-2), 27.564 (C-15), 27.975 (C-23), 28.740 (C-21), 29.061 (C-12), 31.529 (C-29/30), 34.548 (C-7), 35.551 (C-16), 37.073 (C-10), 37.443 (C-13), 38.677 (C-1), 38.833 (C-4), 40.199 (C-22), 41.342 (C-8), 43.514 (C-14), 44.649 (C-17), 48.301 (C-18), 49.930 (C-19), 50.267 (C-9), 55.178 (C-5), 73.521 (C-20), 79.015 (C-3). The assignments of C-2, 12, 15, 21, 24, and 25 in ref 20 were revised as shown here based on HMBC and HMQC data.

**Isolation of 7 and 8.** From the yeast culture harboring PNY Y261H (2000 mL), ~3 mg of triterpene fraction was obtained. The main peak was separated by reverse phase HPLC using a SUPER-ODS column (4.6 × 200 mm) with the same condition as described above. The mixture of **7**, **8**, and **9** were measured for $^1$H and $^{13}$C NMR, and compared with authentic sample prepared as described below, which contains mainly **7**.

**Dammara-18(*E*),21-dien-3$\beta$-ol (7):** $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 0.775 (3H, s, H-24), 0.844 (3H, s, H-25), 0.844 (3H, s, H-27), 0.963 (3H, s, H-26), 0.979 (3H, s, H-23), 1.540 (3H, s, H-28), 1.621 (3H, brs, H-30), 1.689 (3H, brs, H-29), 2.673 (2H, m, H-20), 3.203 (1H, dd, $J$ = 11.3, 4.9 Hz, H-3), 5.085 (2H, m, H-19, 21).

$^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 12.997 (C-28), 15.374 (C-24), 15.604 (C-26), 15.900 (C-27), 16.213 (C-25), 17.693 (C-30), 18.294 (C-6), 21.411 (C-11), 24.858 (C-12), 25.705 (C-29), 27.046 (C-20), 27.317 (C-16), 27.424 (C-2), 28.025 (C-23), 31.595 (C-15), 35.444 (C-7), 37.237 (C-10), 38.981 (C-1), 39.113 (C-4), 40.478 (C-8), 44.320 (C-13), 49.230 (C-14), 50.250 (C-17), 50.982 (C-9), 55.885 (C-5), 78.966 (C-3), 123.211 (C-19), 123.639 (C-21), 131.157 (C-22), 136.891 (C-18).

**Dammara-18(*Z*),21-dien-3$\beta$-ol (8):** $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 0.775 (3H, s, H-24), 0.844 (3H, s, H-25), 0.902 (3H, s, H-27), 0.963 (3H, s, H-26), 0.979 (3H, s, H-23), 1.608 (3H, s, H-28), 1.615 (3H, bs, H-30), 1.680 (3H, bs, H-29), 2.729 (2H, m, H-20), 3.203 (1H, dd, $J$ = 11.3, 4.9 Hz, H-3), 5.048 (1H, m, H-19), 5.152 (1H, m, H-21).

$^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 15.374 (C-24), 15.604 (C-26), 15.991 (C-27), 16.213 (C-25), 17.693 (C-30), 18.294 (C-6), 19.018 (C-28), 21.461 (C-11), 24.916 (C-12), 25.722 (C-29), 26.404 (C-20), 26.552 (C-16), 27.424 (C-2), 28.025 (C-23), 31.825 (C-15), 35.477 (C-7), 37.237 (C-10), 38.981 (C-1), 39.113 (C-4), 40.520 (C-8), 44.460 (C-13), 49.346 (C-14), 50.982 (C-17), 50.982 (C-9), 55.885 (C-5), 78.966 (C-3), 123.869 (C-21), 124.832 (C-19), 130.993 (C-22), 136.965 (C-18).

**Dammara-18(28),21-dien-3$\beta$-ol (9):** $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 0.770 (3H, s, H-24), 0.842 (3H, s, H-25), 0.862 (3H, s, H-27), 0.967 (3H, s, H-26), 0.972 (3H, s, H-23), 1.611 (3H, s, H-30), 1.685 (3H, s, H-29), 3.195 (1H, dd, $J$ = 11.4, 4.8 Hz, H-3), 4.703 (1H, brs, H-28), 4.738 (1H, brs, H-28).

**Synthesis of Authentic 7 and 8.** To a solution of hydroxydammarenone I (370 mg)[23] in pyridine (50 mL), 4 mL of $POCl_3$ was added and the solution was kept at 20 °C for 3 h. The solution was added dropwise into an ice water carefully and was extracted with ether, washed with water to afford an oily product which was separated by silica gel column to give dehydrates (261 mg). Flash chromatography of the dehydrates on 20% $AgNO_3$-impregnated silica gel (eluted with benzene) gave four fractions; DM-2−1 (trace), DM-2−2 (169 mg), DM-2−3 (9 mg), and DM-2−4 (40 mg). The aliquot (64 mg) of DM-2−2 by $LiAlH_4$ was subjected to repeated preparative HPLC (ODS 8 × 250 mm, 100% $CH_3CN$) to furnish a mixture of **7** and **8** (15 mg).

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