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Stereochemical Course in Water Addition during LUP1-Catalyzed Triterpene Cyclization

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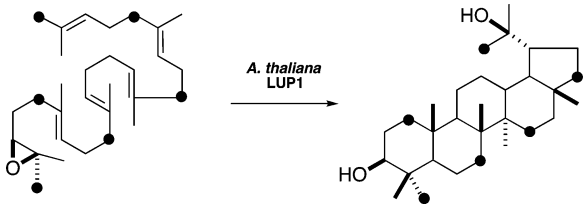
ABSTRACT



Arabidopsis thaliana **LUP1 (At1g78970) catalyzes the cyclization of oxidosqualene into lupeol and 3β,20-dihydroxylupane (lupanediol). The stereochemical course of water addition to the lupanyl cation was studied. The X-ray crystal structure of lupanylepoxide 3,5-dinitrobenzoate established the configuration of epoxide as 20*S*. $LiAlD_4$ reduction of the epoxide enabled the chemical shift assignment of prochiral methyl groups at C20 of lupanediol. Correlation of these methyl groups with biosynthetic lupanediol from [1,2-$^{13}C_2$] acetate established the stereochemical course of water addition.**

The enzymatic cyclization of 2,3-oxidosqualene into various cyclic triterpenes is one of the most fascinating and versatile chemical transformations found in nature. The responsible enzyme, oxidosqualene cyclase (OSC), catalyzes the formation of a multiring system with a number of stereogenic centers through a series of cation−π cyclizations with concomitant Wagner−Meerwein rearrangements including 1,2-methyl and hydride shifts in a single step, only part of which synthetic organic chemistry can mimic.[1] OSCs are widely distributed in nature and play important roles. Lanosterol synthase in mammals is a typical example, as it serves as a key enzyme in cholesterol biosynthesis. In the plant kingdom, nearly 100 of the unique triterpene skeletons are formed by OSCs. Their further metabolites, comprising one of the most diverse groups of natural products, exhibit a range of pharmacological activities.[2] Diverse skeletons of triterpenes suggested the presence of the corresponding number of OSC genes in plants. However, recent molecular cloning studies have revealed that many of the plant OSCs are multifunctional to produce more than one skeletal type of triterpene product.[3,4b,5] Both chimeric and site-directed mutagenesis studies on OSCs have demonstrated that only

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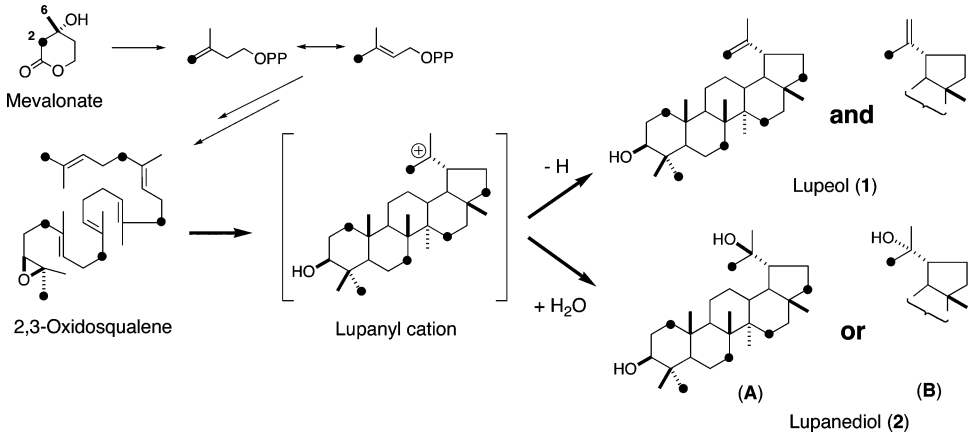

Figure 1. Cyclization of 2,3-oxidosqualene into lupeol and lupanediol by LUP1 and the fate of a carbon atom derived from C2 of mevalonate. Dots indicate the carbons that originate from C2 of mevalonate. LUP1 catalyzes sequences shown with bold arrows.

a limited number of amino acid residues are responsible for controlling their product specificity.[6,7] The major factors which determine the product outcome of cyclization are the folding conformation of the substrate and the position and mechanism of the termination. Generally, OSCs terminate the cyclization by elimination of a proton from a cationic intermediate to yield a product with a double bond. Some enzymes, however, terminate their reactions by quenching the final carbocation with a water molecule to release an alcohol product. The recently reported arabidiol synthase from *Arabidopsis thaliana*[8] and dammarenediol-II synthase from *Panax ginseng*[9] are such examples, where water addition to tri- and tetracyclic cation intermediates terminates their reactions, respectively. In this regard, LUP1 (At1g78970) from *A. thaliana*, one of the first triterpene synthases to be identified for their enzyme functions, offers a unique opportunity to explore the mechanism that controls these processes, as it produces not only lupeol, a deprotonation product, but also 3β,20-dihydroxylupane (lupanediol), a water addition product, in nearly equal amount.[4] We have been interested in how the final deprotonation or hydration of lupanyl cation is controlled by the LUP1 enzyme. To address this interesting and important issue, we took advantage of the fact that two terminal methyl groups of the lupanyl cation have different biosynthetic origins, that is either from C2 or C6 of mevalonate, according to the conventional mevalonate pathway for isoprenoid biosynthesis (Figure 1). These two carbon atoms can be easily distinguished by feeding [1,2-$^{13}C_2$] acetate to the yeast transformant expressing LUP1 and ^{13}C NMR measurements of the products, as a methyl group derived from C6 of mevalonate resonates with an accompanying doublet due to intact incorporation of acetate whereas a methyl group from C2 of mevalonate resonates as an enriched singlet. In our previous studies, LUP1 was demonstrated to eliminate a proton from both terminal methyl groups in nearly equal ratio, indicating a lack of regiospecificity in the deprotonation process.[6] In the same feeding experiment, LUP1 was shown to produce lupanediol with stereospecific addition of water to the lupanyl cation giving rise to either (**A**) or (**B**) in Figure 1.[5] Here, we report the correlation of magnetically and biosynthetically distinguishable prochiral methyl groups of lupanediol that establishes the stereochemical course of water addition during the final termination step of cyclization catalyzed by LUP1.

LUP1 was functionally expressed in *Saccharomyces cerevisiae* GIL77, a yeast mutant strain lacking lanosterol synthase.[10] As previously reported, feeding of [1,2-$^{13}C_2$] acetate to the yeast culture expressing LUP1 yielded ^{13}C-labeled lupanediol.[5] In the ^{13}C NMR spectrum (in $CDCl_3$) of this specimen (Figure 2A), a methyl signal of natural abundance resonating at 31.53 ppm was accompanied by a doublet of J = 39.6 Hz, and a methyl group resonating at 24.76 ppm appeared as an enriched singlet.[5] Accordingly, a quaternary C20 signal at 73.52 ppm was accompanied by a doublet of J = 39.6 Hz.[5] To assign the chemical shifts of the relevant prochiral methyl groups of lupanediol, we relied on the previous report that *m*-chloroperbenzoic acid (*m*-CPBA) oxidation of natural lupanyl acetate gave only one enantiomer of the C20−C29 epoxide, although the stereochemistry has not been established yet.[11] The stereochemistry of the epoxide was determined by X-ray crystallographic analysis of lupanylepoxide 3,5-dinitrobenzoate (**3**) prepared

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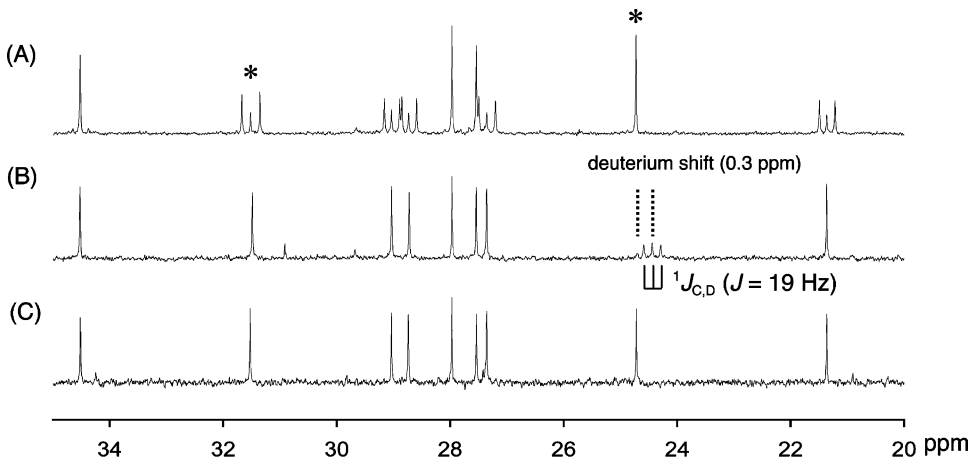

Figure 2. Partial ^{13}C NMR spectra of lupanediol from (A) feeding [1,2-$^{13}C_2$] acetate to LUP1 expressing yeast culture, (B) deuterium labeled by $LiAlD_4$ reduction of (**3**), and (C) an authentic nonlabeled sample. Asterisk indicates the signal of two terminal methyl groups of lupanediol.

from lupenyl 3,5-dinitrobenzoate. The colorless plates suitable for X-ray analysis (monoclinic, $P2_1$, a = 7.942 (5), b = 10.334 (6), c = 19.76 (1) Å, β = 92.16 (5)°, Z = 2) were prepared by crystallization from acetone.[12] The structure was solved by direct methods using SIR97[13] with an R (Rw) value of 0.069 (0.112) and established as shown in Figure 3. The bond lengths and angles are not significantly different

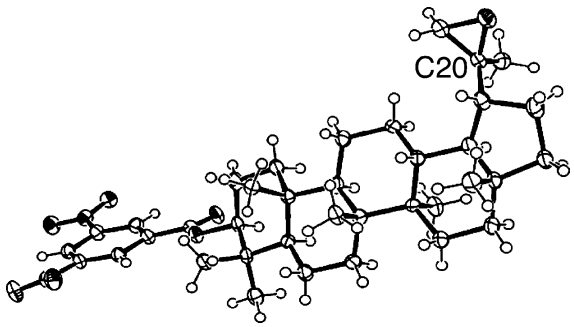

Figure 3. X-ray crystal structure of lupanylepoxide 3,5-dinitrobenzoate (**3**) (ORTEP drawing).

from the expected ones.[14] This structure confirmed that the configuration of the epoxide is 20*S*, as the absolute stereochemistry at C3 is retained from (3*S*)-2,3-oxidosqualene.

$LiAlD_4$ reduction of this epoxide gave deuterated lupanediol. The incorporation of deuterium into the methyl group resonating at a higher field (^1H, 1.121 ppm; ^{13}C: 24.76 ppm) was confirmed by ^1H NMR, ^{13}C NMR, and HMQC analysis. A deuterium shift (0.3 ppm) and direct $^{13}C-^2H$ coupling ($^1J_{C-D}$, J = 19 Hz) were observed on the ^{13}C NMR spectrum (Figure 2B). Therefore, it is now firmly established that the methyl group resonating at a higher field (24.76 ppm) is *pro-R*, whereas the one in the lower field (31.53 ppm) is *pro-S*. By comparing this spectrum with that of lupanediol produced from [1,2-$^{13}C_2$] acetate by LUP1 expressing yeast, it is now clear that the *pro-R* methyl group is derived from C2 of mevalonate, and the *pro-S* methyl group is derived from C6 of mevalonate (Figure 2).

Taken together, these results indicated that water addition to the lupanyl cation takes place stereospecifically from only one face of the plane of the isopropyl cation moiety during lupanediol formation as shown in Figure 4 to produce species

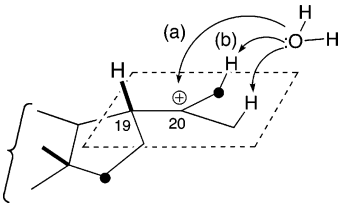

Figure 4. (a) Stereochemical course of final water addition to lupanyl cation. (b) Nonspecific deprotonation during lupeol formation. Carbons originating from C2 of mevalonate are shown with dots.

(**A**) in Figure 1. Simultaneous production of the water addition product and the deprotonation product by LUP1 implies that a water molecule in the active site may be quenching the cation to produce lupanediol and at the same time serving as a base to eliminate a proton to yield lupeol, although participation of any basic residue of the enzyme

(12) Diffraction intensities were collected from a crystal of dimensions 0.25 × 0.15 × 0.03 mm on a Rigaku RAXIS RAPID imaging plate area detector with graphite monochromated Mo Kα radiation. A total of 19 530 reflections, 4983 unique reflections, within a 2θ range of 60.1° were used in the solution and refinement of the structure. The data were collected for Lorenz and polarization effects.

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(14) Lists of atomic parameters, bond lengths, and bond angles will be deposited to the Cambridge Crystallographic Data Centre.

during the deprotonation step is equally possible. Detailed kinetic studies on the formation of both lupeol and lupanediol are necessary to clarify the detailed mechanism regarding proton abstraction by the water molecule. Lack of deprotonation specificity during lupeol formation[6] might suggest a possible rotation of the isopropyl moiety at the nascent lupanyl cation intermediate. However, the observed precise control of the direction of water access with regard to the face of the isopropyl moiety of the lupanyl cation excludes the rotation of the C19−C20 bond before water addition. Nonspecific deprotonation could be achieved by a water molecule approaching both terminal methyl groups from one face of the isopropyl moiety. Therefore, LUP1 seems to control the position of the water molecule with respect to the plane of the isopropyl cation moiety but not accurately enough to let it eliminate a proton from only one methyl group. This was in striking contrast to monofunctional lupeol synthases of OEW from olive (*Olea europaea*) and TRW from dandelion (*Taraxacum officinale*), as they eliminate a proton specifically from the methyl group derived from C6 of mevalonate.[15] These lupeol synthases are monofunctionally producing lupeol as a sole product and no lupanediol or other triterpenes. These high-fidelity enzymes might have evolved to maintain control of deprotonation specificity so as not to produce other byproducts. On the other hand, low-fidelity multiproduct LUP1 might have a different evolutionary path and has lost control in the deprotonation process. In fact, high-fidelity lupeol synthases of the OEW and TRW type form a branch in a phylogenetic tree of plant OSCs,[16] whereas LUP1 does not join this branch and situates more closely to a branch of β-amyrin synthases. One might speculate that LUP1 has evolved from β-amyrin synthase and has lost an ability to control the reaction path after the lupanyl cation stage, which accounts for the observed multiproduct nature to produce multiple lupane triterpenes.

Production of both the deprotonated olefinic product and the hydrated alcohol product by LUP1 is reminiscent of squalene hopene cyclase (SHC) from bacteria, which produces hopene and hopanol in a 5:1 ratio.[17] It has been shown that in a SHC-catalyzed reaction deprotonation takes place, in contrast to a LUP1 reaction, specifically from the (*Z*)-methyl group of the terminal isopropenyl moiety during hopene formation.[18] Water addition to the hopanyl cation is also stereospecific during hopanol formation,[18] although the stereochemistry of this water addition has not been established yet. The present study demonstrated, for the first time, the stereochemical course of the termination of triterpene cyclization with regard to water addition to produce prochiral methyl groups. These results provided a valuable insight into the active site structure of OSCs. In combination with future structural studies, we would be able to draw a precise picture of this fascinating series of enzymatic cation−π cyclization reactions and the observed product diversity.

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Supporting Information Available: Details of the synthetic procedure, ^{1}H and ^{13}C NMR data, and ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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