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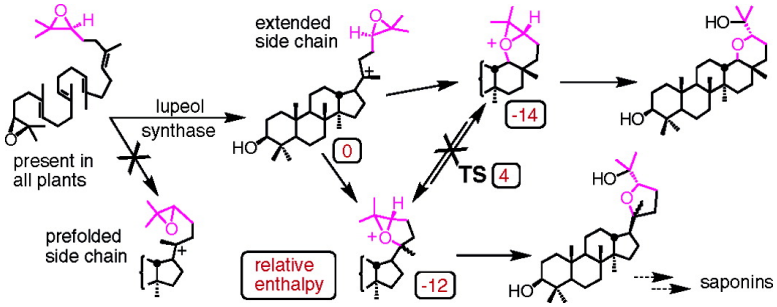

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Enzymatic Cyclization of Dioxidosqualene to Heterocyclic Triterpenes

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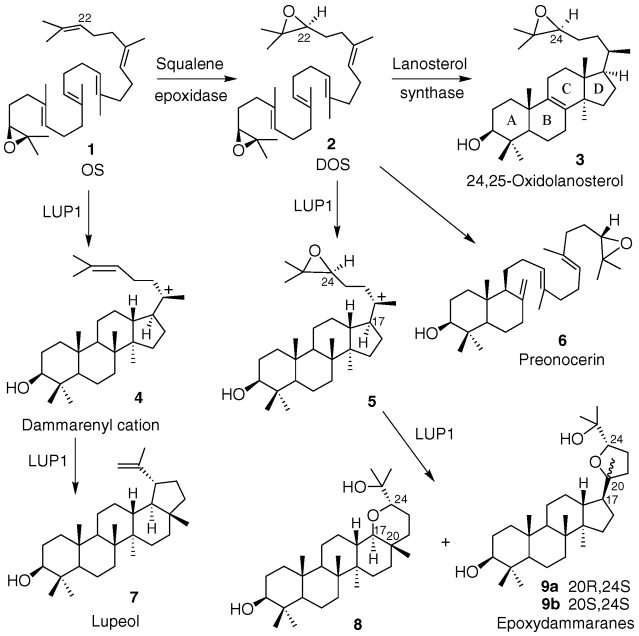
Ginseng and other medicinal plants accumulate biologically active epoxydammarane saponins[1] at high levels (up to 5% of dry weight[1c]). The aglycone precursors, such as **9b**, have been proposed to arise from tetracyclization of (3*S*)-2,3-oxidosqualene (OS, **1**) followed by P450 oxidations en route to the heterocyclic E ring.[1c] We considered that a pathway with oxidation before cyclization might be shorter and evolutionarily more accessible. Herein we demonstrate that a plant triterpene synthase (LUP1) converts (3*S*,-22*S*)-2,3:22,23-dioxidosqualene (DOS, **2**)[2] directly to oxacyclic triterpenoids **8**−**9b** in a single step (Scheme 1). The results establish new roles for DOS in triterpene biosynthesis and illuminate substrate folding during cyclization.

DOS is produced in limited amounts by further epoxidation of OS and is a known substrate of several OS cyclases.[3] These cyclases all exclude the terminal olefin of OS from cyclization and convert DOS to triterpene epoxides (cf. **3** and **6**) rather than heterocycles. In contrast, the biosynthesis of lupeol (**7**) from OS requires that the terminal olefin of the dammarenyl cation (**4**) attack the carbocation to form ring E. We reasoned that in the analogous DOS pathway the distal epoxide in **5** could attack C20 to generate heterocyclic triterpenes.[4] We tested this hypothesis by incubating the natural 3*S*,22*S* isomer of **2** (8 mg) with *Arabidopsis* lupeol synthase (LUP1)[5] expressed from plasmid JR1.16 in RXY6,[3c] a yeast squalene epoxidase/lanosterol synthase double mutant. This construct allowed cyclization of milligram amounts of substrate without further metabolism toward saponins.

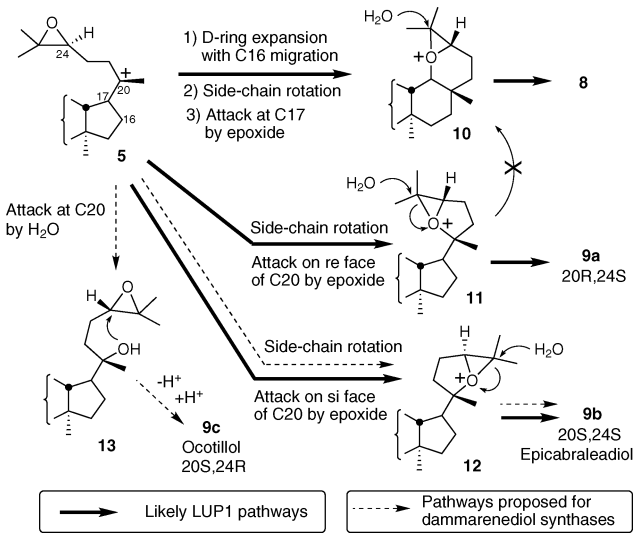
NMR analysis of the crude extract of RXY6[JR1.16] incubation products showed almost complete consumption of substrate **2** and the formation of diols **8**, **9a**, and **9b** in a 3:4:2 ratio, together with olefinic analogues.[6] Silica gel chromatography separated **8** from epoxydammaranes **9a** and **9b**, and reversed phase HPLC resolved **9a** from **9b**.[7] Structure elucidation of these products was nontrivial and required extensive 2D NMR and GC−MS analysis, as described in Supporting Information. The E ring regiochemistry was determined by analyzing the GC−MS fragmentation patterns,[8a] and the stereochemistry was deduced from NOE results[8b] and comparisons with reported spectral data.[1e,9] Our ^{1}H and ^{13}C NMR assignments clarify confusion in the epoxydammarane literature, which lacks definitive NMR data for the 20*R*,24*S* isomer **9a**.[9c] The 20*S*,24*S* isomer **9b** is the known[1e] 3-epicabraleadiol, and **8** has the unusual 17,24-epoxybaccharane skeleton.[10] Each product has 24*S* stereochemistry, derived from the 22*S*-epoxide in **2**.

Products **8**, **9a**, and **9b** are formed via oxonium ion intermediates **10**, **11**, and **12** (Scheme 2). The exothermicity of oxonium ion formation leads irreversibly from **5** to **11** or **12** if side chain rotation brings the distal epoxide oxygen into proximity of the C20 cation.[11] Migration of the positive charge from carbon to oxygen disables the usual mechanistic processes[12] of triterpene synthesis, which are guided by activation of certain bonds through hyperconjugation with the carbocation 2p orbital. DFT calculations predicted an activation

Scheme 1. Enzymatic Cyclization of OS and DOS



Scheme 2. Likely Mechanistic Pathways to **8** and **9a,b**



enthalpy of >16 kcal/mol for the conversion of **11** to **10**.[11] Thus, **8** is formed only when D ring expansion is faster than side chain folding.

The above mechanistic analysis indicates that the side chain of **5** is mobile and not prefolded for E ring formation.[13] A partially extended side chain conformation, as inferred for squalene−hopene cyclase,[14] allows the side chain to slide through the active site tunnel as the substrate contracts during tetracyclization. Assuming that the distal epoxide does not perturb enzyme−substrate interactions, the

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side chain of the dammarenyl cation (**4**) in lupeol synthesis should also have rotational mobility and a partially extended conformation.

Oxacyclic triterpenes, such as **8**−**9b**, are presumably generated by all eukaryotes having pentacyclic triterpene synthases[15] since DOS is ubiquitous as a minor byproduct of OS biosynthesis. Low physiological concentrations of **8** and **9a** are suggested by their rarity in natural product isolations and by the generally limited metabolic flux through the DOS shunt pathway. Variations in trace levels of 24,25-epoxycholesterol, a DOS metabolite, indirectly monitor enzyme activities and are used to regulate transcription in mammalian cholesterol homeostasis.[2a,e,16] Levels of diols **8** and **9a** also reflect epoxidase/cyclase activity and may similarly serve as regulators of triterpenoid synthesis.

Diol **9b** may be produced in nature at much higher levels than **8** or **9a**. Epoxydammarane and dammarenediol saponins commonly occur together and generally have a 20*S* configuration. A dammarenediol synthase could make 3*β*,20*S*-dammarenediol from OS and the 20*S*,24*S*-epoxydammarane **9b** from DOS. The active site of dammarenediol synthases, unlike that of LUP1, evidently obstructs the *re* face of C20 to exclude E ring formation, and this would block formation of **8** and **9a**. DOS cyclization might also produce 24*R*-epoxydammaranes in dammarenediol synthases, as indicated in the conversion of **5** to **9c**.[17] However, our isolation of epoxydammaranes as DOS metabolites does not preclude their origin from other pathways. For example, cycloartenol analogues of epoxydammaranes[18] must arise from post-cyclization oxidation because DOS cyclization cannot generate both cyclopropyl and heterocyclic rings. Similar oxidation by P450s and other oxidases may have evolved to become the major biosynthetic route to epoxydammaranes in many plants.[1c,19] The best current evidence for the DOS pathway in secondary metabolism is the isolation of 17,24-epoxybaccharanes[20a] and olefinic epoxydammaranes,[20b] structures that are unlikely products of P450 pathways.

To model how readily oxacycles could arise in vivo, we expressed JR1.16 in the yeast lanosterol synthase mutant SMY8. Cultures of SMY8[JR1.16] accumulated **8**, **9a**, and **9b** at a level of 2−7% of the OS products.[21] This experimental system evidently made considerable DOS available to LUP1 for oxacycle formation.[22] The crude squalene epoxidase/oxidosqualene cyclase systems that first evolved probably also generated substantial amounts of oxacycles. The oxacyclic triterpenoids produced by these unoptimized early systems may have provided aglycones for saponin synthesis until an efficient cluster of P450s evolved. This could explain how natural selection began assembling saponin synthesis, a multistep process in which the individual components lack biological activity. The use of alternative substrates[23] exemplifies one of several strategies used by plants and fungi to increase the diversity of secondary metabolites.[24] The genetic foundation for artificial and native metabolic engineering may be broader than is evident from natural products surveys and genomic analyses.

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Supporting Information Available: Complete refs 11 and 20a; details of substrate preparation, enzymatic cyclization, molecular modeling, NMR signal assignments, and GC−MS and NMR spectra of **8**, **9a**, and **9b** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) Identification of terminal olefins from NMR spectra was tentative owing to their small amounts and coelution with other substances. The favored quenching of oxonium ions **10**−**12** by attack of water rather than by deprotonation is discussed in Supporting Information.

(7) Silica gel chromatography gave **8** (1.5 mg, R_f 0.31, 1:1 MTBE/hexane) and a mixture of **9a** and **9b** (3 mg, R_f 0.37). Reversed phase HPLC was done with a mobile phase of 9:1 MeOH/H_2O.

(8) (a) GC−MS fragmentation of mono- and bis-TMS derivatives indicated hydroxyl at C25 and an ether linkage at C24. An abundant ion at *m*/*z* 383 in **8** pointed to a stable ring skeleton derived from neutral losses of TMSOH and the hydroxyisopropyl group. Epoxydammaranes **9a** and **9b** coeluted on GC and had identical mass spectra. Their base peak at *m*/*z* 143 is characteristic of epoxydammaranes,[1a] and ion *m*/*z* 383 excluded the possibility of a six-membered E ring. (b) NOE experiments indicated that the C20 methyl and H24 proton are located on the same side of the E ring plane in **9a** and on different sides in **9b**.

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(10) The lack of epimerization at C17 is discussed in Supporting Information.

(11) Side chain rotation and oxonium ion formation were modeled by DFT methods using Gaussian software: Frisch, M. J.; et al. *Gaussian 03*, revision C.02; Gaussian, Inc.: Wallingford, CT, 2003. Bonds from oxygen to C20, C24, and C25 in **11** were 1.59, 1.50, and 1.59 Å (B3LYP/6-31G* geometry). In the absence of enzymatic effects, activation enthalpies for side chain rotation or D ring expansion from **5** were ca. 5 versus 16 kcal/mol for D ring expansion from **11**; see Supporting Information.

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(21) (a) Details are given in Supporting Information. Unlike RXY6[JR1.16], SMY8[JR1.16] contains squalene epoxidase and thus produces OS. (b) Traces of **8** can be seen in the published NMR spectrum of lupanediol.[5b]

(22) Possible factors affecting the relative levels of DOS and OS and the availability of DOS to cyclases are discussed in Supporting Information.

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