

Enzymatic Cyclization of Dioxidosqualene to Heterocyclic Triterpenes

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Ginseng and other medicinal plants accumulate biologically active epoxydammarane saponins¹ 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 3S,22S 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 1H and 13C NMR assignments clarify confusion in the epoxydammarane literature, which lacks definitive NMR data for the 20R,24S isomer 9a.9c The 20S,24S isomer **9b** is the known^{1e} 3-epicabraleadiol, and **8** has the unusual 17,24-epoxybaccharane skeleton.¹⁰ Each product has 24S stereochemistry, derived from the 22S-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 processes¹² 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

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 synthases¹⁵ 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 20S configuration. A dammarenediol synthase could make 3β ,20S-dammarenediol from OS and the 20S,24S-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 24R-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 epoxydammaranes¹⁸ 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 substrates²³ 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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- (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/H₂O.
- (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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- 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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