ORGANIC LETTERS

2008
Vol. 10, No. 21
5071−5074

Biosynthesis of Baccharis Oxide, a Triterpene with a 3,10-Oxide Bridge in the A-Ring

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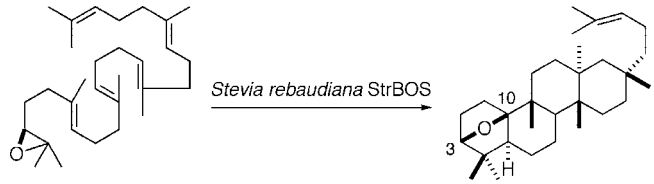
Received September 5, 2008

ABSTRACT



oxidosqualene (**1**) baccharis oxide (**2**)

A new oxidosqualene cyclase (OSC) cDNA was cloned from the roots of *Stevia rebaudiana*. Functional expression in yeast and spectral analyses of the products established that the obtained OSC yields baccharis oxide as the major product. This is the first identification of an OSC yielding baccharis oxide. This result clearly demonstrated that baccharis oxide, a baccharane triterpene with a unique 3,10-oxide bridge in the A-ring, is a direct product from oxidosqualene by an OSC.

Oxidosqualene cyclases (OSCs) build up mono- to pentacyclic triterpene skeletons from a linear substrate, oxidosqualene (**1**). Protonation on an epoxide of **1** first generates a carbocation at C-2 and subsequent cation−olefin cyclizations with or without 1,2-hydride and methyl group shifts, and C−C bond migrations lead to various cyclic carbocation intermediates. Stable neutral products are released either by deprotonation or water addition (Figure 1). For more than 50 years since the general mechanism for triterpene biosynthesis was proposed as the historic biogenetic isoprene rule,[1] extensive studies have been conducted by many research groups to substantiate the mechanism of squalene and oxidosqualene cyclizations.[2] Molecular cloning and heterologous expression of enzymes in the past decade facilitated characterization of more than 30 OSCs with distinct product specificities.[3] Recent cloning of marneral synthase[4] and *seco*-amyrin synthase[5] from *Arabidopsis thaliana* has revealed that OSCs catalyze not only multiring formation but also cleavage of preformed ring systems. In marneral (**11**) formation (Figure 1b), a C−C bond between C-3 and C-4 of the bicyclic intermediate (**4**) is cleaved by Grob fragmentation providing an A-ring-*seco* structure. *seco*-Amyrin synthase cleaves C-8 and C-14 bond of the pentacyclic cation intermediate (**8**) giving a unique C-ring-*seco* triterpene (**16**) (Figure 1d).

Isolation of a *seco*-amyrin (**17**), a regioisomer of **16**, from the roots of *Stevia viscida* and *S. eupatoria*[6] prompted us to try molecular cloning of a new OSC yielding **17** (Figure 1e). Due to the limited availability of these species, *S. rebaudiana*, a more popular and well-known producer of sweet diterpene glycosides, served as the source plant in this study, even though β-amyrin (**15**) from the leaves[7] is the only triterpene constituent reported from this species.

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10.1021/ol802072y CCC: $40.75
Published on Web 10/14/2008

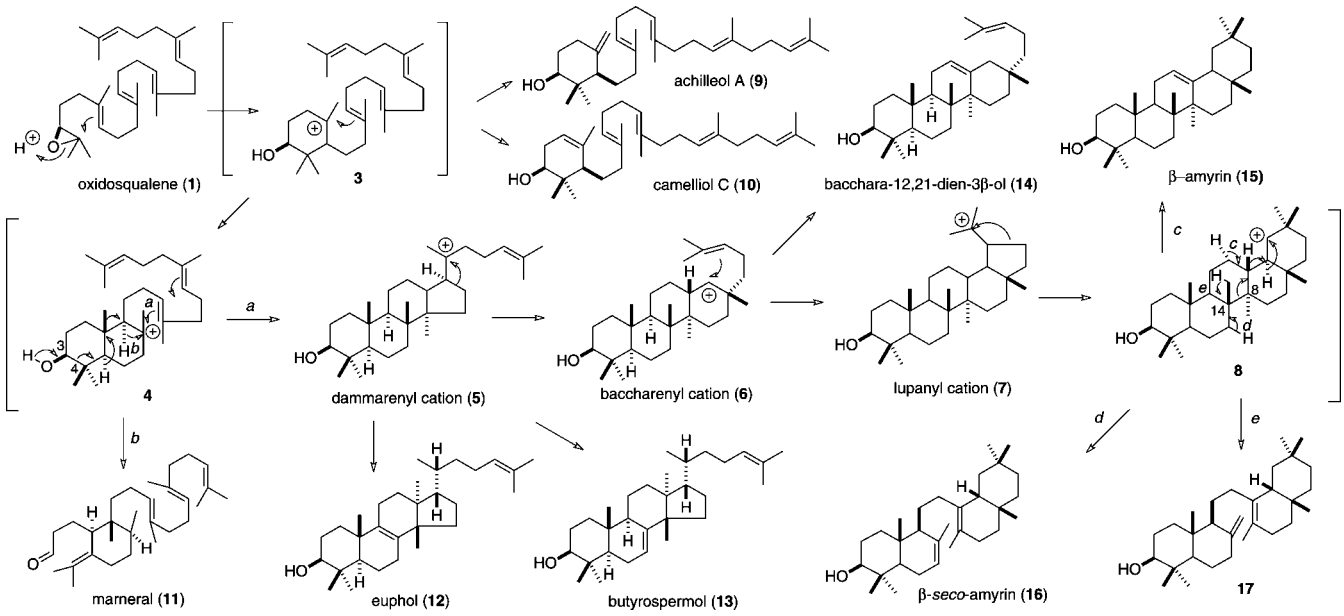

Figure 1. Cyclization and rearrangement of oxidosqualene (**1**) into a variety of cyclic triterpenes.

Homology-based PCR provided a cDNA possibly encoding a new OSC. This cDNA named *Str-3* consists of a 2286 bp length ORF encoding a 762 amino acid long polypeptide and exhibits 65% sequence identity to that of a mixed amyrin synthase PSM from *Pisum sativum*.[8] An ORF of *Str-3* was ligated to the multicloning sites of a yeast expression vector pYES2. With the resultant plasmid, a yeast mutant GIL77 lacking lanosterol synthase[9] was transformed. The hexane extracts of the transformant cells were first analyzed by silica gel TLC. A major spot (**a**) and two minor spots (**b**, **c**) were observed (Figure 2), indicating that *Str-3* is a multifunctional OSC yielding more than three products. A large R_f value of the major product (spot **a)** on normal-phase silica gel TLC showed that this product is less polar than normal triterpene monoalcohols implying the absence of free hydroxyl groups in its structure. From 2 L culture of the transformant, the major product corresponding to spot **a** (10.2 mg) was isolated by silica gel column chromatography followed by recrystallization.

GC/MS analysis of the product showed a single peak in GC and a molecular ion peak at *m*/*z* 426 in MS revealing the net reaction to be isomerization. 1D and 2D NMR analyses indicated the presence of one trisubstituted double bond (125.2 and 130.8 ppm) at the side chain terminal and one each of tertiary (84.3 ppm) and quaternary (93.8 ppm) oxygen-bearing carbons. From these data, the product should have a tetracyclic carbon skeleton with an ether bond. Further detailed HMQC, HMBC analyses (Figure S1, Supporting Information) gave a possible structure of this product. As

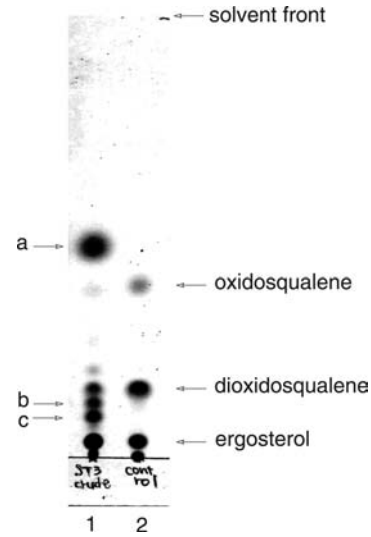

Figure 2. TLC analysis of Str-3 products. Transformant yeast cells were extracted with ethyl acetate after alkaline lysis. TLC plate was developed with the solvent (hexane/ethyl acetate = 93:7), and spots were visualized by heating on hot plate after being sprayed with 10% sulfuric acid. Lane 1 represents the one with the plasmid harboring *Str-3* cDNA and lane 2 with the plasmid without *Str-3* cDNA.

^{1}H and ^{13}C NMR data are completely identical to those reported for baccharis oxide (Figure S1, Supporting Information)[10] and all correlations in HMQC and HMBC are consistent with its structure, the major product was identified as baccharis oxide (**2**) (Figure 3). Two fractions corresponding to spot **b** (1.5 mg) and spot **c** (1.8 mg) on TLC were obtained by silica gel column chromatography. Although they were not separated further, ^{1}H NMR data of spot **b** (e.g.,

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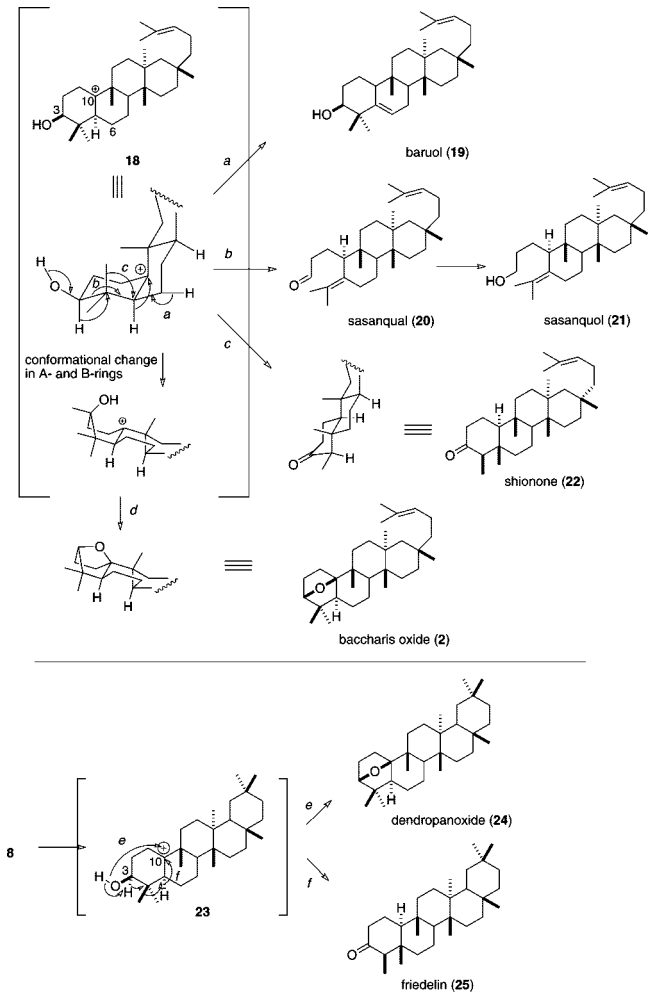

Figure 3. Formation of baruol (**19**), sasanqual (**20**), baccharis oxide (**2**), shionone (**22**), dendropanoxide (**24**), and friedelin (**25**) from the intermediate cations **18** and **23**. Sasanquol (**21**) is a reduction product of sasanqual (**20**) by yeast reductase.

chemical shifts of methyl groups, methine protons at C-3 and olefinic protons) suggested the presence of achilleol A (**9**), camelliol C (**10**), and baruol (**19**) in this mixture. GC/MS analysis of spot **b** indeed showed three peaks (Figure S4, E, Supporting Information) that had identical retention times and MS fragmentations to those of the authentic specimens of the suggested products (Figure S5, J−O, Supporting Information). A similar GC/MS analysis of the fraction corresponding to spot **c** showed seven peaks (Figure S4D, Supporting Information), and four of them were identified as sasanquol (**21**), euphol (**12**), butyrospermol (**13**), and β-amyrin (**15**) in the same manner as in spot **b** (Figure S5, B−I, Supporting Information). From these analyses, the obtained cDNA was characterized to encode an OSC yielding baccharis oxide as the major product and thus named baccharis oxide synthase (StrBOS).

Baccharis oxide (**2**) was first reported from *Baccharis halimifolia* L. in 1970,[11] and its structure was later revised and established by X-ray crystallography to have a 3,10-oxide bridge in A-ring.[12] Recent studies have revealed its macrophage activation activity[13] and trypanocidal activity,[14] thus attracting considerable interests of pharmacologists. The structure of baccharis oxide with 3,10-oxide bridge in A-ring is quite unique among over 100 natural triterpene skeletons.[15] To the best of our knowledge, dendropanoxide (**23**) (synonym; epoxyglutinane, campanulin) reported from *Dendropanax* and *Rhododendron* species[16] is the only natural triterpene with 3,10-oxide bridge besides baccharis oxide. Quenching of C-10 carbocations (**18**, **23**) with hydroxyl group at C-3 during the reactions catalyzed by OSCs has been suggested for formation of their 3,10-oxide bridge (Figure 3c,e).[15]

Tetracyclic baccharenyl cation (**6**) is formed from dammarenyl cation (**5**) by ring expansion and usually generates pentacycles by an additional cyclization and rearrangements (Figure 1). Although β-amyrin having pentacyclic oleanane skeleton is included in the products, successive 1,2-shifts leading to **18** without fifth ring formation are favored by this OSC. Triterpenes with 6−6−6−6 baccharane ring system are rather rare as natural products. Bacchara-12,21-dien-3β-ol (**14**) from *Glycine max* is a deprotonation product of carbocation at C-13 on the way from **6** to **18**.[17] A key branching point of StrBOS reaction is at the C-10 cation (**18**) stage, as three of the products derive from this intermediate (Figure 3). Baruol (**19**), a minor byproduct of StrBOS, is formed from **18** with an additional hydride shift from C-5 followed by elimination of a proton from C-6 (Figure 3a). Sasanquol (**21**), another byproduct, derives from sasanqual (**20**) in yeast by yeast reductase. Compound **20** itself is formed from **18** by the same hydride shift from C-5 followed by Grob fragmentation of A-ring (Figure 3b). An intramolecular attack of 3β-hydroxyl group onto C-10 carbocation with concomitant *chair* to *boat* conformational change in A- and B-rings of **18** yields baccharis oxide (**2**), the major product of StrBOS (Figure 3d). As baccharis oxide shares more than 75% of the total products, StrBOS must be exerting a unique control in favor of intramolecular 1,4-oxide brigde formation.

The DCTAE motif[18] of OSCs (Figure 4) is believed to form deep bottom of their active site pockets to accommodate the *pre*-A ring part of the substrate. The conserved aspartic acid in this motif opens the 2,3-epoxide by protonation to

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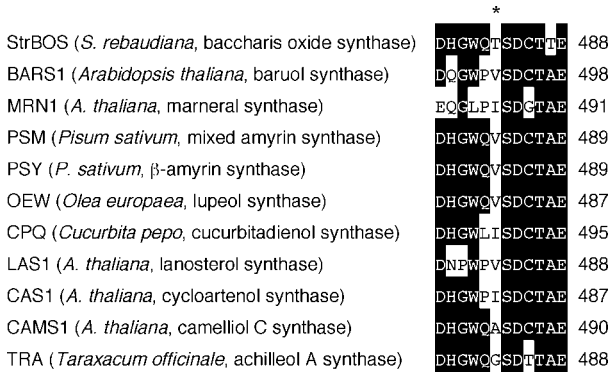

Figure 4. Alignment of amino acid sequences of OSCs around the DCTAE motif. An asterisk (*) designates the position two residues upstream from the DCTAE motif, whose steric bulk is suggested to affect the multiring construction of lanosterol synthase and cycloartenol synthase.

generate C-2 cation for following cyclizations. At two residues upstream from this motif are generally found bulky aliphatic amino acids, valine or isoleucine. The steric bulk at this position has been shown to affect the multiring construction of lanosterol synthase and cycloartenol synthase.[19] In synthases for monocycles, namely camelliol C synthase from *A. thaliana*[20] and achilleol A synthase from *Taraxacum officinale* (our unpublished data), less bulky alanine and glycine are substituted at this position, respectively. The corresponding position of StrBOS is uniquely substituted by hydrophilic threonine. It is tempting to speculate that this threonine is involved in flipping A-ring up and directing a hydroxyl group at C-3 to form an oxide bridge toward C-10 cation. In conclusion, cloning and functional expression of StrBOS in this study clearly demonstrated that baccharis oxide, a triterpene with a unique 3,10-oxide bridge in the A-ring, is a direct product from oxidosqualene by an OSC. In other words, StrBOS catalyzes an intramolecular ether linkage formation by quenching a carbocation with a hydroxyl group at C-3 in the terminating reaction. Although not identified in the StrBOS products, shionone (**22**)[21] must derive from the same carbocation (**18**) as a direct OSC product with additional 1,2-migrations of a β-methyl group from C-4 and an α-hydride from C-3 leaving a ketone at C-3 (Figure 3c). A similar sequence of rearrangements and termination is found in pentacyclic friedelin (**25**)[22] formation (Figure 3f). Cloning of OSCs yielding these 3-keto products is underway in this laboratory, since variety in the termination mechanism would greatly contribute for future generation of diverse unnatural triterpene skeletons by engineered OSCs.

Acknowledgment. We thank Tokiwa Phytochemical Co., Ltd. for a kind gift of *S. rebaudiana* plant. A part of this research was financially supported by grants to M.S. from the Takeda Science Foundation and The Cosmetology Research Foundation and a Grant-in-Aid for Scientific Research (A) (No.20241049) to Y.E. from Japan Society for the Promotion of Science.

Supporting Information Available: Experimental procedures, NMR and GC/MS spectra, and phylogetetic tree. This material is available free of charge via the Internet at http://pubs.acs.org.

OL802072Y

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