DFT Study on the Biosynthesis of Verrucosane Diterpenoids and Mangicol Sesterterpenoids: Involvement of Secondary-Carbocation-Free Reaction Cascades

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ABSTRACT: Some experimental observations indicate that a sequential formation of secondary (2°) carbocations might be involved in some biosynthetic pathways, including those of verrucosane-type diterpenoids and mangicol-type sesterterpenoids, but it remains controversial whether or not such 2° cations are viable intermediates. Here, we performed comprehensive density functional theory calculations of these biosynthetic pathways. The results do not support previously proposed pathways/mechanisms: in particular, we find that none of the putative 2° carbocation intermediates is involved in either of the biosynthetic pathways. In verrucosane biosynthesis, the proposed 2° carbocations (II and IV) in the early stage are bypassed by the formation of the adjacent 3° carbocations and by unusual skeletal rearrangement reactions, and in the later stage, the putative 2° carbocation intermediates (VI, VII, and VIII) are not present as the proposed forms but as nonclassical structures between homoallyl and cyclopropylcarbinyl cations. In the mangicol biosynthesis, one of the two proposed 2° carbocations (X) is bypassed by a C–C bond-breaking reaction to generate a 3° carbocation with a C=C bond, while the other (XI) is bypassed by a strong hyperconjugative interaction leading to a nonclassical carbocation. We propose new biosynthetic pathways/mechanisms for the verrucosane-type diterpenoids and mangicol-type sesterterpenoids. These pathways are in good agreement with the findings of previous biosynthetic studies, including isotope-labeling experiments and byproducts analysis, and moreover can account for the biosynthesis of related terpenes.

KEYWORDS: terpene, biosynthesis, carbocation cascade, DFT study, nonclassical structure

INTRODUCTION

Terpenes/terpenoids are fascinating compounds due to their complicated and diverse structures and wide range of bioactivities. They are biosynthesized from simple unsaturated hydrocarbons via successive carbocation-mediated reactions triggered by terpene cyclases. A detailed knowledge of their biosynthetic mechanisms would be very helpful for understanding the evolution of particular biosynthetic pathways as well as for designing new biosynthetic routes for complex functional molecules. However, the mechanistic issues are extremely difficult to resolve fully by means of experimental studies alone, since terpene cyclization is a domino-type reaction occurring inside a single enzyme (“black box”). We recently established a powerful combination of quantum-chemical calculations with the artificial force induced reaction (AFIR) method2,3 to unveil complicated biosynthetic pathways/mechanisms, such as those leading to trichobrasilenol, quinuclatene,5 and cyclooctatin.6,7 In general, experimental and theoretical studies indicate that a terpene biosynthesis involves a sophisticated exothermic cascade of reactions, in which carbocation intermediates are converted into more stable intermediates such as allyl cations, tertiary (3°) carbocations, and cycloalkylcarbinyl cations. On the basis of experimental observations, it has been proposed that secondary (2°) carbocation intermediates play a role in some cases, such as in the biosynthesis of verrucosane-type diterpenoids and mangicol-type sesterterpenoids, but it remains controversial whether such 2° cations are viable intermediates. The stability of carbocations is one of the most important concepts in organic chemistry, and their stability relationships are fundamental to understanding many aspects of the reactivity of organic molecules/intermediates. For example, experimental studies show that sec-Bu+ is 14.5 kcal mol⁻¹ more unstable than tert-Bu+. Therefore, it is of considerable interest to verify the putative involvement of 2° carbocations in the biosynthesis of natural products.

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**Verrucosane-type Diterpenoids**

Verrucosan-2β-ol (ver) and neoverrucosan-5β-ol (neo), featuring a unique 3,6,6,5-tetracyclic system, are diterpenoids isolated from *Chloroflexus aurantiacus*, a filamentous, gliding, thermophilic phototrophic bacterium.\(^{12}\) Rieder et al. extensively investigated the biosynthetic pathway of verrucosan-2β-ol by means of an *in vivo* incorporation of singly or doubly \(^{13}\)C-labeled acetate, and they proposed a biosynthetic pathway involving the formation of many secondary carbocations (II, IV, VI, VII, and VIII) (Figure 1).\(^{13,14}\) Another important issue is to identify the bifurcation mechanism leading to the ver and neo skeletons.\(^{15}\) At the bifurcation point, a unique C=C bond rearrangement via a cyclopropylcarbinyl cation was proposed. A similar skeletal rearrangement is seen in the cyclooctatin biosynthesis.\(^{6,7}\) However, the key cyclopropylcarbinyl/homoallyl cation intermediates in cyclooctatin biosynthesis are categorized as 3° carbocations, whereas those in the proposed verrucosane-type biosynthesis are all 2° cations (VI, VII, and VIII).

**Mangicol-type Sesterterpenoids**

Mangicols are a family of marine fungal sesterterpenoids with a unique 6,5,5-spirotricyclic skeleton (Figure 2A),\(^{16,17}\) isolated from *Fusarium heterosporum*, and they show weak cytotoxicity toward various cancer cell lines. On the basis of experimental studies, the biosynthetic pathway shown in Figure 2B was proposed,\(^{16,17}\) involving the successive formation of two 2° carbocations (X and XI).

We began our study by evaluating the proposed pathways of verrucosane and mangicol biosynthesis with density functional theory (DFT) calculations. Since the results did not support the involvement of the proposed 2° carbocations, we then comprehensively explored the biosynthetic pathways by means of DFT calculations combined with the AFIR method. On the basis of these findings, we propose new routes that are in good agreement with previous experimental biosynthetic studies and also provide plausible pathways for the formation of other related terpenes/terphenenoids.

**RESULTS AND DISCUSSION**

**Verrucosane-type Biosynthesis**

We first discuss the biosynthetic pathway of verrucosane-type diterpenoids. A systematic search of reaction pathways showed that the reaction IM0 → IM7 depicted in Figure 3A is the most favorable pathway. The dissociation of pyrophosphate followed by an E/Z isomerization\(^ {0,31}\) initiates the exothermic carboxylation cascade of verrucosane biosynthesis and yields an allylic carboxylation (IM0). Then, the C3−C4 single bond rotates with a small activation barrier of 4.9 kcal mol\(^ {−1}\) so that the C1 carbon can interact directly with the C6−C7 π bond to afford the 3° carboxylation intermediate (IM1). As can be seen from the elongated C1−C6 bond distance (1.63 Å) as well as the natural population analysis (NPA) charges on C3 (+0.04) and C11 (+0.04), the 3° carboxylation (+0.48) at the C7 position is partially stabilized by the C1−C6 σ bond and the distal C2−C3/C10−C11 π bonds. Thus, the monocyclic six-membered 3° cation IM1 undergoes a smooth skeletal rearrangement to afford 7,5-bicyclic 3° cation (IM2) in a single step with a large stabilization energy. A close examination of the IRC calculation results revealed that this step is actually concerted but involves two asynchronous events, namely, (1) a reverse (3° → 2° cation) Wagner–Meerwein rearrangement (ring expansion to give the 7,5-membered ring) and (2) an annulation to yield the 7,5-bicyclic system. Notably, the experimentally supported...

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**COMPUTATIONAL METHODS**

All calculations were performed with GRRM11\(^ {2,18–21}\) based on the Gaussian 16 program.\(^ {22}\) Structure optimizations were performed at the M06-2X level in the gas phase using the 6-31+G(d,p) basis set.\(^ {23}\) The vibrational frequencies were computed at the same level to check whether each optimized structure is an energy minimum (no imaginary frequency) or a transition state (single imaginary frequency). Intrinsic reaction coordinates (IRC) calculations\(^ {24–27}\) were performed to track minimum energy paths from transition structures to the corresponding local minima. Single-point energies were calculated at the mPW1PW91/6-31+G(d,p) level\(^ {28,29}\) based on the structures optimized by the M06-2X method. The Gibbs free energy used for discussion in this study was calculated by adding the gas-phase Gibbs free energy correction.

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**Figure 1.** Proposed biosynthetic pathways of verrucosan-2β-ol and neoverrucosan-5β-ol.
monocyclic seven-membered secondary carbocation structure (Figure 1, II) is not a minimum on the potential energy surface (PES). A conformational change of IM2 with an activation barrier of only 1.0 kcal mol\(^{-1}\) yields IM3, in which the 3° cation center is further stabilized by a cation-\(\sigma\) bond interaction assisted by the distal C14--C15 \(\pi\) bond, leading to the second skeletal rearrangement to afford 7,6,5-tricyclic 3° carbocation intermediate (IM4). Again, the experimentally supported 7,6-bicyclic 2° carbocation structure (Figure 1, IV) is not located. Thus, the proposed intermediary 2° carbocations in the early stage of the biosynthesis are bypassed by the formation of the adjacent 3° carbocations together with skeletal rearrangement via a kind of nonclassical carbocation interaction with a through-space participation of the C--C \(\sigma\) bond and the distal \(\pi\) bond. Note that, in these skeletal rearrangement processes (IM1 → IM2/IM3 → IM4), a direct attack of the corresponding (C10--C11/C14--C15) \(\pi\) electrons on the 3° carbocation centers (at C7/C11) does not occur due to the destabilization associated with a four-membered ring formation. From IM4, a 1,5-H shift takes place with a reasonable activation energy of 6.7 kcal mol\(^{-1}\) to migrate the isopropyl 3° cation to a homoallyl cation in a seven-membered ring (IM5) with a slight endothermicity. As judged from the NPA charge distributions and the bond lengths, we consider IM5 to be an intermediate structure between the homoallyl cation and the corresponding cyclopropylcarbinyl cation. The cyclopropylcarbinyl cation has highly distorted cyclopropane C--C \(\sigma\) bonds that can effectively stabilize the adjacent carbocation. Indeed, it has been reported that homoallyl/cyclopropylcarbinyl 3° cations play pivotal roles as stable intermediates in various stages of brasiline-type sesquiterpene\(^{3}\) and cyclooctatin-type diterpene biosynthesis.\(^{5,7}\) It is noteworthy that these were all 3° cations, whereas IM5 is a more unstable 2° cation, presumably reflecting the nonclassical intermediate (equilibrium) structure. Finally, an unusual C--C bond rearrangement takes place via a highly distorted bicyclobutonium 3° cation\(^{32,33}\) (IM6) with very low activation energy to give another homoallyl-cyclopropylcarbinyl intermediate structure (IM7). IM7 is slightly more stable than IM5 by 3.7 kcal mol\(^{-1}\), probably owing to hyper-conjugation from neighboring moieties. The energy diagram (Figure 3B) immediately suggests that this is a thermodynamically and kinetically favorable biosynthetic reaction cascade: (1) the activation barriers are all low enough for the reactions to proceed smoothly at ambient temperature, (2) the overall energy profile descends as the reactions proceed, and (3) the overall exothermicity is very large (ca. 40 kcal mol\(^{-1}\)). Any of the three skeletal rearrangements (IM1 → TS_1→2/IM3 → TS_3→4/IM5 → TS_5→6) could be the rate-determining step.

**Mangicol-type Biosynthesis**

Next, we discuss in detail the biosynthesis of mangicol-type sesterterpenoids. Only a few C20/C25 terpenoids based on 5,11-bicyclic systems, such as ophiobolin,\(^{34,35}\) cotylenin A,\(^{36}\) and fusicoecdane,\(^{5,39}\) have been reported in contrast to those derived from 5,15-systems, such as quiannulatene,\(^{5,40}\) sesterfisherol\(^{39,41}\) (+)-astellatene,\(^{42,43}\) arathanadiene derivatives, sesternobaraenes,\(^{44}\) and aspergiliones.\(^{45}\) In the present study, IM8 was adopted as the simplest chemical model (Figure 4).\(^{36,40−49}\) The reaction pathway for the conversion of IM8 into IM11 is shown in Figure 4A, together with the relative energies with respect to IM8. The generation of allyl cation IM8, upon the loss of pyrophosphate of SM, triggers the carbocation cyclization cascade and affords a 5,11-bicyclic intermediate IM9a, which is a 3° carbocation intermediate, as
generally found in terpene biosynthesis. After the conformational change of IM9a to IM9b with an activation barrier of only 0.2 kcal mol\(^{-1}\), an unusual C–C bond cleavage to afford the monocyclic 15-membered 3° carbocation IM10a proceeds in a single step from the 5/11 bicyclic structure via TS\(_{9b-10a}\) with an activation energy of 11.3 kcal mol\(^{-1}\). Notably, the experimentally supported 6,11-bicyclic 2° carbocation structure (X) is not a minimum on the PES.\(^{16,17}\) We anticipated that the present skeletal rearrangement would involve two asynchronous events, namely, (1) the 1,2-alkyl shift (ring expansion to give 6,11-bicyclic skeleton) and (2) the unusual C–C bond cleavage. Indeed, we found the corresponding peaks (shoulder\(_{9b-10a-1}\) and shoulder\(_{9b-10a-2}\), respectively) located before and after the main peak (TS\(_{9b-10a}\)) upon a close examination of the IRC analysis results (Figure 4B). As judged from the elongated C10–C11 (1.65 Å) and C13–C15 (1.65 Å) bond distances as well as the near linearity of the C10–C14–C15 moiety (dihedral angles of empty orbital of C14 with C10–C11 and C13–C15: 165.8° and 176.5°, respectively), we consider that shoulder\(_{9b-10a-1}\) is close to the putative 2° carbocation structure in which the carbocation at C14 is partially stabilized by hyperconjugative interactions with the C10–C11 and C13–C15 σ bonds. If the C13–C15 bond were broken, a primary (1°) carbocation would be generated at C13, which would be very unstable.\(^{50,51}\) Hence, the C10–C11 bond is elongated (while the C13–C15 bond is shortened) to provide TS\(_{9b-10a}\), leading to the formation of a stable 3° carbocation at C11 (IM10). Note that the distance between the C10–C14 double bond and 3° cation center C11 in IM10a is more than 3 Å, and there are NPA charges at C10 (−0.18) and C14 (−0.25), suggesting little or no interaction between these locations. IM10a has a homoallylic 3° carbocation moiety. Thus, IM10a is converted via TS\(_{10a-10b}\) to a more stable cyclopropylcarbinyl 3° cation IM10b, of a type that is often observed in various stages of brasilane-type sesquiterpene,\(^4\) cyclooctatin-type diterpene,\(^6,7\) ...
and isoafarkanol biosynthesis. Thus, IM10 has three equilibrium structures within ca. 4 kcal mol$^{-1}$, and interconversion (conformational change) between homoallyl carbocations IM10a and IM10c proceeds via via cyclopentane ring cation IM10b. Then, a cation-mediated transannular cyclization takes place from the reactive conformer IM10c to form the 5,12-bicyclic system IM11 with a very low activation energy (2.6 kcal mol$^{-1}$). Intermediate IM11 has an elongated C14−C15 single bond (1.66 Å) and C10−C14 double bond (1.41 Å), reflecting the partial breakdown of the C−C σ bond (1.54 Å) and π bond (1.34 Å), respectively. This suggests a nonclassical structure between the monocyclic 3° cation and bicyclic humulyl 2° cations, leading to the following multiple annulations (vide infra). A similar reaction is also found in variadiene biosynthesis.

We next located the later stage of mangicol biosynthesis, that is, the formation of the spirocyclic structure (Figure 5). Route A is very similar to the previously proposed pathway, in which the annulation of IM11 takes place to give 5,9,5-tricyclic intermediate IM12 having B- and D-rings with a slight endothermicity, followed by 1,2-H shift via TS 12−13 with a large stabilization energy to give the bridgehead 3° carbocation IM13. From IM13, a smooth annulation involving conformational change of the nine-membered ring and 1,2-H shift proceeds to construct the 6,5,5-spirotricyclic skeleton IM14a in a single step with a large exothermicity. Then, IM14a is subjected to deprotonation to give the mangicol core skeleton (PD). The energy profile is consistent with the previously proposed pathway (Figure 2B), with reasonable activation barriers (all low enough for the reactions to proceed smoothly at ambient temperature) and with an overall large exothermicity (over 35 kcal mol$^{-1}$). However, another unprecedented route, Route B (via 5,6,5,5-tetracyclic intermediate IM17 having A- and D-rings), which was located by application of the AFIR method for comprehensive searching of reaction paths, turned out to be the most favorable (Figure 5B). As supported by the NPA charge distribution of IM11, where C10 (+0.26) and C3 (+0.04) are more positive than the...
C6 and C7 carbons, cation-stitching multiple annulation initiates this pathway with a very low activation energy (3.8 kcal mol\(^{-1}\)) to form the 5,5,7,4-tetracyclic intermediate IM15a with a large exothermicity (22.2 kcal mol\(^{-1}\)), in which the highly distorted cyclobutane C3\(\text{--}\)C6 bond effectively stabilizes the adjacent C7 carbocation. Thus, after the interconversion from IM15a to IM15c, a 1,2-H shift takes place smoothly to give a 5,5,9-tricyclic 3° cation (IM16) stabilized by a cation-\(\pi\) interaction with the newly generated C6\(\text{--}\)C7 double bond. This is followed by another smooth cation-mediated annulation to give the 5,5,5,5-tetracyclic skeleton IM17, which undergoes a 1,2-H shift via TS_17\(\text{--}\)18a to give IM18a. Then a C–C bond rearrangement takes place to yield the 5,6,5,5-tetraspirocyclic mangicol core skeleton (IM14b), which is subjected to a deprotonation to form PD.

Notably, this new route is not only kinetically and thermodynamically the most favorable pathway but also appears to provide a versatile biosynthetic pathway leading to the formation of related terpenes/terpenoids, including tsukubadiene, variediene, deoxyconidiogenol/conidiogenone.
none,59,60 and homopompe/ methyl homopompeenate,61,62 (Figure S5B,C). A careful structural comparison of the terpene cyclases responsible for the formation of mangicol, tsukubadiene, variedi diene, deoxyconidiogenol, and homopompe could be helpful to clarify how the structural diversification is controlled.

■ CONCLUSION

In conclusion, the current computational study has uncovered in detail the biosynthetic pathways of the verrucosane-type diterpenoids and mangicol-type sesquiterpenoids as well as provided new insight into the mechanisms of structure diversification in terpene biosynthesis, especially the exquisite skeletal construction processes and conformational changes (the Cartesian coordinates of the three-dimensional structures of all species are given in the Supporting Information).

Remarkably, we found that none of the previously proposed 2° carbocation intermediates was obtained as a minimum on the PES. The verrucosane biosynthetic cascade bypasses the formation of unstable 2° carbocations II and IV by the formation of adjacent 3° carbocations (IM1 and IM3) combined with skeletal rearrangement reactions involving reverse (3° → 2° cation) Wagner-Meerwein rearrangements. Other putative 2° carbons were located in equilibrium with nonclassical carbocation intermediates (IM5 and IM7) via via bicyclobutonium cation (IM6), and this could be the branching point between the verrucosan-2β-ol (ver) and neoverrucosan-5β-ol (neo) biosyntheses. In the mangicol cyclization cascade, the formation of putative 2° carbocation X is bypassed by breaking the adjacent C–C bond to form the more stable 3° carbocation (IM10). Although C–C bond cleavage is an endoergic reaction, the unfavorable energy loss is compensated by the simultaneous generation of a C=C double bond and a more stable 3° carbocation. Another proposed 2° carbocation XI is avoided as a result of a strong hyperconjugative interaction with the adjacent C=C bond, affording a nonclassical structure (IM11) between a 3° carbocation and a humulyl 2° cation. We further found a new, energetically viable pathway for the 6,5,5-spirotricycle formation in the mangicol biosynthesis, and this can also account for the formation of other terpenes/terpenoids. A future comparative study of the terpene cyclases responsible for mangicol, tsukubadiene, variedi diene, deoxyconidiogenol, and homopompe formations could help to establish the molecular basis of the regulation of the branching biosynthetic pathways by these enzymes. Thus, the results presented here are helpful to complete the picture of verrucosane and mangicol biosynthesis and also offer insights into the stability and reactivity of various carbocations and bonds that should be useful not only in terpene biosynthesis but also in fundamental organic chemistry. In particular, this work underlines that great caution is needed in suggesting the involvement of 2° carbocations (even in the cases of humulyl cations and cycloalkylycarbiny1 cations) in the biosynthesis of natural products, since they are very unstable compared with 3° cations. We hope this work will be helpful for future mechanistic investigations of terpenes/terpenoids biosynthesis.

■ ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.1c00178.

Computational details, three-dimensional representation of the structure, coordinates and energies for all computed structures (PDF).

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Notes

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■ REFERENCES

(1) Christianson, D. W. Structural and Chemical Biology of Terpenoid Cyclases. Chem. Rev. 2017, 117, 11570–11648.

(2) Maeda, S.; Ohno, K.; Morokuma, K. Systematic Exploration of the Mechanism of Chemical Reactions: the Global Reaction Route Mapping (GRRM) Strategy Using the ADDF and AFIR methods. Phys. Chem. Chem. Phys. 2013, 15, 3683–3701.

(3) Isegawa, M.; Maeda, S.; Tantillo, D. J.; Morokuma, K. Predicting Pathways for Terpene Formation from First Principles-Routes to Known and New Sesquiterpenes. Chem. Sci. 2014, 5, 1555–1560.

(4) Sato, H.; Hashishin, T.; Kanazawa, J.; Miyamoto, K.; Uchiyama, M. DFT Study of a Missing Piece in Brasilane-Type Structure Biosynthesis: An Unusual Skeletal Rearrangement. J. Am. Chem. Soc. 2020, 142, 19830–19834.

(5) Sato, H.; Mitsuhashi, T.; Yamazaki, M.; Abe, I.; Uchiyama, M. Computational Studies on Biosynthetic Carboxylation Rearrangements leading to Quiannulatene: Initial Conformation Regulates Biosynthetic Route, Stereochemistry, and Type of Skeleton. Angew. Chem., Int. Ed. 2018, 57, 14752–14757.
