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Confirmation of the Stereochemistry of Spiroviolene

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Abstract

We confirm the previously revised stereochemistry of spiroviolene by X-ray crystallographically characterizing a hydrazone derivative of 9-oxo-spiroviolane, which is synthesized from hydroboration-oxidation of spiroviolene followed by oxidation of the resultant hydroxy group. An unexpected thermal boron migration occurred during the hydroboration process of spiroviolene, that have resulted in the production of a mixture

of 1α-hydroxy-spiroviolane, 9α- and 9β-hydroxy-spiroviolane after oxidation. The assertion of the *cis*-oriented 19- and 20-methyl groups has provided further support for the revised cyclization mechanism of spiroviolene.

Keywords

diterpene; stereochemistry; spiroviolene; boron migration

Introduction

Terpenes represent one of the most fascinating family of natural products due to their structural complexity and diversity, as well as their indispensable biological functions that would be potentially applied as fragrances, pharmaceuticals etc. Until now, more than 80,000 terpenoid structures have been reported, which are found in all domains of life [1-3]. Despite their remarkable chemodiversity, the biosynthetic logic of terpenes is relatively neat [4]. All terpenes are originated from two key C5 building blocks, namely isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are biosynthesized via either methylerythritol phosphate (MEP) pathway or mevalonic acid (MVA) pathway by using the primary metabolites. Different numbers of IPP and DMAPP are assembled by prenyltransferases to afford oligoprenyl pyrophosphates, such as farnesyl pyrophosphate (FPP, 3 × C5) and geranylgeranyl pyrophosphate (GGPP, 4 × C5), with varied C5 units. The linear oligoprenyl pyrophosphates are typically converted by terpene synthases in a chemo- and stereo-defined process to form complex terpene skeletons, normally with multiple stereocenters. In this context, the 3D-defined cyclization products retain the rich information of the complex cyclization process. Thus, assignment of the

stereochemistry of terpene skeleton with high confidence is crucial for proposing a reasonable cyclization mechanism [5].

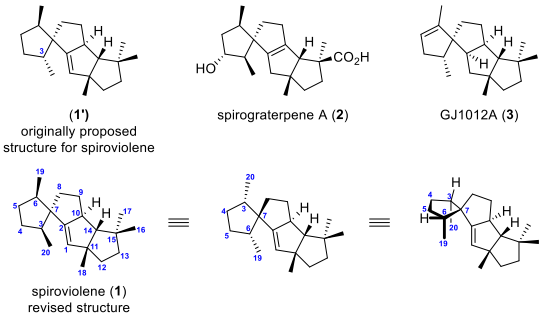
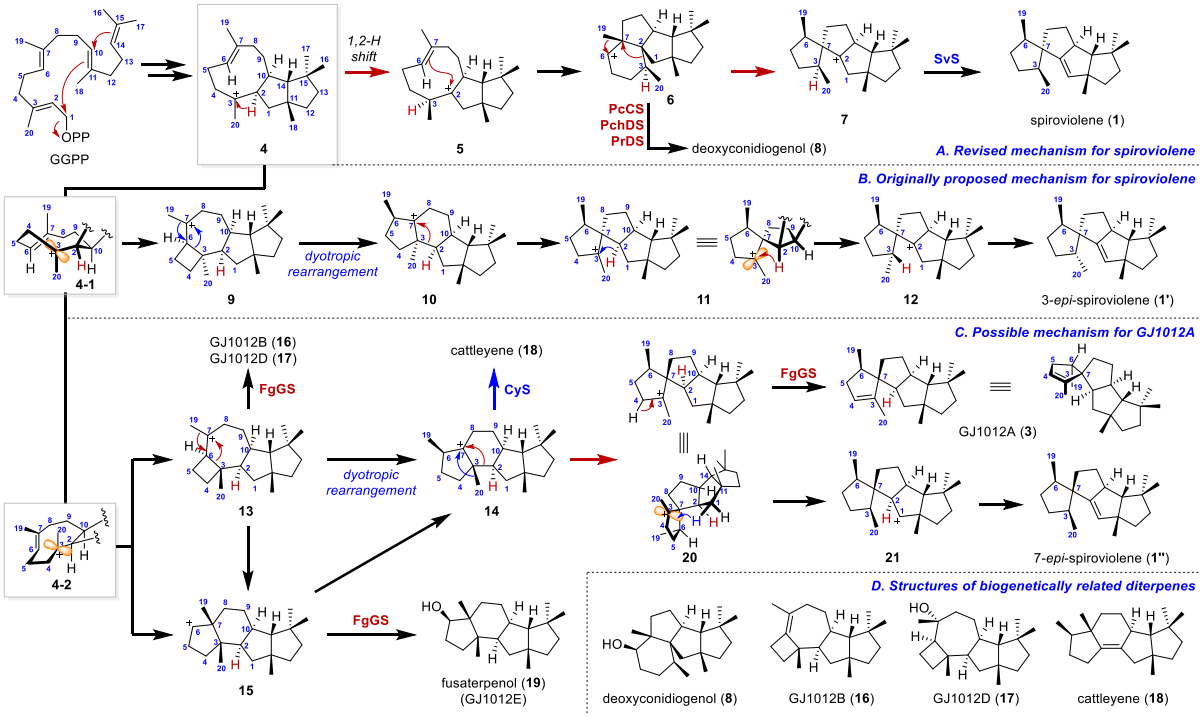


Figure 1: Structures of spiroviolene and related natural products

Spiroviolene (**1**, Figure 1) was identified by Dickschat and co-workers as a nascent cyclization product of spiroviolene synthase (SvS) cloned from *Streptomyces violens* NRRL ISP-5597 [6]. Its unique spiro-fused linear triquinane to cyclopentane skeleton, as well as its stereochemistry, was originally elucidated as **1′** as shown in Figure 1, on the basis of detailed analysis of NMR spectroscopy. Spiroviolene was also found to be produced by several bacterial strains harboring SvS homologs [6,7], as well as putative ancestors of SvS generated by ancestral sequence reconstruction [8,9]. Related natural products with the same 5-5-5-5 tetracyclic ring system, including spirograterpene A (**2**) from *Penicillium granulatum* MCCC 3A00475 [10], and GJ1012A (**3**) from an engineered *E. coli* strain harboring FgGS (FgJ07623) cloned from *Fusarium graminearum* GJ1012 [11], have been reported almost at the same period. The discrepancy of the stereochemistry at C3 between **1′** and **2** was first noticed by Snyder and co-workers [12]. The same stereochemistry at C3 was later confirmed by the conversion of a synthetic intermediate of **2** to spiroviolene. However, the reassignment of spiroviolene to **1** was mainly relied on DFT calculation of the transition state of the key hydroboration reaction and NOE correlation analysis of the resultant product, while direct evidences such as single-crystal X-ray diffraction results were not obtained.

The reassignment of the stereochemistry at C3 have resulted in the revision of the proposed cyclization mechanism [12-14]. The revised mechanism resembled the cyclization process for the formation of deoxyconidiogenol (**8**, Scheme 1A) by several terpene cyclases from fungus (PcCS, PchDS, PrDS) [15,16], which involves a 1,11-10,14 cyclization of GGPP, followed by 1,2-alkyl shift and a 2,10-cyclziation, to give the key C3 cationic intermediate **4**. A key 1,2-hydride shift from C2 to C3, which was observed in the isotope labeling experiments [6], followed by a 2,7-cyclization, afforded C6 cationic intermediate **6** with cyclopiane skeleton. Quench of the cation **6** with water would give **8**, while upon two 1,2-alkyl shifts of **6**, followed by deprotonation of cation **7**, would give spiroviolene (**1**).



Scheme 1: Possible cyclization mechanisms for spiroviolene (**1**) and related natural products. (A) Revised cyclization mechanism for **1** that is related to deoxycondiogenol (**8**); (B) Originally proposed cyclization mechanism for the misassigned structure 3-*epi*-spiroviolene (**1'**); (C) Possible cyclization mechanism for

GJ1012A (**3**); (D) Structures of biogenetically related diterpenes during the proposed cyclization process.

On the other hand, the originally proposed cyclization mechanism (Scheme 1B) involves a 3,6-cyclization of cation **4** through a conformation shown as **4-1** to generated cation **9**, which was proposed to undergo a dyotropic rearrangement, followed by a 1,2-alkyl shift of cation **10** to yield the spirocyclic cation **11**. A key 1,3-hydride shift of 11 from the β-face, followed by deprotonation of the formed C2-cation **12**, would deliver the originally proposed structure **1′**, which we renamed as 3-*epi*-spiroviolene [6]. However, no related natural products that would be derived from the intermediates of this pathway have been found so far.

A third cyclization mechanism (Scheme 3C) leading to the same spirocyclic skeleton of spiroviolene with an altered stereochemistry at C7 found in GJ1012A (**3**) could be proposed [11,17,18]. A 3,6- or 3,7-cyclization of cation **4** through a conformation shown as **4-2** with β-oriented 20-methyl group, would generate either **13** or **15** cations. A direct dyotropic rearrangement, or two stepwise 1,2-alkyl migrations of **13**, are possible pathways en route to cation **14**. The presence of these intermediates **13-15** could be inferred by the identification of GJ1012B/D (**16/17**, Scheme 1D) [11], cattleyene (**18**) [19,20], and fusaterpenol (**19**, GJ1012E) [17]. A similar 1,2-alky shift of **14**, followed by deprotonation of the formed spirocyclic cation **20**, afforded **3**. Furthermore, a hypothetical product 7-*epi*-spiroviolene (**1″**) can be generated if a 1,4-hydride shift of cation **20** followed by deprotonation of **21** occurred. In this case, hydride from C1, which is closer to the C3 cation in the 3D model, rather than that from C2, shifts from the α-face. Although previous isotope labeling experiments did not support this pathway for spiroviolene cyclization, it should be noted that a subtle alteration of stereochemical assignment of spiroviolene would have consequences for a different

mechanistic proposal. We herein report the production of spiroviolene (**1**) in a heterologous host by taking advantage of an artificial isopentenol utilization pathway [21-26], and confirmation of its stereochemistry by X-ray crystallography using a hydrazone derivative of **1**.

Results and Discussion

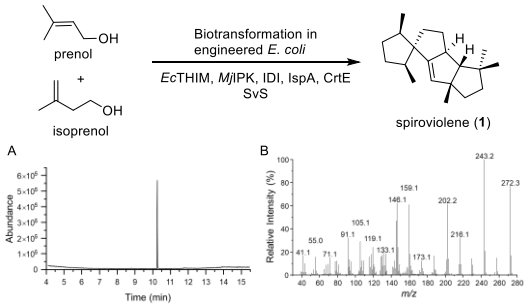
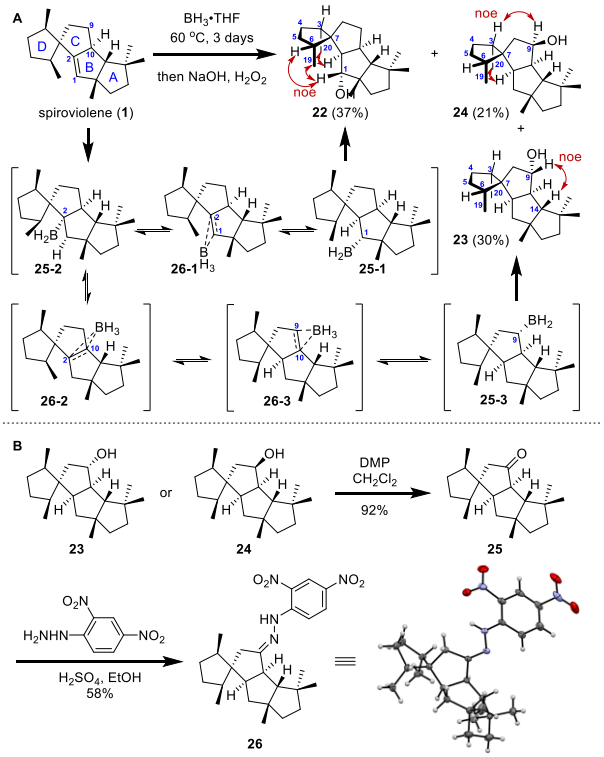


Figure 2: Heterologous production of spiroviolene using isopentenol utilization pathway. (A) GC spectrum of EtOAc-extract of the fermentation broth; (B) EI-MS spectrum of spiroviolene.

Our work commenced with the production of spiroviolene with an engineered *E. coli* by using a recently developed isopentenol utilization pathway for the efficient supply of two C5 precursors for terpene biosynthesis (Figure 2) [21-26]. In this artificial generated pathway, DMAPP and IPP could be easily generated from prenol and isoprenol respectively, by the effect of two kinases, such as hydroxyethylthiazole kinase from *E. coli* (EcTHIM) and isopentenyl phosphate kinase from *Methanocaldococcus jannaschii* (MjIPK). Thus, we have cloned genes coding EcTHIM, MjIPK, IDI, IspA and CrtE into the multiple cloning site-2 of pCDFDeut-1 for GGPP production. Also, we have cloned SvS-coding gene directly from *Streptomyces violens* CGMCC 4.1786 (= NRRL ISP-5597) into pET28a. The two plasmids were then co-transformed into *E. coli* for diterpene production. Spiroviolene could be produced by

feeding prenol and isoprenol to the fermentation broth after the engineered *E. coli* being induced by IPTG, and fermented at 18 ^{o}C for 72 h. GC-MS analysis (Figure 2A) of the EtOAc-extract of fermentation broth gave a single peak, whose EI-MS spectrum (Figure 2B) matches that of spiroviolene. We then carried out large-scale fermentation using shake flasks, and isolated spiroviolene in 40 mg/L yield. The physicochemical data of the isolated material are consistent with those reported for spiroviolene [6].



Scheme 2: Derivatization of spiroviolene for X-ray crystallography. (A) Hydroboration-oxidation reaction of spiroviolene involving a borane migration process; (B) Synthesis of hydrazone derivative of spiroviolene for single-crystal X-ray diffraction.

With sufficient amount of spiroviolene in hand, we next attempted to obtain a crystalline compound suitable for X-ray diffraction by introducing functional groups (e.g. hydroxy group, or ketone group) for further derivatization. Spiroviolene was not

transformed when subjected to conditions for allylic oxidation (SeO_2) even at elevated temperature [27], and the starting material was fully recovered.

We have also tried hydroboration-oxidation conditions for transforming the double bond in a congested environment of spiroviolene (Scheme 2). Low conversion was observed when a tetrahydrofuran (THF) solution of **1** was treated with BH_3·THF at ambient temperature. The hydroboration reaction could be driven to synthetically useful yield when **1** was directly dissolved in 1M BH_3·THF in THF, and heated at 60 ^oC for 3 days. After oxidative treatment of the resultant alkylborane products with $NaOH/H_2O_2$, we have obtained three derivatives (**22-23**) with one hydroxy group in 37%, 30% and 21% isolated yield, respectively, as well as recovery of 10% of the starting material. After detailed analysis of their NMR spectra, we have found that besides the normal hydroboration-oxidation product 1α-hydroxy-spiroviolane (**22**), 9α- (**23**) and 9β-hydroxy-spiroviolane (**24**) resulted from a formal boration at the homoallylic position C9 were also produced. The stereochemistry of the newly generated stereocenters were elucidated on the basis of NOESY spectra. Thus, the key NOE correlations of H-1/H-6, H-1/H_3-19, and H-2/H_3-20 of **22** allowed to assign the 1-OH to be α-oriented, while correlations of H-9/H-14 of **23**, and H-9/H-3 of **24**, supported the assignment of 9α- and 9β-oriented hydroxy groups, respectively.

The formation of all three products **22-24** can be explained as follows (Scheme 1A) [28-31]. Due to the favorable formation of *cis*-5,5-fused B/C ring system, the borane reagent is preferred to approach the double bond of **1** from the α-face, to give either a secondary 1-organoborane intermediate **25-1**, or a tertiary 2-organoborane intermediate **25-2**. Oxidation of **25-1** could stereoselectively furnish the normal hydroboration-oxidation product **22**. On the other hand, the unstable tertiary 2-organoborane **25-2** would undergo a thermal isomerization to give **25-1** through the borane-olefin complex **26-1**, or two consecutive 1,2-boron migrations to yield 9-

organoborane intermediate **25-3** through borane-olefin complexes **26-2** and **26-3**. The suprafacial nature of the boron migration allowed the boron to be α-oriented in intermediate **25-3**, which would give both **23** and **24** after $NaOH/H_2O_2$ oxidation. The low diastereoselectivity in this case might be explained by the epimerization during the oxidation step, which has been reported previously [31].

It is worth noting that significant discrepancies of the NMR data between **23** and **24** are easily to be noticed. To further advance the intermediate to crystalline hydrazone product (Scheme 2B), we have found that both **23** and **24** can be oxidized to the same 9-oxo-spiroviolane **25** in high yield, hence confirming the structural assignment of **23** and **24**. By reacting with 2,4-dinitrohydrazine [32], ketone **25** was further converted to hydrazone derivative **26**, which gave a brownish-yellow crystal suitable for X-ray diffraction [33]. The crystal structure of **26** clearly showed that 19- and 20-methyl groups are *cis*-oriented in D-ring that is consistent with that of spriograterpene A. This structural data reaffirms the revised structure of spiroviolene, and further support the unified cyclization process of fungi-derived deoxyconidiogenol and bacteria-derived spiroviolene by sharing a common C6-cation intermediate **6** with cyclopiane skeleton (Scheme 1A).

Conclusion

The complex structures and congested NMR signals have made the elucidation of terpene structures a challenging task. The same stereochemistry of spiroviolene and spirograterpene A was recently confirmed based on their total synthesis by Snyder and co-workers, that have led to the structural revision of spiroviolene. Stereoselective isotope labelling experiments allowed more precise attribution of the NMR data, and further support the reassignment of the C3 stereochemistry. We have clearly confirmed

this structural revision by obtaining a suitable crystal of hydrazone derivative of 9-oxo-spiroviolane, which is synthesized from hydroboration-oxidation of spiroviolene followed by converting the resultant hydroxy group to a ketone group, for single-crystal X-ray diffraction. The confirmation of the *cis*-oriented 19- and 20-methyl groups of spiroviolene would further support the unified cyclization process that bifurcate at the C6-cation intermediate **6** with a cyclopiane skeleton proposed for spiroviolene and deoxyconidiogenol. In our study, the production of spiroviolene was secured by feeding prenol and isoprenol to an engineered *E. coli* strain harboring an artificial isoprenol utilization pathway for the supply of DMAPP and IPP. In the hydroboration-oxidation reaction of spiroviolene, besides the normal 1-hydroxy-spiroviolane product, an unexpected thermal 1,2-boron migration of the unstable tertiary 2-organoborane occurred, resulted in the formation of both 9α- and 9β-hydroxy-spiroviolane due to the epimerization during the oxidation step.

Supporting Information

Supporting Information File 1: Material and synthetic methods. Copies of NMR spectra for all compounds.

File Name: SI

File Format: PDF

Title: Confirmation of the stereochemistry of spiroviolene

Supporting Information File 2: X-ray crystal structure of 26

File Name: 26

File Format: CIF

Title: crystal structure of 26

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33. Deposition number CCDC 2274944 for **26** contain the supplementary crystallographic data for this paper. These data are provided free of charge by Cambridge Crystallographic Data Centre.