Organic LETTERS

Letter

pubs.acs.org/OrgLett

What Is the True Structure of D609, a Widely Used Lipid Related Enzyme Inhibitor?

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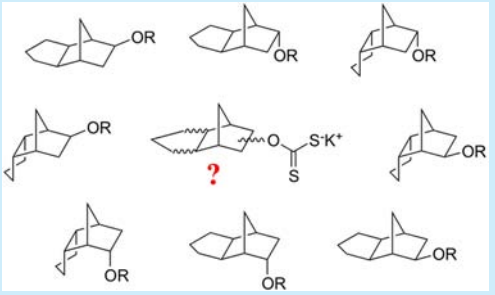
Supporting Information

ABSTRACT: D609 (**1**) has been used as a lipid-related enzyme inhibitor during the past three decades. Although it has eight possible stereoisomers, no systematic research considering its chirality has been performed. In this paper, eight possible chiral alcohols as direct precursors of D609 were synthesized, and their stereochemistries were elucidated by a vibrational circular dichroism (VCD) technique. Phosphatidylcholine-specific phospholipase C and sphingomyelin synthase inhibition assays of these isomers showed considerable differences in their activities.



Tricyclodecan-9-yl xanthogenate (D609, **1**, Figure 1) is a synthetic tricyclic compound having a xanthate group, known as a phosphate group analogue. Though the first synthesis was reported a half century ago,[1] the first biological study of it as antiviral activity started in 1984.[2] After this finding, numerous biological activities, such as antiviral, antitumor, anti-inflammatory, and antiapoptosis properties, have been reported.[3,4] Most of these activities have been attributed to the characteristic competitive inhibitory effect of the D609 on phosphatidylcholine-specific phospholipase C (PC−PLC)[5−7] and indirectly on acidic sphingomyelinase (SMase).[8−10] D609 has also been found to be a sole inhibitor of a sphingomyelin synthase (SMS) whose relationship to metabolic syndrome and amyloid-β peptide clearance was recently reported.[11−16] During the last three decades, more than 700 reports concerning D609's biological activity, especially related to lipid biology, have been published. Currently, D609 is an extremely significant inhibitor for lipid biology and lipid chemical biology.

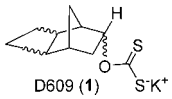


Figure 1. Structure of D609 (**1**) (potassium tricyclodecan-9-yl xanthogenate).

D609 has three asymmetric centers, which lead to eight stereoisomers including their enantiomers (Figure 2). However, to the best of our knowledge, no systematic research has been conducted to investigate the effect the chirality of D609s on their biological activity so far.[17] Furthermore, the commercially available D609s commonly do not show any chiral information and relative stereochemistry because of the enormous difficulty of the assignment of their accurate stereochemistry by standard analytical methods such as NMR techniques.[18] Thus, we attempted to reveal the stereochemistry of the D609 using the emerging vibrational circular dichroism (VCD) technique. VCD measures the differential absorption of left versus right circularly polarized IR radiation on a chiral molecule by a molecular vibrational transition. In the past decade, VCD spectroscopy with ab initio theoretical calculations has been established as a reliable and convenient method to determine its absolute configuration.[19,20] VCD has a great advantage to distinguish between these simple and rigid stereo isomers for hydrocarbons whose NMR signals are severely overlapped, and thus, the assignment of peaks is extremely difficult.

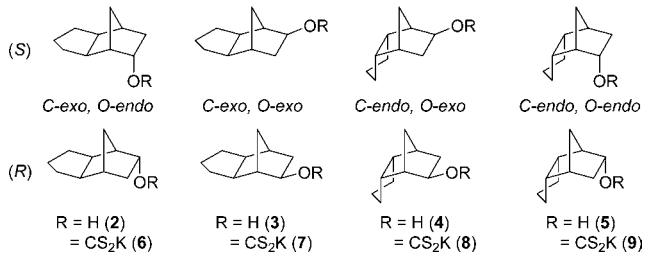


Figure 2. Structures of all possible D609 stereoisomers (**6**−**9**) and their corresponding precursor alcohols (**2**−**5**). Stereochemistry was defined using the *C*-, *O*-, *exo*-, and *endo*-notational system.

In the present paper, we report the first systematic chiral preparation of all possible stereoisomers of D609 and unambiguous determination of their enigmatic absolute configurations as well as their relative stereochemistries by VCD spectroscopy with the aid of their theoretical calculations. In addition, we describe inhibition experiments of PC−PLC

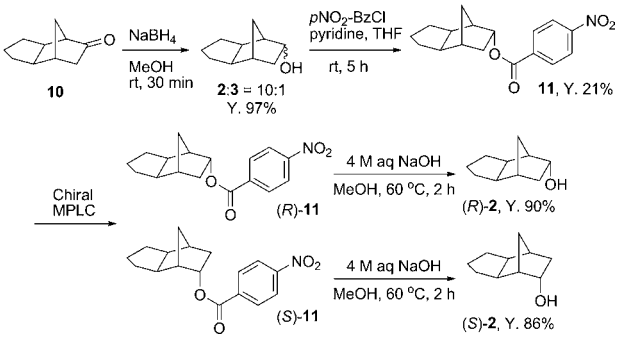
Received: January 4, 2016
Published: February 8, 2016

DOI: 10.1021/acs.orglett.6b00025
Org. Lett. 2016, 18, 768−771

and SMS for all chiral isomers and the determination of stereochemistry of commercial available D609s by utilizing these authentic standard isomers.

A relatively degradable xanthate function should be introduced in the final step to each corresponding alcohol; therefore, four diastereomeric alcohols were planned to be synthesized and followed by its chiral separation leading to totally eight chiral stereoisomers. Each disastereoisomer is defined using the *C-*, *O-*, *exo-*, and *endo*-notational system as shown in Figure 2. First, the synthesis of *C-exo-* and *O-endo*-type diastereomers **2** was conducted via the reduction of the commercially available tricyclo[5.2.1.02,6]decan-8-one (**10**) exhibiting well-confirmed stereochemistry by sodium borohydride in methanol to afford a diastereomeric mixture of alcohols **2** and **3** (ratio = 10:1, respectively) in 97% yield. The resulted alcohols were treated with 4-nitrobenzoyl chloride in THF in the presence of pyridine to give the corresponding ester, which was recrystallized to give the pure *C-exo* and *O-endo* diastereomer **11** in 21% yield (Scheme 1). The chiral

Scheme 1. Synthesis of Chiral *C-exo-* and *O-endo*-Alcohol 2



separation was achieved by using a recently developed middle-pressure chiral liquid chromatography separation system (MPLC, CHIRALFLASH-IF) on relatively medium scale to give enough enantiomers to perform two more reactions toward the D609s. Enantiomerically pure esters (*S*)-**11** and (*R*)-**11** were hydrolyzed using 4 M aqueous sodium hydroxide in methanol to give the corresponding chiral alcohols (*C-exo, O-endo*) (*S*)-**2** and (*R*)-**2** in 86% and 90% yields, respectively. The alcohols (*S*)-**3** and (*R*)-**3** of (*C-exo, O-exo*) were prepared via stereochemical conversion by applying Mitsunobu reaction of **2** with 1-naphthaleneacetic acid to form racemic esters followed by chiral MPLC separation. Other alcohols (*S*)-**4**, (*R*)-**4** (*C-endo, O-exo*) and (*S*)-**5**, (*R*)-**5** (*C-endo, O-endo*) were prepared by the same strategy.[21]

In order to verify their relative configuration and to determine their absolute configuration, we then applied VCD spectroscopy to these enantiomerically pure diastereomers. Figure 3 shows the VCD and IR spectra of (+)-**2**, (−)-**3**, (+)-**4**, and (−)-**5** measured in deuterated chloroform ($CDCl_3$) with a concentration of 0.3 M. The VCD spectra of these diastereomers were sufficiently different to allow distinction by theoretical calculations as described below. Meanwhile, the enantiomers of each isomer exhibited virtually mirror-image VCD and superimposable IR spectra, suggesting that the enantioseparations of these isomers were indeed achieved successfully.[21]

Prior to the theoretical calculations of their VCD and IR spectra, a MMFF conformational search was conducted for each isomer. The following DFT optimization resulted in only

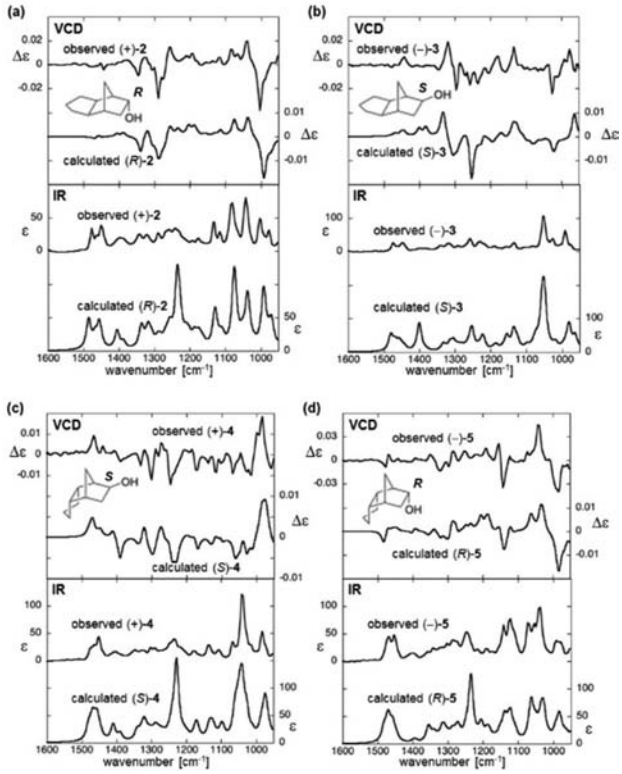


Figure 3. Comparison of VCD (upper frame) and IR (lower frame) spectra of (a) **2**, (b) **3**, (c) **4**, and (d) **5**. Measurement conditions: $CDCl_3$, l = 100 μm, c = 0.3 M, corrected by solvent spectra obtained under the same measurement conditions. Calculation conditions: DFT/B3LYP/6-311G(d,p) without considering solvent effects.

two to six stable conformers that differ in the orientation of the hydroxyl group and the puckering of the terminal 5-membered ring (Figure S2).[21] The theoretical VCD and IR spectra of these conformers were calculated and then averaged on the basis of their Boltzmann populations and compared with the experimental ones (Figure 3). All of the theoretical VCD spectra for each predicted structure showed good agreement with those measured for the isomers with the predicted relative configuration; for example, the negative VCD signals experimentally observed for (+)-**2** at around 1350, 1290, 1000 cm^{-1} were well reproduced in the calculated spectrum for (*R*)-**2**. The theoretical IR spectral features of each isomer also agreed well with the experimental ones, except the sharp intense IR signal predicted at ca. 1230 cm^{-1} (for **2**, **4**, and **5**) ascribed to a C−O−H bending transition, which is easily perturbed by intermolecular interactions and proton exchange under experimental conditions. Overall, the agreement between the theoretical spectra and the experimental spectra not only confirmed the relative configuration of these isomers but also determined their absolute configurations as (*R*)-(+)-**2**, (*S*)-(−)-**3**, (*S*)-(+)-**4**, and (*R*)-(−)-**5**.

To investigate their biological activities in terms of chirality, potassium xanthates of all D609 isomers were synthesized from alcohols in 50−70% yields by treating them with potassium *tert*-butoxide and carbon disulfide. The resulting chiral D609s were subjected to the PC-PLC and SMS inhibition assay. In vitro inhibition assay of the phosphatidylcholine phospholipase C from *Bacillus cereus* of all D609s showed that (*R*)-**8** inhibited the strongest IC_{50} (9 μM), while (*R*)-**6** inhibited the weakest IC_{50} (37 μM), demonstrating a maximum four times difference

DOI: 10.1021/acs.orglett.6b00025
Org. Lett. 2016, 18, 768−771

depending upon their stereochemistries.[22] It is also a significant point that this enzyme could recognize these simple enantiomers of hydrocarbons ((*S*)- and (*R*)-**8**) that are extremely difficult to distinguish by the standard methods. Recent interest in the sphingomyelin synthase (SMS) for drug discovery toward metabolic syndrome prompted us to investigate another inhibition assay of the D609s by using mouse cell lysate system overexpressed SMS1 and SMS2, respectively.[23] The D609 was known as the sole inhibitor of SMS in relatively high concentration. All D609s showed almost the same moderate inhibition (IC_{50} = 70−150 μM) toward SMS1, while toward its isozyme SMS2, a significant difference was observed between the stereoisomers (i.e., (*S*)-**7**; IC_{50} = 150 μM, (*R*)-**9**; IC_{50} = 840 μM) in their IC_{50}.

Most biological studies were performed by using commercially available D609s; therefore, their stereochemistries were studied. An effective GC-analysis system (Zebron ZB-WAX 30 m × 0.25 mm × 0.25 μm) was created by utilizing four standard alcohols whose stereochemistries were unambiguously confirmed by the previously described method. After removal of its xanthate group from the sample D609, a resulting alcohol was submitted to the GC analysis system. Two commercial samples of D609 from two different resources (Sigma-Aldrich and Enzo Life Science) were examined. Surprisingly, it was revealed that these two samples have completely different stereochemistries. In the Sigma case, the sample showed that it contains 84% of the *C-exo,O-exo* isomer (**7**), 3% of the *C-endo,O-exo* isomer (**8**), and unknown impurities, while in the Enzo case, it showed the existence of 92% of the *C-exo,O-endo* isomer (**6**) and 8% of the *C-exo,O-exo* isomer (**7**) in stark contrast with the Sigma case.

All possible stereoisomers of D609s were systematically prepared and enantiometically separated as alcohol precursors for the first time. The stereochemistries of all possible chiral D609s were unambiguously determined by the VCD technique with ab initio calculations. It is also a significant point that VCD can distinguish the possible relative stereochemistries of D609s as well as their absolute configurations, which is extremely difficult to determine by normal methods. These isomers showed significantly different biological activity toward inhibition assay by using the PC−PLC and SMS system. Finally, two D609s from commercial resource showed different stereochemistries, which demonstrated different biological activity. The control and unambiguous characterization of the stereochemistry of D609s was successfully achieved. Our work will contribute to the emerging lipid biology and lipid chemical biology fields.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00025.

Full experimental procedures of all isomers' syntheses, structural data, VCD experimental details, DFT calculations, GC analysis, and inhibition assay (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Dr. Kohei Yuyama, Prof. Susumu Mitsutake, and Prof. Yasuyuki Igarashi at Hokkaido University for helpful advice and discussions of the SMS inhibition assay. This work was partially supported by the Innovation COE Project for Future Medicine and Medicinal Research' and a Grant in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

■ DEDICATION

This paper is dedicated to Prof. Koji Nakanishi at Columbia University on the occasion of his 90th birthday.

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DOI: 10.1021/acs.orglett.6b00025
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(22) The assay kit was obtained from InvitrogenTM EnzChek direct phospholipase C assay kit, which was originally used by Eli Lilly & Co.
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DOI: 10.1021/acs.orglett.6b00025
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