Antitobacco Mosaic Virus Activity of a New Eremophilane Glucoside From the Leaves of Nicotiana tabacum L

Hui Wang, ME1, Li-li Zhang, ME1, Yong-sheng Li, ME1, Shi-tou Li, ME1, Wen-miao He, ME1, Ji-zhong Wu, ME1, Yi-ming Bi, ME1, Lu Dai, ME1, Meng-hao Shen, BE2, Jing Yang, ME2, and Jin-xin Tie, ME1

Abstract
A new eremophilane-type sesquiterpene glucoside, 13-hydroxycapsidiol 3-"β-D-glucoside (1), was isolated from the leaves of Nicotiana tabacum L. The structure of this new metabolite was determined by one-dimensional (1D) and 2D nuclear magnetic resonance (NMR) spectroscopy, high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), and comparison with values reported in the literature. Compound 1 was the second glycosylated eremophilane sesquiterpenoid isolated from plants of the Solanaceae family and exhibited a more potent anti-tobacco mosaic virus (anti-TMV) activity, with an inhibition rate of 39.4% at 20 μM, than the positive control ningnanmycin (34.8%).

Keywords
eremophilane-type sesquiterpene, glucoside, Nicotiana tabacum, NMR, anti-TMV

Introduction
Capsidiol is a terpenoid phytoalexin that accumulates in Solanaceae plants against various pathogens such as the tobacco mosaic virus (TMV), Phytophthora capsica, P. infestans, and Botrytis cinerea. It has an eremophilane-type sesquiterpene structure and its absolute configuration was determined as (2R,3R,4S,5R,7R)-eremophila-9,11(12)-diene-1,3-diol. Eremophilane sesquiterpenoids predominantly exist in non-glycosylated forms in plants, and fewer than three dozen eremophilane glucosides have been identified from plants belonging to the Asteraceae and Lauraceae families. To the best of our knowledge, only one glycosylated eremophilane sesquiterpenoid from the Solanaceae family has been found thus far. As a result of our current study aimed at the discovery of anti-TMV secondary metabolites and expanding the chemical diversity of Nicotiana tabacum, a new eremophilane glucoside (1) was isolated and identified from the leaves of Nicotiana tabacum (Solanaceae). Through spectroscopic methods, including HR-ESI-MS, 1D- and 2D-NMR, as well as comparison with values reported in the literature, the metabolite was determined as 13-hydroxycapsidiol 3-β-D-glucoside (Figure 1). Moreover, the anti-TMV activity of 1 was assessed, and the results demonstrated that 1 exhibited a more potent anti-TMV activity, with an inhibition rate of 39.4% at 20 μM, than the positive control ningnanmycin (34.8%).

Results and Discussion
The molecular formula of 1, obtained as an amorphous white powder, is C_{21}H_{34}O_{8}, determined by HR-ESI-MS (m/z 437.2116 [M+Na]^+), calculated for C_{21}H_{34}O_{8}Na, 437.2146), corresponding to five degrees of unsaturation. The 1H-NMR spectroscopic data (see Table 1) of 1 indicated the presence of a glucose unit, which was verified by HSQC and 1H−1H COSY correlations. Correlation from a characteristic doublet signal of the proton at δ_H 4.37 (1H, d, J = 7.8 Hz, H-1′) to the signal of carbon at δ_C 103.4 (C-1′) in the HSQC experiment and an isolated 1H−1H COSY spin system (H-1′/H-2′/H-3′/H-4′/H-5′/H-6′) were indicative of a glucopyranosyl moiety attached to the aglycone. The 13C-NMR (Table 1), DEPT and HSQC spectra (Supplemental Material) of the aglycone part

1 Technology Center, China Tobacco Zhejiang Industrial Co. Ltd, Hangzhou, China
2 College of Food and Biological Engineering, Zhengzhou University of Light Industry, Zhengzhou, China

Corresponding Author:
Jin-xin Tie, Technology Center, China Tobacco Zhejiang Industrial Co., Ltd, Hangzhou 310000, China.
Email: tiejinxin201@126.com.
showed 15 carbon signals, consisting of two methyl, five methylene (containing a hydroxymethylene and an exo-methylene), five methine (including two oxygenated methines and an olefinic methine), and three nonprotonated carbons (including two olefinic quaternary carbons). The above information suggested that 1 was an eremophilane-type sesquiterpene glucoside,11 which was confirmed by HMBC and 1H−1H COSY experiments (Figure 2a). The 1H−1H COSY correlations of H-1/H-2/H-3/H-4/H-15 and H-6/H-7/H-8/H-9 confirmed the presence of the structural fragments C-1−C-2−C-3−C-4−C-15 and C-6−C-7−C-8−C-9. The HMBC correlations from H-1 to C-5, C-9, and C-10, from H-6 to C-10, from H-8 to C-10, and from H-9 to C-5 demonstrated the presence of the C-1−C-10−C-9, C-4−C-5−C-6 and C-5−C-10 fragments. The singlet methyl (CH3-14) was located at the C-5 position based on the HMBC cross-peaks from H3-14 to C-4 and C-5. The connection of the hydroxyisopropenyl group with a methine carbon (δC 37.0) assigned to C-7 was indicated by the HMBC correlations from H2-12 to C-7, C-11, and C-13, as well as from H2-13 to C-7 and C-11. These characteristic features revealed the aglycone structure of 1, which was similar to that of 13-hydroxycapsidiol.12 The location of the glucose unit was determined to be at C-3, based on the HMBC cross-peak between H-3 and the anomeric carbon C-1′. The β-anomeric form of glucose was determined by its 3J1′,2′ value (7.8 Hz), whereas the D-configuration was obtained by gas chromatographic analysis of its chiral derivatives after snailase hydrolysis of 1. Thus, the planar structure of 1 was established as eremophila-9,11(12)-diene-1,3,13-triol 3-O-β-D-glucoside. The ROESY correlations (Figure 2b) from H-3 to H3-14, as well as from H-7 to H3-15, indicated that H-3 and CH3-14 were cofacial, and H-7 and CH3-15 were in the same orientation. The proton coupling constant (3JH2,3 = 4.2 Hz) between H-3 and H-4 indicated that H-4 had an equatorial position.13

Table 1. 1H- and 13C-NMR Spectroscopic Data of Compound 1 in CD3OD (δ, ppm; J, Hz).

| Position | δH (1H, multiplicity, J, Hz) | δC (ppm) | Position | δH (1H, multiplicity, J, Hz) | δC (ppm) |
|----------|------------------------------|----------|----------|-------------------------------|----------|
| 1        | 4.28 (1H, t, J = 3.0 Hz)   | 75.5     | 12       | 5.02 (1H, s); 4.83 (1H, s)  | 108.4    |
| 2        | 1.98 (1H, m); 1.68 (1H, m) | 34.3     | 13       | 4.04 (2H, s)                 | 65.4     |
| 3        | 4.50 (1H, dt, J = 12.0, 4.2 Hz) | 74.9     | 14       | 1.34 (3H, s)                 | 32.5     |
| 4        | 1.90 (1H, m)               | 48.0     | 15       | 0.91 (3H, d, J = 6.6 Hz)     | 10.5     |
| 5        |                              | 40.4     | 1′        | 4.37 (1H, d, J = 7.8 Hz)     | 103.4    |
| 6        | 1.83 (1H, m); 1.30 (1H, m) | 47.0     | 2′        | 3.14 (1H, m)                 | 75.1     |
| 7        | 2.28 (1H, m)               | 37.0     | 3′        | 3.33 (1H, m)                 | 78.2     |
| 8        | 2.11 (1H, m); 1.90 (1H, m) | 31.9     | 4′        | 3.30 (1H, m)                 | 71.6     |
| 9        | 5.89 (1H, dd, J = 7.2, 1.2 Hz) | 129.2    | 5′        | 3.25 (1H, m)                 | 77.8     |
| 10       | 141.7                       |          | 6′        | 3.83 (1H, m); 3.67 (1H, m)   | 62.7     |
| 11       |                              | 154.8    |          |                               |          |

Figure 2. (a) 1H−1H COSY and key HMBC correlations of compound 1. (b) Key ROESY correlations of compound 1.
like signal of H-1, with small coupling constants ($\tilde{J} = 2$−4 Hz), indicated that H-1 had an equatorial position and the hydroxy group at C-1 an axial position, which was supported by the ROESY correlation between H-1/H-9.11

The absolute configuration of 1 was confirmed by comparing the $^1$H and $^{13}$C NMR data of the aglycone (1A), specifically the chemical shifts and coupling patterns, with those of 13-hydroxycapsidiol.12 NMR spectroscopic data for aglycone (1A) and 13-hydroxycapsidiol (1B) reported in the literature are shown in Supplemental Material. As can be seen, the chemical shifts and coupling patterns were indistinguishable from those of 1A on comparison with those of 1B. Moreover, the optical rotation of 1A was measured and the dextrogyre form ([α]D = +4.2 (c 0.2, CHCl₃)) was consistent with that of capsidiol, which had been isolated from a Nicotiana species and determined by X-ray analysis. Hence, 1A was tentatively determined as (1R,3R,4S,5R,7R)-eremophila-9,11(12)-diene-1,3,13-triol 3-O-β-D-glucoside and named as 13-hydroxycapsidiol 3-O-β-D-glucoside.

Previous studies found that capsidiol could be accumulated in N. tabacum leaves after infection with TMV.4,5 Thus, 1A and its aglycone (1A) were tested for their inhibitory activities against TMV replication at a concentration of 20 μM using the half-half method.13 The results showed that 13-hydroxycapsidiol 3-O-β-D-glucoside (1) exhibited a more potent anti-TMV activity, with an inhibition rate of 39.4% at 20 μM, than the positive control ningnanmycin (34.8%) and 13-hydroxycapsidol (31.3%).

Several hundred eremophilanes have been identified to date, but only a few of these have been isolated in glycosylated forms.9 Our finding of a new sesquiterpenoid glucoside not only enriched the chemical diversity of glycosylated eremophilane-type natural compounds, but suggested that N. tabacum could be a source of chemically diverse compounds. 13-Hydroxycapsidiol was first isolated from Capsicum frutescens Linn. in 1977,12 and its structure contained three hydroxy groups, which were potential binding sites of glycosides. However, the glycosidic compounds of 13-hydroxycapsidiol have, to our knowledge, never been described in the literature, indicating that 1 is the first glycoside example of a 13-hydroxycapsidiol.

Experimental

General Experimental Procedures

NMR spectra data were recorded in deuterated methanol (CD$_3$OD) using a Bruker Ascend 600 MHz spectrometer (Billerica, MA, USA), and HR-ESI-MS analyses were conducted on an AB SCIEX Triple TOF 6600 mass spectrometer (Tokyo, Japan). A Waters Prep 150 LC system (Manchester, UK) equipped with a YMC-Pack ODS column (250 × 10 mm, 5 μm; Kyoto, Japan) was used to carry out semi-preparative liquid chromatography. Amberlite XAD-2 macroporous resin (Rohm & Haas, Philadelphia, PA, USA) and Sephadex LH-20 (GE Healthcare, Chicago, IL, USA) were used for column chromatography. The column fractions were guided by a Waters Arc high-performance liquid chromatography (HPLC) system equipped with either an ultraviolet or evaporative light scattering detector. All other chemicals and solvents were of analytical grade.

Plant Material

The cured leaves of N. tabacum were provided by the China Tobacco Zhejiang Industrial Co., Ltd in October 2020 and authenticated by Shen Huang, an associate professor of the Zhengzhou University of Light Industry. The Duobin Mao research group was the custodian of the voucher specimen (20200008).

Extraction and Isolation

The cured leaves (1.2 kg) of N. tabacum were powdered and subjected to ultrasound-assisted extraction with MeOH (3 × 1 L). After filtration, the MeOH extracts were concentrated under reduced pressure to obtain the crude residue (260 g). The brown crude extract was dissolved in water (1 L) and partitioned sequentially with light petroleum (3 × 1 L), ethyl acetate (EtOAc, 3 × 1 L), and butyl alcohol (n-BuOH, 4 × 1 L). The n-BuOH-soluble fraction (126 g) was subjected to chromatography on an XAD-2 macroporous adsorption resin column, eluting successively with water, diethyl ether/hexane (1:1, v/v), and MeOH. The MeOH solution (104 g) was fractionated on a Sephadex LH-20 column (150 × 10 cm i.d.) and eluted with a MeOH/H₂O gradient system (0%, 15%, 30%, 50%, 70%, 85%, and 100%, v/v, 1 L each) to provide seven fractions (fractions A–G) on the base of HPLC profiles. Fraction D was purified by reverse preparative HPLC using MeCN/H₂O (10:90, v/v) as the mobile phase at 5 mL/min to yield 1 (5 mg). The isolate was dried under reduced pressure and the spectroscopic data were collected.

Enzymatic Hydrolysis and Determination of the Absolute Configuration of the Monosaccharide

Compound 1 (3 mg) was mixed with snailase (w/w = 1:3) and then dissolved in H₂O (2 mL). The mixed solution was maintained at 37 °C for 48 h. Next, the reaction mixture was separated by preparative HPLC eluting with CH₃CN–H₂O (v/v = 1:4) to yield the corresponding aglycone (1A, 1.2 mg) and sugar residue. The $^1$H- and $^{13}$C-NMR spectra of 1A are presented in the Supplemental Material. The dried sugar residue was dissolved in anhydrous pyridine (2 mL), followed by adding 2 mg L-cysteine methyl ester hydrochloride. The mixture was reacted at 60 °C for 1 h. After drying the solution, N-trimethylsilylimidazole (0.2 mL) was added and heated at 60 °C for 2 h. The reaction mixture was partitioned between n-hexane and H₂O (2 mL each). Then the n-hexane extract
was subjected to GC analysis using the same previously reported method.\textsuperscript{14}

**Anti-TMV Assays**

The protective effects of 13-hydroxycapsidiol 3-O-β-D-glucoside (1) and its aglycone (13-hydroxycapsidiol) against TMV replication were examined at a concentration of 20 μM using the same half-half method as reported.\textsuperscript{15} *N. glutinosa* was used as a local lesion host. The experiments were conducted when the plants grew to the 5–6 leaf stage. The virus was inhibited by mixing with the solution of the compounds. After 30 min, the mixture was inoculated on the left side of the leaves of *N. glutinosa*, whereas the right side of the leaves was inoculated with a mixture of dimethyl sulfoxide (DMSO) solution and the virus as the control. The local lesion was inoculated with a mixture of dimethyl sulfoxide (DMSO) solution and the virus as the control. The local lesion numbers were recorded 3 or 4 days after inoculation. The measurements were obtained in triplicate, and the inhibition rates were calculated according to the formula: inhibition rate (\%) \(=\frac{[(C-T)/C]\times 100\%}{\text{where } C \text{ is the average number of local lesions for the control, and } T \text{ is the average number of local lesions for the treatment.}}\)

**Conclusion**

A new eremophilane-type glucoside, 13-hydroxycapsidiol 3-O-β-D-glucoside (1), was isolated and identified from the cured leaves of *N. tabacum*. It was the second glycosylated eremophilane sesquiterpenoid that has been isolated from plants of the Solanaceae family. Compound 1 exhibited a more potent anti-TMV activity, with an inhibition rate of 39.4% at 20 μM, than the positive control ningnanmycin (34.8%).

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**Declaration of Conflicting Interests**

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**ORCID iD**

Jin-xin Tie https://orcid.org/0000-0002-5464-7024

**Supplemental Material**

Supplemental material for this article is available online.

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