Three Sulfur-Containing Natural Products From *Rubus Hirsutus* Thunb

Na Lin\(^1,2\), Sihui Li\(^2\), Xuexia Chen\(^2\), Jianfeng Song\(^3\), Yichao Ge\(^2\), Xiaodan Wu\(^4\), Huifang Zhang\(^5\), and Bin Wu\(^2\)

Abstract

Chemical investigation of the leaves and stems of *Rubus hirsutus* Thunb has resulted in the isolation and characterization of a rare new tricyclic thienocyclopentapyran (1) and 2 known thiophenes, 5-(3-buten-1-ynyl)-2,2'-bithiophene (2) and α-terthienyl (3). The structure of compound 1 was unambiguously determined by nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry and time-dependent density functional theory electronic circular dichroism calculations. The new compound 1 exhibited impressive antibiotic properties against methicillin resistant *Staphylococcus aureus* (MRSA), with an MIC value of around 1 µg/mL.

Keywords

thienocyclopentapyran, sulfur-containing natural products, *Rubus hirsutus* Thunb, antibiotic, α-terthienyl

Received: February 5th, 2021; Accepted: March 30th, 2021.

Sulfur contributes significantly to the chemical diversity of natural compounds thanks to its particular features which allow fundamental biological reactions.\(^4\) Plant-derived sulfur-containing secondary metabolites constitute a small group of low-molecular weight natural products, which play a vital role in plant-pest interactions in numerous plant families and represent major defense molecules in the Asteraceae, Alliaceae, and Brassicaceae families.\(^2\) Both primary and secondary S-containing compounds have great effects on plant health and regulatory mechanisms when under stress.\(^3\) In addition to antibacterial activity, some sulfur-containing natural products have shown promising antifungal activities against a wide range of plant pathogenic fungi. For instance, the phytoalexins sinalbins A and B were proved to have antifungal activity against the plant pathogenic fungus *Leptosphaeria maculans*.\(^4\) Sulfur-containing monoterpenoids have been reported as potential antithrombotic drugs.\(^5\) Sulfur-containing natural products have emerged as a new class of compounds that have the potential to exert strong anti-inflammatory effects and may, therefore, stand as a potential “goldmine” for the discovery of future anti-inflammatory drugs.\(^8\) In this study, we investigated the chemical composition of the leaves and stems of *Rubus hirsutus* Thunb, leading to the isolation and characterization of a rare new tricyclic thienocyclopentapyran (1) and 2 known thiophenes, 5-(3-buten-1-ynyl)-2,2'-bithiophene (2) and α-terthienyl (3). The antibacterial activities of these 3 sulfur-containing compounds were also evaluated.

The MeOH extracts of the leaves and stems of *R. hirsutus* were subjected to preparative HPLC (C18), affording a new sulfur-containing compound 1 and the known compounds 5-(3-buten-1-ynyl)-2,2'-bithiophene (2)\(^7\) and α-terthienyl (3) (Figure 1).\(^8\)

Results and Discussion

Compound 1 was isolated as a brown powder. The molecular formula was determined to be C\(_{17}\)H\(_{16}\)O\(_5\)S by analysis of the HR-TOF-MS ion peak at \(m/z\) 331.0639 [M - H] (calcld. 331.0640). The formula was supported by the 13C NMR data, which showed 10 degrees of unsaturation. The NMR data (Table 1) in the downfield region of 1 were similar to those of (+) and (-) xanthienopyran.\(^9\) This type of molecule contains 2 distinct domains, a tricyclic thienocyclopentapyran and a conjugated diene chain, connected by an oxygenated methine carbon.\(^9\) The 13C NMR spectrum showed the presence of 10 signals for the tricyclic thienocyclopentapyran domain, including a cyclopent[4]thiophene basic skeleton and a hydroxylated pyran lactone moiety, with the remaining 7 resonances...
corresponding to a conjugated diene chain and a hydroxymethyl moiety. When compared with the NMR data of (-) xanthienopyran, isolated from the seeds of Xanthium strumarium, compound 1 showed similar chemical shifts for the main tricyclic thienocyclopentapyran skeleton. However, the chemical shifts for the substructures of the thiophene and attached methyl unit at the adjacent position of the sulfur atom were less similar to those of xanthienopyran.

The 10 signals for the tricyclic thienocyclopentapyran domain comprised 2 aromatic methines at δC 115.2 (C-4), and 120.8 (C-3), an oxygenated methine at δC 82.8 (C-8), six quaternary aromatic carbons at δC 151.5 (C-5), δC 121.5 (C-4a), δC 136.7 (C-8a), δC 127.8 (C-8b), δC 144.9 (C-3a), and δC 150.6 (C-2), corresponding to 3 sets of double bonds, and a lactone carbonyl carbon at δC 171.8 (C-6). Among the six quaternary aromatic carbons, an enol carbon at δC 151.5 (C-5) was observed. In the 1H, 1H COSY spectrum of 1 (Figure 2), the oxygenated methine at δH 6.06 (d, H-8) was coupled with the olefinic proton at δH 5.68 (dd, H-9), which further coupled with another olefinic proton at δH 6.49 (dd, H-10), revealing that the side chain was positioned at C-8 of the thienocyclopentapyran main skeleton. The 1H, 1H COSY spectrum of 1 also showed a diagnostic cross peak between 2 double bonds, demonstrating that a chain of 2 conjugated double bonds was attached at C-8. A sequence of HOCH2-CH2- was deduced by the observation of the 1H, 1H COSY correlations between the proton signals at δH 3.59 (t, H-14) and δH 2.31 (q, H-13). From the above analysis of the COSY correlations, a side chain of HOCH2-CH2-CH = CH-CH=CH was inferred, attached at C-8 of the main backbone. The stereochemistry of both C-9 and C-11 was deduced as E on the basis of J values (J9,10= 15.2 Hz, J11,12= 15.4 Hz).

HMBC analyses showed the formation of the tricyclic thienocyclopentapyran skeleton with a characteristic (E)-hexa-3,5-dien-1-ol pedant (Figure 2). HMBC cross peaks between olefinic H-9,10 and the oxygenated methine C-8 confirmed that the side chain was located at C-8 of ring A. In the 1H NMR spectrum of 1, 2 olefinic protons at δH 7.23 (s, H-3) and δH 7.40 (s, H-4) displayed 2 singlet signals, both of which showed HMBC correlations with 2 quaternary carbon, C-3a and C-8a, 2 fusing atoms between ring B and ring C. As such, these 2 singlet aromatic protons were assigned to C-3 of ring B and C-3 of ring C. A diagnostic long range correlation from H-8 to the carbonyl at C-6 was observed, showing that a
The leaves and stems of *Rubus hirsutus* were collected in Zhejiang Province, China, in September 2018 and identified by Prof. Changxi Zhang (Jinhua Medical College, Jinhua, China). A voucher specimen (No. zju 7541) is kept in the College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China.

**Extraction and Isolation**

The shade-dried leaves and stems (5 kg) were extracted with methanol (20 L), and 134 g extract was obtained, which was partitioned with light petroleum (1200 ml), EtOAc (1500 ml) and n-BuOH (1000 ml), successively. The light petroleum extract (27 g) was subjected to column chromatography (4 × 40 cm, 700 g, 200-300 mesh) over silica gel, eluting with light petroleum/EtOAc (10:1-0:10, gradients, 3000 ml) to afford 4 fractions. Fraction 3 was purified preparative HPLC (flow rate 8 mL/min, UV detector 250 nm), using CH₃OH-H₂O (75:25) as eluent, to afford compound 1 (5.3 mg, δt 33.6 minutes). The EtOAc extract (30 g) was fractionated by column chromatography (5 × 45 cm, 850 g, 200-300 mesh) over silica gel, eluting with light petroleum/EtOAc (8:1-0:10, gradients, 3000 ml) to afford six fractions. Fraction 4 (8.4 g) was applied to a Sephadex LH-20 column (4 × 150 cm, 100 g, Amersham), and eluted with acetone (1200 ml) at 15 °C for 1 day to give pure 2 (6.3 mg) and 3 (4.9 mg).

**Computation Section**

The geometry was optimized starting from initial conformations, with DFT calculations at the B3LYP/6-31 + G(d) level using the Gaussian 09 program. Frequency analysis was made at the same level of theory to verify that these optimized structures are real minima on the potential energy surface. Time-dependent DFT calculations were performed on the lowest-energy conformations for each configuration using 30 excited states and in methanol solution. ECD spectra were generated using the program SpecDis by applying a Gaussian band shape with 0.2 eV width, from dipole-length rotational strengths.

**Antibiotic Activity Screening of Stress Metabolites**

The conventional broth dilution assay was used to evaluate the antibacterial activities. Three clinical pathogens namely MRSA, *P. aeruginosa* [CMCC(B)10104], and *K. pneumoniae* [CMCC(B)46117] were cultured and left overnight to grow. Each pathogenic culture was then diluted in Hinton broth with a starting concentration of 512 µg/mL and n-BuOH (1000 ml) successively. The light petroleum extract (27 g) was subjected to column chromatography (4 × 40 cm, 700 g, 200-300 mesh) over silica gel, eluting with light petroleum/EtOAc (10:1-0:10, gradients, 3000 ml) to afford 4 fractions. Fraction 3 was purified preparative HPLC (flow rate 8 mL/min, UV detector 250 nm), using CH₃OH-H₂O (75:25) as eluent, to afford compound 1 (5.3 mg, δt 33.6 minutes). The EtOAc extract (30 g) was fractionated by column chromatography (5 × 45 cm, 850 g, 200-300 mesh) over silica gel, eluting with light petroleum/EtOAc (8:1-0:10, gradients, 3000 ml) to afford six fractions. Fraction 4 (8.4 g) was applied to a Sephadex LH-20 column (4 × 150 cm, 100 g, Amersham), and eluted with acetone (1200 ml) at 15 °C for 1 day to give pure 2 (6.3 mg) and 3 (4.9 mg).

**Experimental**

**General**

The melting point (uncorrected); Reichert apparatus. Optical rotations: Perkin-Elmer-341, polarimeter. IR spectra: Nicolet Avatar-360FT-IR spectrometer. ¹H NMR (600 MHz) and ¹³C NMR (150 MHz) spectra: Agilent 600 NMR spectrometer with TMS as internal standard (at 25 °C). Mass spectra: AB Sciex 5500 Q-TRAP and LC/MS Q-Orbitrap (Q Exactive Focus, ThermoFisher Scientific). TLC: Merck precoated plates (silica gel 60 F254) of 0.25 mm thickness. Chromatography: Waters 600 Preparative HPLC, with a Shim-pack PRP-OADS (250 × 20 mm) column; Sephadex LH-20 (Amersham).
samples was dispensed into well 1 and serially diluted across the plate. Ultimately, addition of 125 µL of the bacterial inoculum was made and the plates were incubated at 37 °C for 18 hours. The bacteriostatic abilities of the compounds were noted as MICs, which were made in triplicate. Five mg per mL of a methanolic solution of 3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyltetrazolium bromide (MTT; Lancaster) was used to detect bacterial growth by a change in color from yellow to blue.13

Hirsuthienopyran (1)

Brown powder

[α]D20 −23 (c 0.1, MeOH).

Rf : 0.40 (CHCl3-MeOH, 2:1).

UV/Vis λmax (MeOH) nm (log ε): 210 (4.24), 265 (2.47), 324 (3.14).

ECD (c 50 ppm, MeOH) λmax (Δε) 224 (-1.56), 215 (+0.01), 203 (−3.57) nm.

ESI MS m/z 331.0639 [M - H]- (calcd. 331.0640).

Product yield: 0.02%.

Acknowledgments

We thank Dr Yaqin Liu from the Department of Chemistry for the NMR measurement and Prof. Hujun Xie from Zhejiang Gongshang University for his help on ECD calculation.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Science Foundation of Lishui Science and Technology Bureau (2017ZDYF17) and Jinhua Science and Technology Project (2020-2-019).

ORCID ID

Bin Wu https://orcid.org/0000-0002-7638-2696

References

1. Milito A, Brancaccio M, D’Argenio G, Castellano I. Natural sulfur-containing compounds: an alternative therapeutic strategy against liver fibrosis. Cells. 2019;8(11):1356. doi:10.3390/cell 8113156

2. Abdalla M, Mühling K. Plant-derived sulfur containing natural products produced as a response to biotic and abiotic stresses: a review of their structural diversity and medicinal importance. J Appl Bot Food Qual. 2019;92:204-215. doi:10.5073/JABFQ.2019. 092.029

3. Chan KX, Phua SY, Van Breusegem F, Van BF. Secondary sulfur metabolism in cellular signalling and oxidative stress responses. J Exp Bot. 2019;70(16):4237-4250. doi:10.1093/jxb/erz119

4. Pedras MSC, Yaya EE. Phytoalexins from Brassicaceae: news from the front. Phytochemistry. 2010;71(11-12):1191-1197. doi:10. 1016/j.phytochem.2010.03.020

5. Nikitina LE, Kiselev SV, Startseva VA, et al. Sulfur-Containing monoterpenoids as potential antithrombotic drugs: research in the molecular mechanism of coagulation activity using pinanyl sulfoxide as an example. Front Pharmacol. 2018;9:116. doi:10. 3389/fphar.2018.00116

6. Cao X, Cao I, Zhang W, Lu R, Bian J-S, Nie X. Therapeutic potential of sulfur-containing natural products in inflammatory diseases. Pharmacol Ther. 2020;216:107687. doi:10.1016/j.pharmthera.2020.107687

7. Margl I, Eisenreich W, Adam P, Bacher A, Zenk MH. Biosynthesis of thiophenes in Tagetes patula. Phytochemistry. 2001;58(6):875-881. doi:10.1016/S0031-9422(01)00360-0

8. Guo D-A, Liu X-Y, Qiao I, et al. Chemical constituents of Echi nops talassicus root. J Chinese Pharmaceutical Sci. 1992;1(1):82-83.

9. Lee C-I, Huang P-C, Hsieh P-W, et al. (−)-Xanthienopyran, a new inhibitor of superoxide anion generation by activated neutrophils, and further constituents of the seeds of Xanthium strumarium. Planta Med. 2008;74(10):1276-1279. doi:10.1055/s-2008-1081295

10. Frisch MJ, Trucks G, Schlegel HB, et al. Gaussian 09 revision A.1. Gaussian Inc. 2009

11. Xie H-J, Lei Q-F, Fang W-J. Intermolecular interactions between gold clusters and selected amino acids cysteine and glycine: a DFT study. J Mol Model. 2012;18(2):645-652. doi:10.1007/s00894-011-1112-6

12. Xie H, Ren M, Lei Q, Fang W. Nitric oxide adsorption and reduction reaction mechanism on the Rh7(+) cluster: a density functional theory study. J Phys Chem A. 2011;115(49):14203-14208. doi:10.1021/jp2044652

13. Appendino G, Gibbons S, Giana A, et al. Antibacterial cannabinoids from Cannabis sativa a structure-activity study. J Nat Prod. 2008;71(8):1427-1430. doi:10.1021/np8002673