Bioactive isopimarane and 3,4-seco isopimarane diterpenoids from *Isodon amethystoides*

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**Abstract**

*Isodon amethystoides* (Lamiaceae) is a popular plant in folk medicine in the southern provinces of China. Our phytochemical investigation of the twigs and leaves of this plant led to the discovery of five new diterpenoids with isopimarane and 3,4-seco isopimarane scaffolds [isoamethinols A–E (1–5)], along with the known compound 3,4-seco isopimara-4(18),7,15-triene-3-oic acid methylester (6). The chemical structures of these compounds, including the absolute configurations of the new diterpenoids, were determined by comprehensive spectroscopic analyses and single crystal X-ray diffraction measurements. These compounds were evaluated for their biological activities against a panel of human cancer cell lines, gram-positive bacterial strains and HIV. Notably, the 3,4-seco-isopimarane isoamethinol D (4) showed toxicity to the cervical Hela cancer (Hela) cells with an IC₅₀ value of 27.21 μM and the lung (A549) cancer cells with an IC₅₀ value of 21.47 μM. Compound 4 also exhibited mild antimicrobial activity against the oral bacterial strain *Streptococcus mutans*. These findings suggested that the diterpenoids with a 3,4-seco-isopimarane diterpenoids isolated from *I. amethystoides* could provide a novel structure scaffold for the discovery of anticancer and antimicrobial compounds.

**Keywords:** *Isodon amethystoides*, Lamiaceae, Isopimarane diterpenoids, Cytotoxicity, Antibacterial

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Introduction
Plants are known to produce abundant small molecules of secondary metabolites with novel and unique chemical structures and a wide range of bioactivities, thus providing an important source for the discovery and development of new drugs.

The plant genus *Isodon* (Lamiaceae), mainly distributed in the tropical and subtropical regions of Asia, has been increasingly studied in the recent years due to its structurally diverse constituents with medical applications [1–3]. As popular Chinese folk medicines, some species in the genus *Isodon* have been traditionally used for the treatment of gastritis, arthritis, hepatitis, cholecystitis, enteritis, and amenorrhea in the southern provinces of China [4–8]. Diterpenoids are a major class of natural compounds that are abundantly found in *Isodon* plants. They have been discovered to have a broad-spectrum of pharmacological activities, including anticancer, antiviral, and antibacterial properties [1–3, 5, 7].

*Isodon amethystoides* is a medicinal plant belonging to the genus *Isodon*. Its leaf extract has been patented as a commercial drug with the trade-name “Wei Fu Chun Pian” for the treatment of precancerous lesions of gastric cancer lesions and also serves as an adjuvant therapy after gastric cancer surgery, as recorded in the Chinese Pharmacopoeia 2020 edition [9]. Previous phytochemical investigations of this species revealed significant differences in the chemical profiles of the secondary metabolites among the plants collected from various geographical areas [4–6]. Our recent research on a sample of this species collected from Libo County in Guizhou Province also led to the identification of a novel bioactive diterpene with 6/5/7 carbon skeleton, which showed inhibitory activity against the autoimmune disease-associated RORγt [10]. These prior findings encouraged us to carry out further phytochemical studies to discover additional new bioactive diterpenoids from this plant. As a result, a total of five new isopimarane and 3,4-seco isopimarane diterpenes [isoamethinols A–E (1–5)], along with the known compound 3,4-seco isopimar-4(18),7,15-triene-3-oic acid methylester (6) [11], were isolated from the leaves of *I. amethystoides* (Fig. 1). The newly isolated compounds were subsequently tested for their cytotoxic, antibacterial, and antiviral activities. Herein, we describe the isolation, structural determination, and biological activity evaluation of the compounds obtained from *I. amethystoides*.

Result and discussion
Compound 1 was obtained as a colorless cubic crystal (MeOH). It has 6 degrees of unsaturation according to the deduced molecular formula of C<sub>20</sub>H<sub>30</sub>O<sub>2</sub> by analysis of the HR-ESI-MS data ([m/z 325.2133 [M+Na]<sup>+</sup> (calcld for 325.2138)]. By interpreting the chemical shift signals in the high and middle field regions of the <sup>1</sup>H-NMR spectrum (Table 1), 1 was found to possess four methyl
Fig. 1 The chemical structures of compounds 1–6

Table 1 1H NMR data for 1–5 (δ in ppm)

| Position | δ_H, mult, (J in Hz) | 1 | 2 | 3 | 4 | 5 |
|-----------|----------------------|---|---|---|---|---|
| 1α        | 1.60, dt (13.2, 8.5) | 1.75, td (14.2, 4.3) | 1.57, overlap | 1.75, ddd (17.4, 11.2, 5.4) | 2.26, dd (12.0, 4.1) |
| 1a        | 1.98, overlap        | 2.17, dd (5.5, 3.2)  | 1.97, td (8.1, 1.9) | 2.28, dt (11.3, 2.7) | 2.45, ddd (15.3, 11.9, 5.7) |
| 1β        | 1.98, overlap        | 2.17, dd (5.5, 3.2)  | 1.97, td (8.1, 1.9) | 2.28, dt (11.3, 2.7) | 2.45, ddd (15.3, 11.9, 5.7) |
| 2α        | 2.48, dddd (16.1, 8.0, 4.1) | 2.31, td (14.5, 3.7) | 2.65, ddd (16.8, 8.6, 1.5) | 2.22, ddd (14.7, 11.4, 5.3) | 1.36, ddd (14.0, 11.9, 5.7) |
| 2β        | 2.53, dddd (16.1, 9.8, 7.6) | 2.78, dt (14.7, 5.4) | 2.73, ddd (16.7, 11.3, 1.7) | 2.70, ddd (14.3, 11.0, 2.0) | 2.01, overlap |
| 5α        | 1.95, overlap        | 1.59, dd (11.5, 4.4) | 1.80, dd (11.8, 4.1) | 1.65, ddd (11.8, 4.5) | 1.77, s  |
| 6α        | 1.78, m              | 2.07, t (5.0)  | 2.03, dtt (16.5, 6.0, 2.1) | 1.98, br d (13.7) | 2.05, dt (15.3, 2.9) |
| 6β        | 1.73 dt (13.9, 2.5)  | 2.22, td (5.4, 3.1) | 1.94, td (8.5, 3.1) | 1.94, dd (13.6, 2.5) | 2.20, overlap |
| 7β        | 3.94, s              | 5.47, d (5.2)  | 5.38, dt (3.9, 1.6) | 5.30, dd (5.3, 1.9) | 5.37, dd (4.7, 2.3) |
| 9α        | –                    | –               | 1.79, d (3.9)  | 1.89, overlap | –         |
| 11α       | 1.95, overlap        | 5.41, t (4.0)  | 1.36, dq (13.7, 3.3) | 1.40, ddd (13.4, 3.4) | 1.56, overlap |
| 11β       | 1.95, overlap        | 1.61, overlap | 1.54, ddd (12.6, 6.5, 2.8) | 1.56, overlap | 1.56, overlap |
| 12α       | 1.37 dddd (12.8, 9.8, 5.9) | 2.13, overlap | 1.37, dt (143, 12.5) | 1.35, d (7.4) | 1.45, dt (5.7, 2.7) |
| 12β       | 1.55 dddd (12.8, 5.7, 4.2, 1.7) | 1.99, dd (17.9, 3.5) | 1.51, dt (9.4, 2.9) | 1.49, dt (9.2, 2.7) | 1.52, overlap |
| 14α       | 1.76 dd (12.6, 4.1)  | 2.13, overlap | 1.94, d (2.5)  | 1.98, brd (13.7) | 2.23, overlap |
| 14β       | 2.39, d (17.1)       | 2.13, overlap | 1.99, overlap | 1.94, overlap | 2.23, overlap |
| 15         | 5.72 dd (17.5, 10.8) | 5.78, dd (17.5, 10.7) | 5.80, dd (17.5, 10.7) | 5.80, dd (17.5, 10.7) | 5.60, dd (17.6, 10.9) |
| 16α        | 4.96 dd (17.5, 1.3)  | 4.92, dd (17.5, 1.4) | 4.92, dd (14.1, 1.2) | 4.91, dd (17.5, 1.2) | 4.98, dd (17.6, 1.4) |
| 16β        | 4.93 dd (10.8, 1.3)  | 4.88, dd (10.8, 1.4) | 4.90, dd (11.9, 1.2) | 4.87, dd (10.7, 1.2) | 4.92, dd (10.9, 1.4) |
| 17         | 1.00, s              | 0.98, s       | 0.87, s       | 0.86, s       | 0.96, s       |
| 18         | 1.12, s              | 1.06, s       | 1.42, s       | 1.21, s       | 1.28, s       |
| 19         | 1.06, s              | 1.12, s       | 1.55, s       | 1.34, s       | 1.27, s       |
| 20         | 1.01, s              | 1.19, s       | 1.03, s       | 1.0, s        | 1.13, s       |
| OCH3       |                     | 3.65, s       | 3.66, s       |               |               |

The 1H NMR (600 MHz) spectral data of 1 and 3–5 were measured in CDCl3, and the 1H NMR (400 MHz) spectral data of 2 were measured in CDCl3.
groups (3H, δH 1.00, s; 1.01, s; 1.06, s; 1.12, s) and three olefinic groups (1H, δH 5.72, dd, J = 17.5, 10.8; 4.96, dd, J = 17.5, 1.3; 4.93, dd, J = 10.8, 1.3). Moreover, analyses of the 1D 13C and DEPT-135 NMR data (Table 2), combined with the HSQC correlation data, allowed to identify the 20 carbon resonances in 1 as one carbonyl carbon (δC 217.5), five quaternary carbons including two non-protonated olefinic carbons (δC 127.5 and 140.0), one methine (δC 45.3), one oxygenated methine (δC 68.8), six methyl- enes (δC, 34.7; 34.4; 29.5; 21.3; 34.6; 39.2), two protonated olefinic carbons (δC 145.8 and 111.6), and four methyl groups (δC 28.1; 26.7; 21.3; 17.9). The above NMR spectral data suggested that compound 1 belongs to a pimarane or an isopimarane diterpenoid. Based on the analysis of the correlations obtained from the 1H-1H COSY and HMBC spectra (Fig. 2), the ketone carbon at δC 217.5 is assigned at C-3, the four olefinic carbons at δC 127.5/140.0 and δC 111.6/145.8 are assigned as the two double bonds of Δ8,9 and Δ15,16 respectively, and the oxygenated methine carbon at δC 68.8 is assigned at C-7. The relative configuration of 1 was established through the analysis of the NOE correlations obtained from the NOESY spectrum of 1. First of all, the presence of the NOE correlations between H-5 and H3-18 and between H3-19 and H3-20 indicated the possibility of a trans-fused A/B ring system (Fig. 3). In addition, the observed NOEY cross-peaks between H-7 and H-14β and between H-14β and H3-17 determined the co-facial relationship of the protons of H-7 and H3-17. The resulted Flack number of 0.07 (10) from the refinement of the X-ray crystallographic data on CuKα radiation allows the determination of the absolute configuration of 1 to be the same as an isopimarane diterpenoid (Fig. 4). The structure of compound 1 was thus assigned as isopimara-7α-hydroxy-8,15-diene-3-one, and given the trivial name isoamethinol A.

Compound 2, colorless oil, was found to possess a molecular formula of C20H28O by the HR-ESI-MS data [m/z 285.2206 [M+H]+ (calcd for 285.2213)]. The similar coupling pattern and chemical shifts of the 1H and 13C NMR spectroscopic data to 1 were observed for 2 (Tables 1 and 2). Compound 2 differs from 1 by lacking the hydroxyl group at C-7 but having three olefinic double bonds in the structure. The three double bonds in 2 are assigned as Δ7,8, Δ9,11 and Δ15,16, respectively, by analyzing the 2D correlation data of the 1H-1H COSY, HMQC and HMBC NMR spectra (Fig. 2). Compound 2 shared the similar key NOE correlation pattern (H-1/H-20, H-18/H-5, H-19/H-20, H-12β/H-17) to 1, revealing that 2 is also an isopimarane diterpenoid. The structure of 2 was thus established as

| Position | δC type | 1 | 2 | 3 | 4 | 5 |
|----------|----------|---|---|---|---|---|
| 1        | 34.7     | 36.5 | CH2 | 33.3 | CH2 | 32.3 | CH2 | 31.7 | CH3 |
| 2        | 34.4     | 34.9 | CH2 | 31.9 | CH2 | 29.3 | CH2 | 30.9 | CH2 |
| 3        | 217.5    | C   | 216.7 | C | 175.8 | C | 175.7 | C | 174.7 | C |
| 4        | 46.8     | C   | 47.8 | C | 864 | C | 751 | C | 81.6 | C |
| 5        | 45.3     | CH  | 50.2 | CH | 51.7 | CH | 49.5 | CH | 49.7 | CH |
| 6        | 29.5     | CH2 | 24.3 | CH2 | 27.0 | CH2 | 27.3 | CH2 | 28.3 | CH2 |
| 7        | 68.8     | CH  | 122.6 | CH | 121.1 | CH | 121.4 | CH | 123.3 | CH |
| 8        | 127.5    | C   | 131.6 | C | 135.8 | C | 135.9 | C | 137.9 | C |
| 9        | 140.0    | C   | 145.2 | C | 49.4 | CH | 44.5 | CH | 81.8 | C |
| 10       | 37.9     | C   | 36.7 | C | 38.5 | C | 38.9 | C | 46.5 | C |
| 11       | 21.3     | CH2 | 117.1 | CH  | 204 | CH2 | 19.5 | CH2 | 25.3 | CH2 |
| 12       | 34.6     | CH2 | 38.3 | CH2 | 36.2 | CH2 | 36.2 | CH2 | 34.5 | CH2 |
| 13       | 35.1     | C   | 36.1 | C | 36.9 | C | 36.9 | C | 36.5 | C |
| 14       | 39.2     | CH  | 43.5 | CH2 | 46.2 | CH2 | 46.1 | CH2 | 41.7 | CH2 |
| 15       | 143.8    | CH  | 146.4 | CH | 150.0 | CH | 150.3 | CH | 145.4 | CH |
| 16       | 111.6    | CH2 | 110.9 | CH2 | 109.1 | CH2 | 109.5 | CH2 | 113.4 | CH2 |
| 17       | 28.1     | CH2 | 26.5 | CH2 | 21.6 | CH2 | 21.6 | CH2 | 30.0 | CH2 |
| 18       | 26.7     | CH2 | 24.5 | CH2 | 34.1 | CH2 | 34.2 | CH2 | 31.2 | CH2 |
| 19       | 21.3     | CH2 | 22.4 | CH2 | 24.3 | CH2 | 27.0 | CH2 | 27.4 | CH2 |
| 20       | 17.9     | CH2 | 20.8 | CH2 | 15.4 | CH2 | 18.2 | CH2 | 22.6 | CH2 |
| OCH3     |         | 51.7 | CH3 | 51.7 | CH3 |

The 13C NMR (150 MHz) spectral data of 1 and 3–5 were measured in CDCl3 and the 13C NMR (100 MHz) spectral data of 2 were measured in CDCl3.
isopimara-7,9(11),15-triene-3-one, and given the trivial name isoamethinol B.

Compound 3 was obtained as colorless crystals (MeOH). Given the HR-ESI–MS molecular ion peak at $m/z$ 325.2129 [M + Na]$^+$ (calcd 325.2138) and the molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_{2}$, 3 is calculated to have one less degree of unsaturation than 2. By interpretation of the correlation data of the HMBC and $^1\text{H}$$-$$^1\text{H}$ COSY,
spectra (Fig. 2), 3 was elucidated to be a ring-expanded ω lactone congener of 2. Specifically, the absence of the HMBC correlations of H₃-18 to C-3 and H₃-19 to C-3 in 3 (Additional file 1: Figs. S25 and S26 combined with the observation of the upfield chemical shift of C-3 (δ_C 175.8) and a downfield chemical shift of C-4 (δ_C 86.4) in 3 in comparison of 2, revealed that the ω lactone is formed via an oxygen insertion at the position between C-3 carbonyl carbon and C-4. Compared with 2, compound 3 contains a less olefinic double bond, which is determined to occur at C-8 and C-9. These structure features in 3 are evidenced by the disappearance of the characteristic double bond Δ⁹,¹¹ (δ_C 117.1, 145.2) resonance signals (Table 2), the upfield shift of the carbonyl carbon (from δ_C 216.6 to 175.8) and the downfield shift of the gem methyl group connected quaternary carbon (from δ_C 47.8 to 86.4) in comparison with 2. It is worthy to mention that a pimarane diterpene with ring A being oxidated to a lactone like 3 is rarely discovered in nature.

The relative stereochemistry of 3 was determined by a NOESY experiment. The presence of the NOE cross-peaks of H₃-20/H-11α, H₃-20/H₃-19 and H-11α/H₃-17 demonstrated that the three methyl groups of C-17, C-19 and C-20 are co-facial and β-orientated. H-5 and H-9 were established as α-oriented by the observation of the NOE correlations between H-5 and H₃-18 and between H-9 and H₃-18. In addition, the X-ray diffraction results further confirmed the structure elucidated from the above deduction (Fig. 4). As a result, 3 was identified as isopimara-7,15-diene-3,4-lactone and given the trivial name isomethinol C.

Compound 4, colorless needle crystals (MeOH), was determined to possess 5 degrees of unsaturation calculated by the molecular formula of C₂₁H₃₄O₃, which was obtained through the analysis of its HR-ESI-MS data [m/z 357.2397 [M + Na]⁺ (calcd for C₂₁H₃₄O₃Na, 357.2400)]. It has one unsaturation degree less than 3. The complete assignments of the 1D and 2D NMR signals (Tables 1

Fig. 4 X-ray crystallographic structures of 1, 3 and 4
and 2, and Fig. 2) allowed us to identify a methyl propaneate ester group \([\delta_H \text{ } 3.65 \text{ (3H, s, OCH}_3\text{)}, \text{ 1.75 (1H, ddd, } J = 17.4, 11.2, 5.4, \text{ H-1a)}, \text{ 2.28 (1H, dt, } J = 11.3, 2.7, \text{ H-1}\beta])	ext{, 2.22 (1H, ddd, } J = 14.0, 11.9, 5.7, \text{ H-2}\alpha), \text{ 2.70 (1H, ddd, } J = 14.3, 11.0, 2.0, \text{ H-2}\beta), \text{ } \delta_C \text{ 51.7 (OCH}_3\text{)}, \text{ 32.3 (C-1), 29.3 (C-2), 175.7 (C-3, the ester carboxyl carbon)]} \) linked to C-10. The analysis of the NMR data also assigned an oxygenated tertiary carbon at C-4, which is attached with two gem methyl groups to form a 2-hydroxy propanoyl group. Collectively, compound 4 was elucidated as a ring A opened methyl ester derivative. The key NOE correlations observed between H-5 and H-9 and between H-20 and H-11β determined that the propanoate ester group and the 2-hydroxy propanoyl group are trans-oriented with each other. Moreover, the presence of the cross-peaks of H3-17/H-11β and H3-20/H-11β revealed the β-orientation of the methyl groups at C-10 and C-13. Finally, a single-crystal X-ray diffraction study (CuKa radiation) with a Flack parameter of \(-0.01(6)\) determined the absolute configuration of 4 shown in Fig. 4. Accordingly, 4 was determined as 3,4-seco-isopimara-4-hydroxy-7,15-diene-3-oic acid methylester, and given the trivial name isoamethinol D.

Compound 5 was obtained as yellow oil. The analysis of the molecular ion peak \([M+H]^+\) at \(m/z 333.2420\) (calcd 333.2424) measured in the HR-ESI-MS spectrum rendered the molecular formula of 5 as \(C_{21}H_{32}O_3\), which has 6 degrees of unsaturation and two protons less than that of 4. Compound 5 showed very similar \(^1H\) and \(^{13}C\) NMR spectral data to 4. The one more degree of unsaturation of 5 than 4 suggested that additional ring could be formed in 5. Through the total analysis of the 1D and 2D NMR data (Tables 1 and 2, and Fig. 2), the additional ring was found to be formed by the bond connection between C-4 and C-9. Moreover, 5 shared high similarity of the proton NMR spectral data to our previously identified compound fladin A, except for an additional methoxy group (\(3H, \delta_H 3.66, s, \delta_C 51.7\)) observed in 5 [17]. Herein, compound 5 is a methyl ester congener of fladin A. The absolute configuration of 5 was assigned the same as 4, which was deduced by the consideration of the biosynthetic origins and the similar NOE correlation patterns observed between the two compounds. Compound 5 was thus identified as 3,4-seco isopimara-4,9β-epoxy-7,15-diene-3-oic acid methylester, and given the trivial name isoamethinol E.

Compound 6 was identified as the known 3,4-seco isopimara-4(18),7,15-triene-3-oic acid methylester based on the comparison of the proton coupling patterns and chemical shifts of the \(^1H\) and \(^{13}C\) NMR data (Additional file 1: Figs. S50 and S51) with the reported literature data [11].

Compounds 1–5 were evaluated for their in vitro cytotoxic activities against a panel of human cancer cell lines (HL-60 promyelocytic leukemia cells, SMMC-7721 hepatocellular cancer cells, MCF-7 breast cancer cells, SW480 colon cancer cells, and Hela cervical carcinoma cells). Among these compounds, only 4 showed moderate cytotoxicities against Hela and A549 cells with IC\text{50} values of 27.21 and 21.47 μM, respectively (Fig. 5). These compounds were also evaluated for their activities against the gram-positive bacteria including methicillin-resistant strain \(Staphylococcus aureus\) (MRSA), \(Streptococcus sobrinus\), and \(S. mutans\). As a result, only 4 was demonstrated with mild antibacterial effect on \(S. mutans\) with an inhibitory rate of 42.1% at the concentration of 149.48 μM. In addition, the anti-HIV activities of these compounds were further explored for their antiviral potential using our previously established “One-Stone-Two-Birds” protocol [12]. However, no significant antiviral activities were observed for these compounds at a concentration of 25 μM.

**Conclusion**

Five new isopimarane and 3,4-seco isopimarane diterpenes, along with a known 3,4-seco isopimarane diterpene, were isolated from the leaves of \(I. amethystoides\). Their chemical structures and absolute configurations were determined by analysing their spectroscopic and X-ray crystallographic data. To the best of our knowledge, isoamethinol C (3) is the first naturally occurring 3,4-seco isopimarane diterpenoid containing a A-ring ω lactone ring, which expands the chemical diversity of isopimarane diterpenoid family. Isoamethinol D (4) showed
Extraction and isolation

The air-dried leaves of *I. amethystoides* (29 kg) were ground into powder and further extracted by MeOH at room temperature (3 times, 7 days each time) to yield a crude extract (3.2 kg), which was suspended in water (8 L), and partitioned with petroleum ether (PE) (3 × 8 L, 48 h each time) and ethyl acetate (EtOAc) (3 × 8 L, 48 h each time). The PE fraction (1.5 kg) was subjected to a silica gel column separation with gradient elution of PE/EtOAc (1:0 to 1:1) to provide fractions A–D. Fraction B (234 g) was further separated on a silica gel column using a gradient elution of PE/dichloromethane (CH₂Cl₂) (99:1 to 1:1) to afford compound 1 (156 mg) and 4 (305 mg). Fraction C (345 g) was separated on a silica gel column, eluting with gradient PE/EtOAc (49:1 to 1:1) to obtain 2 (18 mg) and 5 (200 mg). The EtOAc fraction (880 g) was subjected to fractionation over an MCI gel column using gradient MeOH/H₂O (50:50 to 90:10) to yield fractions E–I. Fraction G (179 g) was subjected to a silica gel chromatographic separation using gradient (EtOAc/MeOH) (50:1 to 5:1) to produce sub-fractions G1–G4. G1 was subjected to a Sephadex LH-20 chromatographic column eluting with CH₂Cl₂/MeOH (1:1) to give 1 (23 mg). Fraction H (191 g) was further purified by a silica gel column, eluting with gradient CH₂Cl₂/MeOH (50:1 to 1:1) to afford 3 (156 mg) and 4 (90 mg).

**Isoamethinol A (1)**

Colorless cubic crystal, mp 88.6–89.1 °C; [α]²⁸.⁶D+ 46.67 (c 0.6, CH₂Cl₂); IR (KBr) ν max 3522, 2935, 2921, 2873, 1690, 1458, 1383, 1245, 1040, 932, 909 cm⁻¹; ¹H and ¹³C NMR (CDCl₃), see Tables 1 and 2; HR-ESI-MS m/z 325.2133 [M + Na]+ (calcd. for C₂₀H₃₀O₂Na, 325.2138).

**Isoamethinol B (2)**

Colorless oil; [α]²⁸.⁶D+ 69.54 (c 0.3, CH₂Cl₂); IR (KBr) ν max 3404, 2964, 1709, 1458, 1386, 1271, 1116, 1006, 910, 803 cm⁻¹; ¹H and ¹³C NMR (CDCl₃), see Tables 1 and 2; HR-ESI-MS m/z 285.2218 [M + H]+ (calcd. for C₂₀H₂₉O, 285.2218).

**Isoamethinol C (3)**

Colorless needle crystal, mp 83.3–83.6 °C; [α]²⁸.⁶D− 15.38 (c 0.26, CH₂Cl₂); IR (KBr) ν max 3421, 2957, 2918, 1726, 1639, 1389, 1371, 1225, 1108, 986, 910 cm⁻¹; ¹H and ¹³C NMR (CDCl₃), see Tables 1 and 2; HR-ESI-MS m/z 325.2129 [M + Na]+ (calcd. for C₂₀H₂₉O₂Na, 325.2138).

**Isoamethinol D (4)**

Colorless needle crystal, mp 84.36–85.3 °C; [α]²⁸.⁶D− 17.39 (c 0.23, CH₂Cl₂); IR (KBr) ν max 3491,
Crystallographic data of 1, 3 and 4
Crystals of 1, 3, and 4 were obtained from MeOH. Single-crystal X-ray crystallographic analyses of 1 and 4 were obtained on a Bruker D8 VENTURE diffractometer (Cu Kα radiation, \( \lambda = 1.54178 \) Å). The crystal structure of 3 was determined on a Bruker D8 D8 VENTURE diffractometer (Mo Kα radiation, \( \lambda = 0.71076 \) Å). The crystals of 1, 3, and 4 were kept at 160.0 K, 293.0 (2) K, and 170.0 K during data collection, respectively. Using OLEX2, the structures were solved with the Shelix structure solution program using direct methods, dual space, or intrinsic phasing, and refined with the Shelix refinement package using least-squares minimization [13–15].

Crystallographic data of 1
\[ \text{C}_{20}\text{H}_{30}\text{O}_2, ~ M = 302.44, ~ a = 9.1730 ~ (2) \, \text{Å}, ~ b = 11.5454 ~ (3) \, \text{Å}, ~ c = 16.8349 ~ (4) \, \text{Å}; ~ a = 90°, ~ \beta = 90°, ~ \gamma = 90°, ~ V = 1782.92 ~ (6) \, \text{Å}^3, ~ T = 160.0 \, \text{K}, \text{space group P2}_1\text{2}_1\text{2}_1, \text{Z} = 4, \mu (\text{Cu Kα}) = 0.54 \, \text{mm}^{-1}; 49814 \text{ reflections collected, 3644 independent reflections (R}_{\text{int}} = 0.0617, \text{R}_{\text{sigma}} = 0.0275). \]

The final R1 values were 0.0438 \( > 2\sigma (I) \). The final wR \( (F^2) \) values were 0.1161 \( > 2\sigma (I) \). The final R1 values were 0.0484 (all data). The final wR \( (F^2) \) values were 0.1188 (all data). The goodness of fit on \( F^2 \) was 1.049. Flack parameter = 0.07 (10).

Crystallographic data of 3
\[ \text{C}_{20}\text{H}_{30}\text{O}_2, ~ M = 302.44, ~ a = 6.238 ~ (2) \, \text{Å}, ~ b = 9.613 ~ (4) \, \text{Å}, ~ c = 29.043 ~ (13) \, \text{Å}; ~ a = 90°, \beta = 90°, \gamma = 90°, \text{V} = 1782.92 ~ (6) \, \text{Å}^3, ~ T = 293.0 ~ (2) \, \text{K}, \text{space group P2}_1\text{2}_1\text{2}_1, \text{Z} = 4, \mu (\text{Mo Kα}) = 0.07 \, \text{mm}^{-1}; 18262 \text{ reflections collected, 3072 independent reflections (R}_{\text{int}} = 0.0203, \text{R}_{\text{sigma}} = 0.0133). \]

The final R1 values were 0.0290 \( > 2\sigma (I) \). The final wR \( (F^2) \) values were 0.0720 \( > 2\sigma (I) \). The final R1 values were 0.0297 (all data). The final wR \( (F^2) \) values were 0.0725 (all data). The goodness of fit on \( F^2 \) was 1.055. Flack parameter = −0.2 (3).

Cytotoxicity assay
A panel of human cancer cell lines (HL-60, SMMC-7721, MCF-7, SW480, and Hela) were utilized to determine the cytotoxic effects of compounds 1–5 using MTS method as previously reported [16], HL-60, MCF-7, and Hela cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), where the SMMC-7721 and SW480 cells were cultured in RPMI-1640. Cells were seeded at 4000 cells/190 µL per well in 96-well plates in their corresponding medium before adding 10 µL test samples. Serial concentrations (1.25–40 µM) of each tested compound were added in wells, and the plates were incubated for 48 h. Spent media was decanted after 48 h incubation. To each well, 100 µL of fresh media and 20 µL of 2 mg/mL MTS stock solution were added and the cells were incubated for 3 h at 37 °C. Afterwards, the optical density was measured at 515 nm using an ELISA plate reader. Both cisplatin and paclitaxel were used as positive controls.

Antibacterial assay
The bacteriostatic activity assay for compounds 1–5 was carried out using the standard microdilution method in 96-well plates as previously described [17]. Each well contained 50 µL two-fold serially diluted test agents, 50 µL growth medium (Tryptic Soy Broth Medium for MRSA, Brain Heart Infusion for S. mutans and S. sobrinus, respectively), and 10 µL of an overnight culture of strains, representing approximately \( 5 \times 10^7 \) CFU mL\(^{-1}\). The controls comprised inoculated growth medium without test agents and sample blanks in growth medium only. The plates were incubated overnight at 200 rpm, 37 °C. The \( \text{OD}_{600} \) values were obtained using the microplate reader.

Anti-HIV assay
Compounds 1 to 5 were also evaluated for their anti-HIV activity using our previously established “One-Stone-Two-Birds” protocol [18]. Human embryonic kidney cells 293 T were transiently transfected with
0.5 μg VSV-G envelope expression plasmid and 2 μg Env-deficient HIV vector (pNL4.3. Luc-R-E-) in 10 cm plates via PEI (polyethyleneimine). Sixteen hours post-transfection, all media was replaced with fresh, complete DMEM. Forty-eight hours post-transfection, the supernatants were collected and filtered through a 0.45 μm-pore size filter, and the pseudovirions were directly used for infection. Targeted A549 cells were seeded at 4000 cells/190 μL per well in 96-well plate in DMEM supplemented with 10% FBS. The tested samples were added, 10 μL each, with a final concentration of 5 and 25 μM. Forty-eight hours post-infection, cells were lysed and prepared for luciferase assay.

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Author contributions

ZH and ZJ designed the overall experiments. ZC, ZL and XW performed most of the chemistry-related experiments including separation and structure determination of the reported compounds with support of ZC. ZL and YJ performed most of the biology-related experiments with support of ZJ. HK and PL collected the plant materials, and prepared the plant extracts. ZC, ZL and XW are major contributors in writing the manuscript. ZH, ZJ and PL edited and revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its Additional file 1.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no competing interest to others.

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