Lonimacranaldes A–C, three iridoids with novel skeletons from Lonicera macranthoides†

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Lonimacranaldes A and B (1 and 2), along with one biogenetically related intermediate, lonimacranalde C (3), were isolated from the flower buds of Lonicera macranthoides. Characterized by an iridoid structure and an additional C-6 unit with an aldehyde group, compounds 1 and 2 are the first examples of hybrid iridoids possessing an unexpected 6/5/6 fused tricyclic ring system, while compound 3 serves as an important precursor for their generation. The structures of lonimacranaldes A–C (1–3) were revealed by extensive spectroscopic and X-ray diffraction analyses. A plausible biogenetic pathway for them was proposed. Compound 3 showed anti-inflammatory activities by inhibiting the production of IL-6 on LPS-induced RAW 264.7 cells with an IC₅₀ value of 6.33 μM.

In our continuous efforts to discover new bioactive iridoids from the flower buds of Lonicera macranthoides, three iridoids with novel skeletons (lonimacranaldes A–C, 1–3) were isolated unexpectedly. The common structural feature among 1–3 was biogenetically derived from two precursors, an iridoid (secoiridoid), and a volatile component with C-6 unit (hexanal, 5), both of which had been reported in Lonicera genus before.16,17 Obviously, the aldol condensation of the two precursors led to 3, while 1 and 2 might be derived from 3 by an oxidative reaction and an intramolecular Diels–Alder reaction to form an unexpected 6/5/6 fused tricyclic ring skeleton system (Fig. 1, rings A/B/C). Herein, we reported the isolation, structure elucidation, and plausible biosynthetic pathway of 1–3, and their potential anti-inflammatory activities.

Results and discussion

Lonimacranalde A (1) was obtained as white powder and subsequently crystallized from EtOH/H₂O as colorless needles with negative rotation ([α]D²⁰ = 18.7, c = 0.6, in CH₃OH). The UV
spectrum showed a characteristic absorption maximum of iridoid at 232 nm (log ε 3.9), while the IR spectrum showed an absorption band of hydroxyl (3366 cm⁻¹) and carbonyl (1700 cm⁻¹). Its molecular formula was determined as C_{12}H_{20}O_{10} based on HR-ESI-Q-TOF-MS (m/z 477.1747 [M + Na]⁺, calecd for C_{12}H_{20}O_{10}Na, 477.1737). The ¹H NMR spectrum of 1 exhibited characteristic signals of iridoid glycosides, including an acetal proton [δ_H 5.21 (1H, d, J = 7.1 Hz, H-1)], a trisubstituted olefinic proton [δ_H 7.48 (1H, d, J = 1.2 Hz, H-3)], and an anomeric proton [δ_H 4.72 (1H, d, J = 7.9 Hz, H-1')]. In addition, a proton signal assignable to an aldehyde group [δ_H 9.38 (1H, s, H-1')] was also observed. The sugar moiety of 1 was determined to be β-D-glucose by HPLC analysis after acid hydrolysis and glycosyl derivatization. Combined with the J value of the anomeric proton mentioned above, a β-D-glucosyl residue was deduced.

Besides signals attributable to a glucosyl residue, the rest carbons were revealed as two carbonyls (δ_C 196.4, 170.9), two trisubstituted double bonds (δ_C 158.1, 153.5, 145.1, 112.2), and ten sp³ carbons, which comprised six methines (δ_C 98.6, 46.1, 38.4, 36.1, 35.7, 35.2), three methylenes (δ_C 38.9, 32.1, 28.7), and a methyl (δ_C 11.8) according to ¹³C NMR and DEPT 135 spectra. All of the proton and carbon signals were assignable by COSY, HSQC, and HMBC spectra (Table S1†).

The aglucone of compound 1 was determined by the COSY correlations of H-1/H-9(H-8)/H-5/H-3/H-7/H-8/H-10/H-4(H-3’)/H-5’/H-6’, together with the HMBC correlations of H-3/C-1, 4, 5, 11, H-1/C-3, 5, 8, 9 and H-1’/C-7, 2’, 3’, (Fig. 2). Moreover, other HMBC correlations of H-1’/C-1 and H-1/C-1’ revealed that the β-D-glucosyl was located at C-1. Hence, the planar structure of compound 1 was established.

The cyclopentanopyran ring in iridoids was commonly H-5/H-9 β,β-cis-fused, while H-1 α-oriented. Since the C-1 resonance was at δ 98.6 (compared to δ 101–103 for trans-fused iridoids) and the allylic coupling of the H-3/H-5 resonances was 1.2 Hz (compared to 2.0–2.5 Hz for trans-fused iridoids)†, the H-5/H-9 in 1 could be preliminarily assumed as β,β-cis-fused. Then, based on the NOE correlations of H-1/H-1/H-7 and H-1/H-8, A/B and B/C rings in 1 were determined as H-5/H-9 β,β-cis-fused, and H-7/H-8 α,α-cis-fused, respectively. Furthermore, the NOE correlations of H-4’/H-9 indicated that H-4’ was β-oriented. For the deductions above, the relative configurations in 1 were all consistent with the absolute configurations determined by X-ray (Fig. 3). Consequently, the structure of 1 was unmistakably and completely confirmed and named lonimacranalde A.

Lonimacranalde B (2), isolated as colorless needle ([α]²⁰⁰ D = 9.7, c = 0.6, in CH₂OH), has the same molecular formula with 1 based on the HR-ESI-MS data. The ¹H NMR and ¹³C NMR data of 2 were assignable by COSY, HSQC, and HMBC spectra (Table S1†), which were highly similar to 1, suggesting that they were two isomers.

### Table 1 ¹H NMR and ¹³C NMR data for compounds 1–3

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*Recorded at 600 (¹H) and 150 MHz (¹³C) in CD₃OD. Multiplets or overlapped signals are reported without designating multiplicity.*
There is no single crystal data of 2, so its configurations were solved by analyzing the coupling constants and NOESY data, as well as the CD spectrum. Similarly, for the NOE correlations of H-6 (δH 2.23)/H-1, H-1/H-7 and H-1/H-8, A/B and B/C rings in 2 were determined as β,β-cis-fused and α,α-cis-fused, respectively, as well. The coupling constants of H-10a, H-4', and H-8 (J10a,4' = 11.0 Hz, J10a,8 = 11.0 Hz) indicated that H-10a/H-4' and H-10a/H-8 were a/a coupling. Since that, H-4' was β-oriented as H-8. It also meant that the dominant conformation of ring C in compound 2 was the same as 1 with 4'-ethyl in equatorial bond. The NOE correlations of H-9/H2-10 and H-10b (δH 1.88)/H3-6 further provided more evidences for such deduction (Fig. 4). Obviously, 2 was a C-4’ epimer of 1, which can further be validated by their experimental CD spectra with the same Cotton effects (Fig. 5). In conclusion, compound 2 was determined and named lonimacranalde B.

Lonimacranalde C (3), obtained as light yellow powder ([α]D25 - 150.4, c = 0.3, in CH3OH), its molecular formula was determined as C22H32O10 on the basis of the HR-ESI-MS data (m/z 479.1895 [M + Na]⁺, calcd for C22H32O10Na: 479.1893). Compound 3 was considered as a secoiridoid-type glycoside for the typical protons attributed to an acetal [δH 5.55 (1H, d, J = 5.6 Hz, H-1)], a tri-substituted olefinic proton [δH 7.52 (1H, d, J = 1.1 Hz, H-3)], and a mono-substituted vinyl group [δH 5.77 (1H, ddd, J = 17.1, 10.4, 8.9 Hz, H-8), 5.27 (2H, m, H2-10)], together with an anomeric proton [δH 4.69 (1H, d, J = 7.9 Hz, H-1')]. Additionally, the sugar moiety in 3 was identified as β-D-glucosyl by comparing the NMR data with 1.

According to the COSY correlations of H-1/H-9/H-5/H2-6/H-7 and H-9/H-8/H2-10, as well as the HMBC correlations of H-1/
to generate 1 and 2 through a [4 + 2] cycloaddition. The cycloaddition has endo preference and occurs with remote stereocontrol syn to the substituent at the stereogenic center, which can explain the epimerization of 1 and 2 in a certain extent. This plausible biogenetic pathway of 1–3 has not been validated, but it provides a reference for their biosynthesis and total synthesis.

Anti-inflammatory activities of lonimacralandes A–C (1, 2 and 3) were evaluated in LPS-stimulated RAW 264.7 cells, including NO (nitric oxide), IL-6 (interleukin 6) and TNF-α (tumor necrosis factor) inhibitory activities. As a result, compound 3 exhibited inhibitory effect on IL-6 with an IC₅₀ value of 6.33 μM (Fig. 6). Unfortunately, none of them displayed TNF-α and NO inhibitory activity at 100 μM.

In order to confirm that compounds 1–3 are indeed natural products, the crude methanol extract of dried flower buds of L. macranthoides was analyzed by UPLC-Q-TOF-MS (see the ESI†). The ion peaks in accord with those of 1–3 were detected, which confirmed the natural occurrence of these compounds.

Conclusions
In conclusion, we have isolated three novel hybrid iridoids, lonimacralandes A–C (1–3) from the flower buds of Lonicera macranthoides. Their unique architectures and remarkable anti-inflammatory activities of compound 3 should receive considerable attention from synthetic chemists and pharmacologists.

Experimental
General experimental procedures
$^1$H and $^{13}$C NMR spectra were measured on a Bruker AV 600 (Bruker Co. Ltd., Bremen, German) at 600 and 150 Hz with solvent signals (CD₃OD, δ_H 3.10/δ_C 49.0) as internal reference. IR spectra were taken on a JASCO FT/IRBCA plus spectrometer (JASCO International Co. Ltd., Tokyo, Japan) with KBr. UV spectra were acquired using a JASCO V-550 UV/Vis spectrometer (JASCO International Co. Ltd., Hachioji, Tokyo, Japan). Optical rotation was measured on JASCO P-1020 digital polarimeter (JASCO International Co. Ltd., Hachioji, Tokyo, Japan). ECD spectra were recorded in methanol using a JASCO J-810 spectrophotometer (Jasco Internal Co. Ltd, Tokyo, Japan). HRESIMS spectra were measured on a Waters Synapt G2 mass spectrometer (Waters, Manchester, U.K.) with a RP-18 column (1.7 m, φ 3.0 × 150 mm; BEH). HPLC analyses were performed on a Waters 2695 separations module (Waters, Manchester, U.K.) equipped with a 2998 photodiode array (PDA) detector and an Alltech 3300 evaporative light scattering detector (ELSD; Alltech Inc., Deerfield, Illinois, U.S.A.) using a Phenomenex Gemini C18 column (5 μm, φ 4.6 × 250 mm; FLM Inc., Guangzhou, China). The semi-preparative HPLC analyses were performed on a Waters 1515 isocratic HPLC pump (Waters, Manchester, U.K.) coupled with a 2489 UV/Vis detector (Waters, Manchester, U.K.) and a Phenomenex Gemini C18 column (5 μm, φ 10 × 250 mm; FLM Inc., Guangzhou, China). The methanol of HPLC grade was purchased from BCR International Co. Ltd. (Shanghai, China), while the acetonitrile was purchased from Merck (Darmstadt,
Germany). Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan), silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Shandong, China), ODS-silica gel (12 nm, S-50 μm, YMC Ltd., Tokyo, Japan) and Sephadex LH-20 (Amersham Pharmacia Biotech, Sweden) were used for chromatography column (CC). TLC was performed on pre-coated silica gel plate (SGF54, 0.2 mm, Yantai Chemical Industry Research Institute, Shandong, China).

Plant material

Dried flower buds of *L. macranthoides* were collected in Shandong Province and purchased from Kunyuan Pharmaceutical Co., Ltd. in Nov, 2014, and identified by Prof. G. X. Zhou of Jinan University. A sample (no. LM2014111) is deposited in Jinan University, Guangzhou, China.

Extraction and isolation

The dried flower buds of *L. macranthoides* (35 kg) were extracted twice with 70% ethanol (280 L, for 2 h each time) under reflux. The ethanol was evaporated under vacuum, affording a crude extract (8.5 kg), which was suspended in water and then subjected to an HP-20 macroporous resin CC (φ 20 × 85 cm) eluted with EtOH–H₂O (0:100, 30:70, 50:50, 5:95, v/v/v) to offer four fractions (LM-1–LM-4). LM-3 (700 g) was chromatographed over a silica gel CC (φ 110 × 420 cm) by using a CHCl₃–CH₂OH–H₂O gradient (95:5:0 to 60:40:8, v/v/v), yielding sixteen fractions (3A-3P). Fraction 3E (17.7 g) was separated by ODS (φ 3.3 × 33 cm) eluted with CH₂OH–H₂O (30:70 to 100:0, v/v) to offer eleven subfractions E1–E11. Fractions E6, was separated on a silica gel CC (CHCL₃–CH₂OH–H₂O, 80:20:2, v/v/v), Sephadex LH-20 CC (CH₂OH–H₂O, 50:50, v/v), followed semipreparative HPLC (CH₂CN–H₂O–HCOOH, 23:75:0.1, v/v/v) respectively, to furnish t₂ (tₚ: 15.3 min, 7.7 mg) and t₃ (tₚ: 17.0 min, 4.7 mg), while fractions E10 was purified by semipreparative HPLC (CH₂CN–H₂O–HCOOH, 30:70:0.1, v/v/v) to furnish t₃ (tₚ: 22.3 min, 2.0 mg).

**Lonimacranalde A (1).** White powder; [α]D₂⁰ = -18.7 (c 0.6, CH₂OH); ESI-MS: m/z 477 [M + Na]+; HR-ESI-MS: m/z 477.1747 [M + Na]+ (calcd for C₂₂H₃₀O₁₂Na, 477.1737); UV (CH₂OH) λmax (log e): 204 (3.9), 232 (3.9), IR (KBr) νmax 3366, 2880, 2357, 1700, 1414, 1070 cm⁻¹; ¹H and ¹³C NMR data see Table 1.

**Lonimacranalde B (2).** White powder; [α]D₂⁰ = -9.7 (c 0.6, CH₂OH); ESI-MS: m/z 477 [M + Na]+; HR-ESI-MS: m/z 477.1738 [M + Na]+ (calcd for C₂₂H₃₂O₁₂Na, 477.1737); UV (CH₂OH) λmax (log e): 206 (4.1), 230 (3.9); IR (KBr) νmax 3389, 2891, 2345, 1678, 1621, 1274, 1070 cm⁻¹; ¹H and ¹³C NMR data see Table 1.

**Lonimacranalde C (3).** Light yellow powder; [α]D₂⁰ = -150.4 (c 0.3, CH₂OH); ESI-MS: m/z 455 [M - H]; HR-ESI-MS: m/z 479.1895 [M + Na]+ (calcd for C₂₂H₂₂O₁₂Na, 479.1893); UV (CH₂OH) λmax (log e): 204 (3.9), 234 (4.2); IR (KBr) νmax 3400, 2926, 2357, 1675, 1627, 1272, 1070 cm⁻¹; ¹H and ¹³C NMR data see Table 1.

**X-ray crystallographic analysis**

**Crystal data for compound (1).** Data were collected using a Sapphire CCD with a graphite monochromated Cu Kα radiation, λ = 1.54184 Å at 150 K. Crystal data: C₂₂H₃₀O₁₂, M = ~454, space group P2₁; unit cell dimensions were determined as a = 13.1270(2) Å, b = 7.9802(1) Å, c = 13.2754(2) Å, α = 90.00°, β = 116.561(2)°, γ = 90.00°, V = 1243.90(4) Å³, Z = 2, Dₐ = 1.336 g cm⁻³, F(000) = 536.0, μ (Cu Kα) = 0.888 mm⁻¹. 23 017 unique reflections were collected until θmax = 74.16°, in which 4804 reflections were observed [F² > 4σ(F²)]. The structure was solved by direct methods using the SHELXS-97 program, and refined by the program SHELXL-97 and full-matrix least-squares calculations. In the structure refinements, non-hydrogen atoms were placed on the geometrically ideal positions by the “ride on” method. Hydrogen atoms bonded to oxygen were located by the structure factors with isotropic temperature factors. The final refinement gave R = 0.0360(4804), Rwr = 0.0899(4915), S = 1.042, and fack = -0.01(8). Crystallographic data for structure 1 has been deposited at the Cambridge Crystallographic Data Centre (CCDC 1877498).

**Anti-inflammatory activity assays**

**Cell culture and viability.** RAW 264.7 murine macrophage cell line was obtained from Chinese Academy of Sciences. The cells were grown in DMEM (Gibco, USA) containing 10% FBS (Gibco, USA), 100 U mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin. They were cultured at 37 °C in 5% CO₂. Cell viability was analyzed using MTT assay. Compounds was added to the cells and incubated for 1 h and then cells were treated with or without LPS for 18 h at 37 °C with 5% CO₂. MTT solution (5 g L⁻¹) was added to each well and incubated for 4 h at 37 °C. The formazan dyes in the cells were dissolved in 100 μL 10% SDS-HCl solution. The optical density was read at 570 nm (reference, 650 nm) using a microplate UV/VIS spectrophotometer (Tecan, Mannedorf, Switzerland). The cell viability in the control group (cells were not treated by compounds and LPS) was set as 100%.

**Effect of compounds 1–3 on the PGE₂, NO, TNF-α and IL-6 production.** Cells were seeded in a 96-well plate at a density of 1 × 10⁴ cells per well in DMEM and incubated for 24 h. The cells were pretreated with the compounds for 30 min and treated with LPS (1 μg mL⁻¹) for 24 h in the presence or absence of different concentrations of the compounds. The supernatant of the cell culture (100 μL) was harvested, and the concentration of NO was mixed with Griess reagent (100 μL, Sigma) at room temperature for 10 min. Absorbance was recorded at 550 nm with a microplate reader (Varioskan LUX, Thermo Fisher Scientific Inc., Waltham, MA, USA). The concentration of PGE₂, TNF-α and IL-6 in the culture medium was determined using commercially ELISA kits according to the instructions. The IC₅₀ values were calculated from calibration curves of the absorbance inhibition by each compound at three concentrations against the increasing absorbance of macrophage cells stimulated only with LPS (without the compounds). Data were obtained from three independent experiments (n = 3).

**Data analysis.** The data obtained are presented as the means ± SD of three independent experiments. A one-way analysis of variance (ANOVA) test was used for statistical analysis, followed by a Dunnett’s post hoc test for multiple comparisons. GraphPad
Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA) was used to perform the analyses.

Conflicts of interest
There are no conflicts to declare.

Acknowledgements
This work was supported by grants from the National Natural Science Foundation of China (program no. 81630097, 81602984 and 81803347). The authors are grateful to State Key Laboratory of New-Tech for Chinese Medicine Pharmaceutical Process, Jiangsu Kanion Pharmaceutical Co. Ltd. for their assistance of activity test.

Notes and references