Mumic acids A–E: new diterpenoids from mumiyo

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Abstract Five new diterpenoids belonging to labdane and isopimarane skeletons, mumic acids A–E (1–5), have been isolated from mumiyo. Their structures and absolute configurations were elucidated on the basis of spectroscopic data and chemical derivatization.

Keywords Mumiyo · Mumic acids A–E · Diterpenoid · Labdane

Introduction

Mumiyo, also known as mumijo, mumie, or shilajit, is a material often found as crusts in rock cracks or interstices in the alpine region of Central Asia. Mumiyo has been used as a traditional medicine in the former Soviet Union, India, and Tibet for more than 3000 years, and is currently available in numerous countries as a food supplement [1]. Although there are many claims on the activity of mumiyo, scientific evidences on the chemical components and bioactivity are lacking [1]. In our search for new bioactive compounds [2–11], the mumiyo in Kyrgyzstan was investigated, resulting in the isolation of five new diterpenoids, mumic acids A–E (1–5) and agathic acid (6) [12]. The structure elucidation of 1–5 are reported herein (Fig. 1).

Mumic acid A (1, [α]D{28} +9 (c 0.4, MeOH)) was isolated as a colorless oil, with molecular formula C_{22}H_{32}O_{6}, as determined by HRESITOFMS [m/z 415.2072 (M + Na) – 2.5 mmu]. IR absorptions suggested the presence of carbonyl (1737 and 1715 cm\(^{-1}\)) and hydroxy (3420 cm\(^{-1}\)) groups. The 1H NMR data (Table 1) of 1 suggested the presence of an oxygenated methine (\(d_H 5.30, \text{br s}\)) and 4 methyls (\(d_H 0.70, \text{s}; d_H 1.18, \text{s}; d_H 2.10, \text{s}; d_H 2.12, \text{s}\)). The 13C NMR data (Table 2) revealed 22 carbon resonances due to 3 carbonyls, 2 sp\(^2\) quaternary carbons, 2 sp\(^3\) quaternary carbons, 1 sp\(^2\) methines, 3 sp\(^3\) methines, an sp\(^2\) methylene, 6 sp\(^3\) methylenes, and 4 methyls. The 1H and 13C NMR data (Tables 1 and 2) of 1 showed similarities to those of agathic acid (6) isolated in this study, suggesting the structure of 1 as a labdane type of diterpenoid related to 6.

Analysis of the 1H–1H COSY of 1 (Fig. 2) revealed 3 partial structures, a (C-1–C-3), b (C-5–C-7), and c (C-9, C-11 and C-12). HMBC correlations of H-3 to C-1, C-5, C-9, and C-10 revealed the connectivity of partial structures a, b, and C-20 through C-10. The connectivity of partial structures a, b, C-18, and C-19 through C-4 was suggested by the HMBC correlations of H$_2$-18 to C-3, C-4, C-5, and C-10 of 1. The connectivity of partial structures a, b, C-18, and C-19 through C-4 was suggested by the HMBC correlations of H$_2$-18 to C-3, C-4, C-5, and C-19. HMBC correlations of H$_2$-17 to C-7, C-8, and C-9 indicated the connectivity of partial structures b, c, and C-17 through C-8. The presence of an acetoxy group at C-3 was deduced from the HMBC correlations of H-3 and a methyl (\(d_H 2.10, \text{s}\)) to a carbonyl (\(d_C 172.4\)). Finally, the HMBC correlations of H$_3$-16 to C-12, C-13, and C-14 and...
Table 1 $^{13}$H NMR data of mumic acids A–E (1–5) in CD$_3$OD at 300 K

|   | 1$^a$ | 2$^b$ | 3$^b$ | 4$^a$ | 5$^b$ |
|---|------|------|------|------|------|
| 1a | 1.43, m | 1.41, td, 13.7, 3.7 | 1.11, td, 13.4, 3.7 | 1.05, dd, 12.3, 4.3 | 1.29, dt, 13.4, 3.2 |
| 1b | 1.65, m | 1.63, br d, 12.8 | 1.85, br d, 12.7 | 2.15, t, 12.3 | 1.78, m |
| 2a | 1.71, m | 1.72, m | 1.52, m | 4.12, m | 1.60, m |
| 2b | 2.25, m | 2.22, br t, 14.6 | 1.96, m | 1.65, m |
| 3a | 5.30, br s | 5.33, br s | 1.11, br d, 13.4 | 1.02, d, 12.3 | 4.00, dd, 11.3, 3.1 |
| 3b | 2.22, td, 13.4, 3.7 | 2.39, t, 12.3 |
| 5 | 1.75, m | 1.82, dd, 12.2, 2.2 | 1.39, dd, 11.7, 4.4 | 1.35, d, 12.0 | 1.82, br d, 12.2 |
| 6a | 1.94, m | 1.93, m | 1.83, m | 1.25, m |
| 6b | 2.00, m | 2.00, m | 2.04, m | 2.00, m | 1.50, m |
| 7a | 1.98, m | 1.95, m | 1.93, m | 1.95, br t, 13.2 | 2.07, dd, 14.2, 3.8 |
| 7b | 2.44, m | 2.44, m | 2.43, dd, 10.7, 3.5 | 2.41, m | 2.24, br t, 14.2 |
| 9 | 1.71, m | 1.69, m | 1.63, br d, 11.0 | 1.80, m | 1.76, d, 9.2 |
| 11a | 1.55, m | 1.54, m | 1.54, m | 2.10, m | 1.51, m |
| 11b | 1.72, m | 1.70, m | 1.72, m | 2.34, m | 1.64, m |
| 12a | 2.00, m | 2.02, m | 2.02, m | 5.39, br t, 5.4 | 1.33, m |
| 12b | 2.30, m | 2.31, ddd, 13.6, 9.8, 4.2 | 2.30, ddd, 13.8, 9.6, 4.3 | 1.50, m |
| 14 | 5.66, s | 5.62, s | 5.60, s | 3.92, dd, 7.4, 4.4 | 5.32, s |
| 15a | 3.44, dd, 11.3, 4.4 | 3.50, dd, 11.3, 7.4 |
| 15b | 3.30, dd, 8.8, 2.2 |
| 16a | 2.12, s | 2.13, s | 2.12, s | 1.64, s | 3.36, dd, 11.2, 8.8 |
| 16b | 2.18, m | 2.10, m | 2.12, s | 1.64, s | 3.70, dd, 11.2, 2.2 |
| 17a | 4.57, s | 4.56, s | 4.54, s | 4.52, s | 0.96, s |
| 17b | 4.92, s | 4.91, s | 4.89, s | 4.87, s |
| 18 | 1.18, s | 1.25, s | 1.26, s | 1.26, s |
| 19 | 1.07, s | 1.07, s | 1.07, s | 1.07, s |
| 20 | 0.70, s | 0.65, s | 0.62, s | 0.68, s | 0.82, s |
| Ac | 2.10, s | 2.09, s | 5.49, d, 7.7 | 5.47, d, 8.0 |
| 1$'$ | 3.40, dd, 8.9, 7.7 | 3.40, dd, 9.1, 8.0 |
| 2$'$ | 3.42, dd, 8.9, 8.7 | 3.42, dd, 9.3, 9.1 |
| 3$'$ | 3.51, dd, 9.3, 8.7 | 3.52, dd, 9.6, 9.3 |
| 4$'$ | 3.73, d, 9.3 | 3.77, d, 9.6 |

$^a$ 400 MHz; $^b$ 700 MHz
analyses of the NOESY correlations (Fig. 3) and 1H–1H group at C-3 and carboxylic acids at C-4 and C-14. deduced to be a new labdane diterpenoid with an acetoxy H-14 to C-15 completed the structure of 1.

Selected 2D NMR correlations for mumic acid A (Fig. 2) and hydroxy (3393 cm−1) groups. The 1H NMR data (Table 1) of 1 suggested the presence of a sugar moiety. Except for the signals assigned to the sugar moiety, the 1H and 13C NMR data (Tables 1 and 2) of 2 are highly similar to those of 1, suggesting 2 to be a glycoside derivative of 1. The sugar moiety was identified as δ-glucuronic acid on the basis of HPLC analysis with chiral detector of the acid hydrolysate of 2. The HMBC correlation of H-1′ to C-19 and C-5′ suggested the pyranose form of the sugar moiety and the connectivity of C-19 and C-1′ through an oxygen atom. The ROESY correlations for H-1′/H-3′ and H-5′, and the 1H–1H coupling constant value of H-1′/H-2′ (7.7 Hz) indicated the α-orientation of H-1′. Further analysis of the 2D NMR data confirmed the structure of 2 as 19-O-β-δ-glucuronic acid-1.

Mumic acid C {3, [α]D 28 +5 (c 0.2, MeOH)} was isolated as a colorless oil and had molecular formula C26H38O10, as determined by HRESITOFMS [m/z 533.2398 (M + Na)⁺, Δ +3.5 mmu]. IR absorptions suggested the presence of carbonyl (1732 and 1716 cm−1) and hydroxyl (3420 cm−1) groups. The 1H NMR data (Table 1) of 3 suggested the presence of a sugar moiety. The differences in the 1H and 13C NMR data (Tables 1 and 2) of 3 are reminiscent to the differences observed between 1 and 2. Thus, 3 was assumed to be a 19-O-β-glucuronic acid derivative of agathic acid (6). Acid hydrolysis of 3 gave 6 and a sugar, which was identified as δ-glucuronic acid on the basis of HPLC analysis with chiral detector.

Mumic acid D {4, [α]D 25 +28 (c 2.0, MeOH)} was isolated as a colorless oil, with molecular formula C20H32O5, as determined by HRESITOFMS [m/z 375.2151 (M + Na)⁺, Δ +0.4 mmu]. IR absorptions suggested the presence of carbonyl (1694 cm−1) and hydroxyl (3370 cm−1) groups. The 13C NMR data (Table 2) of 4 revealed 20 carbon resonances due to 1 carbonyl, 2 sp² quaternary carbons, 2 sp³ quaternary

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**Table 2** 13C NMR data of mumic acids A–E (1–5) in CD3OD at 300 K

| No. | 1a | 2b | 3b | 4b | 5b | No. | 1a | 2b | 3b | 4b | 5b |
|-----|----|----|----|----|----|-----|----|----|----|----|----|
| 1   | 34.2| 34.0| 40.4| 49.1| 38.35| 15  | 172.1| 170.3| 170.3| 65.9| 80.7|
| 2   | 25.6| 25.5| 21.2| 65.5| 27.9 | 16  | 18.9 | 18.9 | 18.9 | 12.7| 63.9|
| 3   | 75.8| 74.8| 39.1| 47.6| 76.6 | 17  | 107.0| 107.3| 107.0| 108.6| 23.1|
| 4   | 47.0| 48.4| 45.7| 46.0| 54.9 | 18  | 24.6 | 24.4 | 29.3 | 29.6| 182.5|
| 5   | 51.3| 51.6| 57.9| 56.6| 51.4 | 19  | 180.3| 175.5| 177.2| 180.7| 12.1|
| 6   | 27.1| 26.7| 27.3| 26.8| 25.4 | 20  | 13.1 | 13.6 | 13.8 | 12.3| 15.5|
| 7   | 39.8| 39.7| 39.9| 39.4| 36.5 | COMe| 172.4| 172.1|     |     |     |
| 8   | 149.3| 148.9| 149.3| 148.9| 138.6| COMe| 21.2 | 21.1|     |     |     |
| 9   | 56.5| 56.4| 56.6| 57.6| 52.2 | 1′  | 95.4 | 95.3 |     |     |     |
| 10  | 41.2| 41.2| 41.6| 42.2| 38.39| 2‘  | 73.8 | 73.8 |     |     |     |
| 11  | 23.0| 22.9| 22.9| 23.8| 19.5 | 3’  | 78.2 | 78.1 |     |     |     |
| 12  | 40.6| 40.8| 40.8| 128.7| 31.1 | 4’  | 73.3 | 73.2 |     |     |     |
| 13  | 158.8| 161.8| 162.0| 135.1| 39.1 | 5’  | 78.0 | 77.5 |     |     |     |
| 14  | 118.7| 116.8| 116.7| 78.7 | 129.4| 6’  | 174.0c| 173.7c|     |     |     |

*a 100 MHz; b 175 MHz; c assigned from HMBC correlations

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Fig. 2 Selected 2D NMR correlations for mumic acid A (1)

H-14 to C-15 completed the structure of 1. Thus, 1 was deduced to be a new labdane diterpenoid with an acetoxy group at C-3 and carboxylic acids at C-4 and C-14.

The relative configuration of 1 was determined by analyses of the NOESY correlations (Fig. 3) and 1H–1H coupling constant data. The α-orientation of H-5, H-9, and H-18 was deduced from the NOESY correlations of H-5/H-9 and H-18, and those of H-20/H-2b and H-2-11 suggested the β-orientation of C-20. The small 1H–1H coupling constant value of H-3/H-2a and H-2b indicated H-3 to be β-oriented. Finally, the C-13–C-14 double bond was deduced to be of the E configuration based on the NOESY correlation of H-12a/H-14. Thus, the relative configuration of 1 was elucidated to be as shown in Fig. 1.

Mumic acid B {2, [α]D 223 −7 (c 0.3, MeOH)} was isolated as a colorless oil and had molecular formula C22H34O12, as determined by HRESITOFMS [m/z 591.2438 (M + Na)⁺, Δ +2.1 mmu]. IR absorptions suggested the presence of carbonyl (1743 and 1721 cm−1) and hydroxy (3393 cm−1) groups. The 1H NMR data (Table 1) of 2 suggested the presence of a sugar moiety.
carbons, 1 sp² methine, 4 sp³ methines, an sp² methylene, 6 sp³ methylenes, and 3 methyls. Analysis of the 2D NMR correlations of 4 (Fig. 4) revealed the structure of 4 to be a new labdane diterpenoid with hydroxyl groups at C-2, C-14, and C-15, and carboxylic acids at C-19.

The configuration of 4 was determined as follows. The α-orientation of H-5, H-9, and C-18 was deduced from the NOESY correlations of H-5/H-9 and H-3-18, and the ROESY correlation of H-3-20/H-2 and H-2-11 suggested the β-orientation of H-2 and C-20. The double bond of C-12–C-13 was deduced to be of the E configuration based on the NOESY correlation of H-12/H-14. C-2 was determined to be of the R configuration based on the advanced Mosher’s method [13]. The absolute configuration of C-14 of the terminal 1,2-diol was deduced to be S configuration based on the vicinal coupling constant value of H-14/H-15a and H-15b (5.5 Hz) and the Cotton effect (CE) signs [238 (Δε: -12.5), 229 (0), and 221 (+6.9) nm] of the 3,15,16-tribenzoyl-18-methyl-derivative of 5 [14] indicated the absolute configuration of C-15 of the terminal 1,2-diol to be R. Thus, 5 was deduced to be a new isopimarane diterpenoid with hydroxyl group at C-3, C-15, and C-16, a carboxylic acid at C-18, and C-8–C-14 double bond.

Compounds 1–5 were tested for cytotoxic activity against the HL-60 (human promyelocytic leukemia) cell line, LPS-induced NO production inhibitory activity on the RAW264.7 (murine leukemic monocyte macrophage) cell line, melanin-production inhibitory activity on the B16F10 (murine melanoma) cell line, lipid-droplet accumulation inhibitory activity on the MC3T3-G2/PA6 (mouse pre-

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adipocyte) cell line, and vasorelaxant activity on rat aortic artery. All compounds gave negative results for these bioactivity assays.

Experimental section

General experimental procedures

Optical rotations were measured on a JASCO DIP-1000 polarimeter. UV spectra were recorded on a Shimadzu UV-mini-1240 spectrophotometer and IR spectra on a JASCO FT/IR-4100 spectrophotometer. CD spectra were recorded on a JASCO J-820 polarimeter. High-resolution ESI MS were obtained on an LTQ Orbitrap XL (Thermo Scientific). 1H and 2D NMR spectra were measured on a 125- or 175-MHz spectrometer at 300 K, while 13C NMR spectra were measured on a 125- or 175-MHz spectrometer at 300 K. UV mini-1240 spectrophotometer and IR spectra on a ZnSe). Optical rotations were measured on a JASCO DIP-1000 polarimeter. UV spectra were recorded on a Shimadzu UV mini-1240 spectrophotometer and IR spectra on a JASCO FT/IR-4100 spectrophotometer. CD spectra were recorded on a JASCO J-820 polarimeter. High-resolution ESI MS were obtained on an LTQ Orbitrap XL (Thermo Scientific). 1H and 2D NMR spectra were measured on a 125- or 175-MHz spectrometer at 300 K, while 13C NMR spectra were measured on a 125- or 175-MHz spectrometer at 300 K. UV mini-1240 spectrophotometer and IR spectra on a ZnSe).

Material

The natural *mumiyo* samples were collected from Kyrgyz. To 1 kg of natural *mumiyo*, 4 L of water was added, and the mixture was then stirred and heated to boiling for about 40 min. The mixture was cooled, and the precipitates were separated from the supernatant. The precipitates were then extracted with water repeatedly to obtain the water extract. The supernatant and the water extract were combined and subjected to centrifugation for 10 min. The supernatant was collected and concentrated by an evaporator. The concentrated solution was again subjected to ODS silica gel column chromatography (CHCl3/MeOH, 1:0→1:1; retention time of authentic L- and D-glucuronic acid was as follows: L (4.9 min with negative intensity) and D (4.9 min with positive intensity). The retention time of

Acid hydrolysis of 2 and 3

2 (2.0 mg) was treated with 2 M aqueous HCl (400 μL) at 100 °C for 1 h. After neutralization with 2 M aqueous NaOH, the mixture was extracted with CHCl3. The aqueous layer was submitted to HPLC analysis (GL science NH2 column φ 4.6 × 250 mm, eluent: 70% aqueous MeCN, flow rate 1.0 mL/min, JASCO OR-1590 chiral detector). Retention times of authentic L- and D-glucuronic acid were as follows: L (4.9 min with negative intensity) and D (4.9 min with positive intensity). The retention time of
glucuronic acid in the aqueous layer of hydrolysate of 2 was 4.9 min, with positive intensity. 3 (1.0 mg) was subjected to a similar treatment as 3, and the retention time of glucose in the aqueous layer of hydrolysate of 3 was 4.9 min, with positive intensity.

Synthesis of 2,14,15-tri-O-acyl-19-methyl-4 and 3,14,15-tri-O-acyl-18-methyl-5

To a solution of 4 (0.8 mg in 100 μL MeOH), 20 μL of TMS-diazomethane (10% in n-hexane) was added and left at room temperature. After 10 min, the reaction mixture was dried under an N₂ stream, and the resulting residue (0.8 mg) was dissolved in 150 μL of CH₂Cl₂. To the CH₂Cl₂ solution, a catalytic amount of 4-(dimethylamino)pyridine and 2 μL of triethylamine were added, and the mixture was then separated into three containers (50 μL each). Into the container, (R)-MTPA chloride, (S)-MTPA chloride, or benzoyl chloride was added, and the solutions were allowed to stand at room temperature overnight. The residue obtained under an N₂ stream was subjected to SiO₂ column chromatography (CHCl₃) to obtain the tri-(R)-MTPA, tri-(R)-MTPA, and tri-benzoyl derivatives of 19-methyl-4. The same procedure was used to obtain tri-(S)-MTPA, tri-(R)-MTPA, and tri-benzoyl derivatives of 19-methyl-5.

2,14,15-tri-O-[(R)-MTPA]-19-methyl-4

1H NMR (CDCl₃, 400 MHz) δ 1.11 (dd, 12.3, 4.3; H-1a), 2.20 (t, 12.3; H-1b), 5.57 (m; H-2), 1.30 (d, 12.3; H-3a), 2.55 (t, 12.3; H-3b), 1.86 (m; H-7a), 2.38 (m; H-7b), 2.08 (m, H-2-11), 5.51 (br t, 5.4; H-12), 5.60 (dd, 7.4, 4.4; H-14), 4.27 (dd, 11.3, 7.4; H-15a), 4.49 (dd, 11.3, 4.4; H-15b), 1.63 (s; H_3-16), 4.38 (s; H-17a), 4.84 (s; H-17b), 1.29 (s; H-3-18), 0.62 (s; H_3-20), and 3.68 (s; 19-OMe).

2,14,15-tri-O-[(S)-MTPA]-19-methyl-4

1H NMR (CDCl₃, 400 MHz) δ 1.20 (dd, 12.3, 4.3; H-1a), 2.22 (t, 12.3; H-1b), 5.57 (m; H-2), 1.21 (d, 12.3; H-3a), 2.49 (t, 12.3; H-3b), 1.86 (m; H-7a), 2.38 (m; H-7b), 2.08 (m, H-2-11), 5.34 (br t, 5.4; H-12), 5.50 (dd, 7.4, 4.4; H-14), 4.27 (dd, 11.3, 7.4; H-15a), 4.54 (dd, 11.3, 4.4; H-15b), 1.53 (s; H-3-16), 4.36 (s; H-17a), 4.85 (s; H-17b), 1.28 (s; H-3-18), 0.63 (s; H-3-20), and 3.67 (s; 19-OMe).

2,14,15-tri-O-benzoyl-19-methyl-4

UV (MeOH) λ_max (log ε) 229 (4.75) nm; CD (MeOH) λ_max (Δε) 237 (+13.7), 229 (0), and 222 (−6.8) nm. 1H NMR (CD₂OD, 400 MHz) 5.50 (m; H-2), 5.58 (br t, 5.4; H-12), 5.66 (t, 5.5; H-14), 4.53 (d, 5.5; H-2-15), 1.81 (s; H-3-16), 4.50 (s; H-17a), 4.82 (s; H-17b), 1.29 (s; H-3-18), 0.65 (s; H-3-20), and 3.66 (s; 19-OMe).

3,14,15-tri-O-[(R)-MTPA]-18-methyl-5

1H NMR (CDCl₃, 400 MHz) δ 1.43 (dd, 13.4, 3.2; H-1a), 1.78 (m; H-1b), 1.72 (m; H-2a), 2.01 (m; H-2b), 5.46 (dd, 11.3, 3.1; H-3), 1.80 (m; H-5), 1.10 (m; H-6a), 1.50 (m; H-6b), 1.93 (m; H-7a), 2.14 (br d, 14.2; H-7b), 1.73 (m; H-9), 5.21 (s; H-14), 5.22 (d, 8.8; H-15), 4.19 (dd, 11.2, 8.8; H-16a), 4.80 (br d, 11.2; H-16b), 1.00 (s; H-3-17), 1.17 (s; H-3-19), 0.80 (s; H-3-20), and 3.58 (s; 18-OMe).

3,14,15-tri-O-[(S)-MTPA]-18-methyl-5

1H NMR (CDCl₃, 400 MHz) δ 1.42 (dd, 13.4, 3.2; H-1a), 1.76 (m; H-1b), 1.60 (m; H-2a), 1.93 (m; H-2b), 5.46 (dd, 11.3, 3.1; H-3), 1.82 (m; H-5), 1.12 (m; H-6a), 1.51 (m; H-6b), 1.95 (m; H-7a), 2.19 (br d, 14.2; H-7b), 1.73 (m; H-9), 5.26 (s; H-14), 5.22 (d, 8.8; H-15), 4.07 (dd, 11.2, 8.8; H-16a), 4.81 (br d, 11.2; H-16b), 1.05 (s; H-3-17), 1.19 (s; H-3-19), 0.80 (s; H-3-20), and 3.66 (s; 18-OMe).

3,14,15-tri-O-benzoyl-18-methyl-5

UV (MeOH) λ_max (log ε) 228 (4.84) nm; CD (MeOH) λ_max (Δε) 238 (−12.5), 229 (0), and 221 (+6.9) nm. 1H NMR (CD₂OD, 400 MHz) 5.39 (br d 11.0; H-3), 5.50 (s; H-14), 5.35 (dd, 8.4, 2.2; H-14), 4.47 (dd, 11.0, 8.4; H-15a), 4.77 (br d, 11.0; H-15b), 0.94 (s; H-3-17), 1.09 (s; H-3-19), 0.78 (s; H-3-20), and 3.67 (s; 18-OMe).

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