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Ustalic acid as a toxin and related compounds from the mushroom *Tricholoma ustale*

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A toxin and related compounds were isolated from a poisonous mushroom *Tricholoma ustale*. Their structures were determined by analyses of the spectral data and synthesis.

In Japan, people eat various kinds of wild mushrooms in the fall and a lot of them get poisoned by eating toxic mushrooms accidentally. Every year the three mushrooms responsible for most cases of poisoning in Japan are *Lampteromyces japonicus*, *Rhodophylllus rhodopolius*, and *Tricholoma ustale*.[1] The toxic principles of the former two mushrooms have been isolated and characterized,[2,3] but that of *T. ustale* had remained unknown. *T. ustale* (Kakishimeji in Japanese) is widespread and common throughout temperate regions in the world and human ingestion of this mushroom causes gastro-intestinal poisoning accompanied by vomiting and diarrhoea. In this paper, we report the isolation, structure determination, and biological activity of the toxin, ustalic acid (**1**), and related compounds (**2**–**5**) from this mushroom.

The isolation of the toxin was guided by toxicity against mice; hesitancy to move, tremors, and death were observed by oral force-administration of fractions into their stomachs.

The aqueous ethanol extract from the mushroom was separated by solvent partition between chloroform and water. Since the chloroform soluble part showed the toxicity, this part was fractionated by repeated silica gel flush column chromatography and finally ODS HPLC to furnish ustalic acid (**1**) (191.4 mg from 30.3 kg of the fresh mushroom) and its analogues **2** to **5** (**2**, 10.0 mg; **3**, 4.3 mg; **4**, 3.1 mg; **5**, 3.6 mg).

FAB-MS analysis of ustalic acid (**1**) produced pseudo-molecular ions at *m*/*z* 339 $[M + H]^+$ and 361 $[M + Na]^+$. HR-FAB-MS of the *m*/*z* 339 ion gave an exact mass value of *m*/*z* 339.0869 (calcd 339.0868), suggesting a molecular formula of $C_{19}H_{14}O_6$. Since ^{1}H NMR measurement gave very simple signals [δ 5.45 (2H, s) and 7.30–7.48 (10H, m)], the other NMR experiments such as COSY, HMQC, and HMBC did not give significant information for determination of the structure.[4] Therefore, we elected for single-crystal X-ray diffraction techniques (Fig. 1).[5] The compound was a dimer linked with hydrogen bonds of each other's carboxy groups.

The molecular formula, $C_{24}H_{21}NO_7$, of compound **2** was determined by HR-FAB-MS of the ion at *m*/*z* 436.1401 (calcd 436.1396). The signals other than those from ustalic acid's moiety in NMR (^{13}C NMR, a carboxy group, and signals at δ 60.7, 50.1, 30.7, 26.1; ^{1}H NMR δ 4.54 (1H, dd, 7.6, 7.6), 3.43 (1H, m), 2.68 (1H, m), 2.35 (1H, m), 2.00 (1H, m), 1.82 (1H, m), 1.79 (1H, m)), HMBC, COSY, and HMQC data suggested that **2** was an amide of ustalic acid and proline (Fig. 2).[6] The structure was confirmed by synthesis and the $[\alpha]_D$ of synthetic **2** was consistent with that of the natural compound.[7]

The structures of compounds **2** to **5** were also determined by analyses of their spectral data and synthesis (Fig. 2).[8–10]

All the compounds except for **2** were optically inactive.[6–10]

Compound **1** was orally force-fed to mice at three concentrations, 2, 5 or 10 mg per mouse, by using a catheter (one group, 3 mice). All doses caused them to sit in a crouched position, to be hesitant to move, and caused tremors and abdominal contractions. The onset of these reactions occurred consistently in 3 h and continued up to 2 days, often resulting in death; at 10 mg per mouse, all the mice died. These reactions were very similar to those caused by injection into the peritoneal cavity

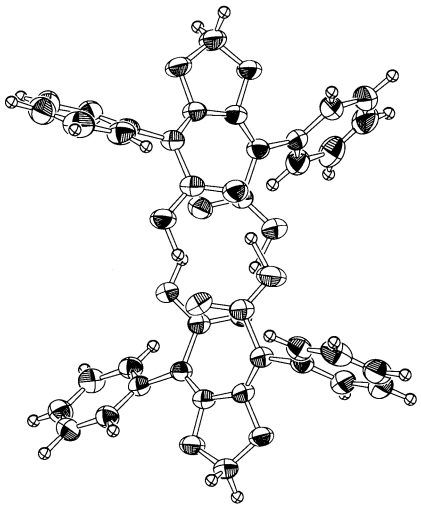

Fig. 1 A stereo ORTEP of ustalic acid (**1**).

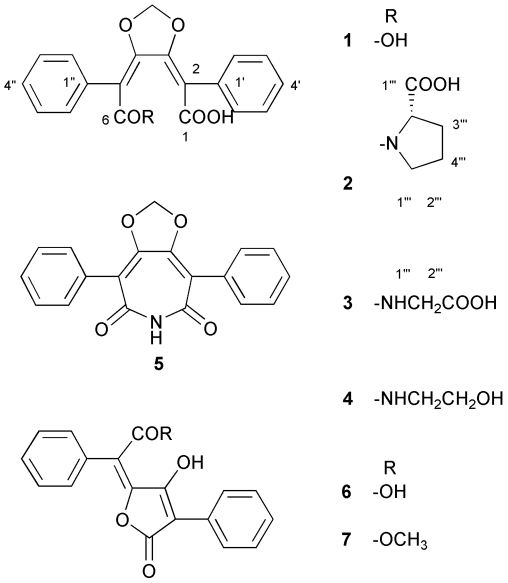

Fig. 2 Structures of compounds **1** to **7**.

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and subcutaneous injection of proteinous toxins from two mushrooms, *R. rhodopolius* and *Lepiota morganii*.[3,11] However, those toxins did not cause such symptoms by oral administration, unlike **1**. Human diarrhoea is variously caused. After following some trails for the elucidation of the mechanism, we found that this compound inhibited Na^+,K^+-ATPase; IC_{50} of **1** against the commercially available enzyme purified from porcine cerebral cortex and the crude enzyme from mouse intestinal mucosal cells were 5.2 and 0.77 mM, respectively.[12] Compounds **2** to **5** also inhibited the commercially available enzyme and IC_{50} of the compounds were 5.7, 1.8, 1.7, and 25 mM, respectively. In general, absorption of water from the intestines is suppressed by inhibition of intestinal Na^+,K^+-ATPase, resulting in diarrhoea. This mushroom produces HCN.[13] However, the toxicity of this mushroom was not due to HCN, because the acid was completely destroyed by culinary preparation such as boiling or frying[13] and the toxicity of this mushroom was not affected by those treatments.

The keto-form of dihydroxy analogue of **1**, 2,5-diphenyl-3-hydroxy-4-oxo-2-hexendioic acid has been obtained as a metabolite of pulvinic acid (**6**) and vulpinic acid (**7**) by an unclassified soil bacterium.[14] In addition, 4,5-methylenedioxy-3,6-diphenyl-1,2-benzoquinone, phlebiarubrone, has been isolated from cultured mycelia of a toadstool *Phlebia strigosozonata*.[15] Biogenetically, **1** is likely to be derived from the quinone by oxidative ring opening as happens during the biosynthesis of **6** and **7**.[16]

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4 IR (NaCl, neat) ν_{max} 1693, 1630 cm^{-1}; ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.4 (C1,6), 149.4 (C3,4), 133.5 (C1′,1″), 130.0, 128.1 (C3′,5′,3″,5″ or C2′,6′,2″,6″), 128.4 (C4′,4″), 115.1 (C2,5), 96.4 (-OCH_2O-); mp 200–202 °C; $[\alpha]^{20}_D$ 0° (*c* 1.0, MeOH).

5 *Crystal data* for **1**: $C_{19}H_{14}O_6$, M = 338.32, monoclinic, *a* = 12.699(2), *b* = 12.3295(8), *c* = 20.909(2) Å, β = 92.241(9)°, *U* = 3271.3(6) $Å^3$, *T* = 296 K, space group $P2_1/n$ (no. 14), *Z* = 8, μ(Cu-Kα) = 0.868 mm^{-1}, 12624 reflections measured, 6008 unique (R_{int} = 0.022) which were used in all calculations. The final *wR*(*F*) was 0.062 (all data). CCDC 182250. See http://www.rsc.org/suppdata/cc/b2/b202607d/ for crystallographic data in .cif or other format.

6 IR (NaCl, neat) ν_{max} 1725, 1630, 1564 cm^{-1}; ^{13}C NMR (125 MHz, CD_3OD) δ 175.5, 170.8, 169.4 (C1‴ or C1 or C6), 149.9, 146.1 (C3 or C4), 136.6, 135.5 (C1″ or C1′), 131.1, 131.0, 129.0, 128.8 (C3′,5′ or C3″,5″ or C2′,6′ or 2″,6″), 128.9, 128.6 (C4′ or C4″), 119.7, 115.7 (C2 or C5), 97.2 (-OCH_2O-), 60.7 (C2‴), 50.1 (C5‴), 30.7 (C3‴), 26.1 (C4‴); ^1H NMR (500 MHz, CD_3OD) δ 7.64 (2H, br.d 7.0, H2′,6′ or H2″,6″), 7.25–7.37 (8H, m, H2′6′,2″6″ except for H2′,6′ or H2″,6″), 5.48 (1H, s, -OCH_2O-) 5.38 (1H, s, -OCH_2O-), 4.54 (1H, dd, 7.6, 7.6, H2‴), 3.43 (1H, m, H5‴), 2.68 (1H, m, H5‴), 2.35 (1H, m, H3‴), 2.00 (1H, m, H4‴), 1.82 (1H, m, H3‴), 1.79 (1H, m, H4‴) mp 200–202 °C; $[\alpha]^{20}_D$ −28° (*c* 0.10, MeOH).

7 Compound **1** (1.0 equiv), EDC (0.8 equiv), and DMAP (0.8 equiv) were dissolved in pyridine and stirred at 50 °C for 0.5 h. To the solution, L-proline (1.5 equiv) was added and the reaction mixture was further stirred at the same temperature for 2 h. After drying the reaction mixture under reduced mixture, the residue was separated by ODS HPLC, affording **2** in 19% yield. $[\alpha]^{20}_D$ −29° (*c* 0.10, MeOH).

8 Compound **3**. $C_{21}H_{17}NO_7$ from HR-FAB-MS of $[M + H]^+$ ion at *m*/*z* 396.1099 (calcd 396.1083); IR (NaCl, neat) ν_{max} 1714, 1644 cm^{-1}; ^{13}C NMR (125 MHz, CD_3OD) δ 173.2, 170.7, 170.5 (C2′ OR C1 OR C6), 149.8, 146.8 (C3 or C4), 136.6, 136.4 (C1′ or C1″), 131.6, 131.2, 128.9, 128.8 (C3′,5′ or C3″,5″ or C2′,6′ or 2″,6″), 129.0, 128.6 (C4′ or C4″), 119.9, 115.7 (C2 or C5), 97.2 (-OCH2O-), 42.5 (C1‴); ^1H NMR (500 MHz, CD_3OD) δ 7.53 (2H, br.d 7.6, H2′,6′ or H2″,6″), 7.22–7.44 (8H, m, H2′6′,2″6″ except for H2′,6′ or H2″,6″), 5.41 (2H, s -OCH_2O-), 3.94 (2H, s, H1‴). mp 149–152 °C; $[\alpha]^{20}_D$ 0° (*c* 0.16, MeOH). Treatment of **1** with glycine, EDC, and DMAP gave **3** in 24% yield.

9 Compound **4**. $C_{21}H_{19}NO_6$ from HR-FAB-MS of $[M+H]^+$ ion at *m*/*z* 382.1283 (calcd 382.1291); IR (NaCl, neat) ν_{max} 1671 cm^{-1}; ^{13}C NMR (125 MHz, acetone-d_6) δ 168.6, 168.4 (C1 or C6), 149.5, 145.8 (C3 or C4), 136.8, 136.6 (C1′ or C1″), 131.0, 131.0, 128.6, 128.4 (C3′,5′ or C3″,5″ or C2′,6′ or 2″,6″), 128.5, 128.0 (C4′ or C4″), 120.8, 114.9 (C2 or C5), 96.4 (-OCH_2O-), 61.1 (C2‴), 42.9 (C1‴); ^1H NMR (500 MHz, acetone-d_6) δ 7.24–7.43 (10H, m, H2′6′,2′6″), 5.41 (2H, s, -OCH_2O-), 3.66 (2H, m, H2‴), 3.37 (2H, m, H1‴). mp 128–131 °C; $[\alpha]^{20}_D$ 0° (*c* 0.10, MeOH). Treatment of **1** with ethanolamine, EDC, and DMAP gave **4** in 15% yield.

10 Compound **5**. $C_{19}H_{13}NO_4$ from HR-FAB-MS of $[M + H]^+$ ion at *m*/*z* 320.0913 (calcd 320.0923); IR (NaCl, neat) ν_{max} 1642. 1531 cm^{-1}; ^{13}C NMR (125 MHz, $CDCl_3$) δ 162.5 (C1,6), 153.2 (C3,4), 132.1 (C1′,1″), 129.9, 128.2 (C3′,5′,3″,5″ or C2′,6′,2″,6″), 128.7 (C4′,4″), 115.4 (C2,5), 99.0 (-OCH_2O-); ^1H NMR (500 MHz, $CDCl_3$) δ 7.36–7.46 (10H, m, H2′6′,2″6″), 5.73 (2H, s, −OCH_2O-). mp 239–242 °C. $[\alpha]^{20}_D$ 0° (*c* 0.10, MeOH). Treatment of **1** with aqueous NH_3, EDC, and DMAP gave **5** in 30% yield.

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