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# SECONDARY METABOLITES OF ENDOPHYTIC FUNGUS *Xylaria* sp. YC-10 OF *Azadirachta indica*

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Endophytic fungi grow within their plant host without causing apparent disease symptoms. In their symbiotic association, the host plant protects and feeds the endophyte, which in return produces bioactive metabolites to enhance the growth and competitiveness of the host and to protect it from herbivores and plant pathogens [1]. They are recognized as producers of a vast array of secondary metabolites, many of them with promising bioactivities [1–3]. *Azadirachta indica* A. Juss (Meliaceae), commonly known as "neem," is of considerable economic importance. Our previous investigation of endophytic fungi from *A. indica* resulted in some new bioactive compounds [4, 5]. In continuous work, we have studied the secondary metabolites of *Xylaria* sp. YC-10 from *A. indica* and obtained 11 compounds.

The fungal strain *Xylaria* sp. YC-10 was isolated from the stem of *A. indica* collected in Yuanjiang County, Yunnan Province, P. R. China. It was classified as a *Xylaria* species by its morphological characteristics and its rDNA sequence analysis. The strain was deposited in Yunnan Institute of Microbiology, Kunming, P. R. China.

The fungus was cultured in 500 mL Erlenmeyer flasks (× 200) containing 100 mL of PDB medium at 200 rpm at 28°C for 6 days on a rotary shaker. The culture broth was filtered to remove mycelia. The filtrate was concentrated under reduced pressure to 5 L and then exhaustively extracted with EtOAc (3 × 5 L). After removal of the solvent in vacuum, the resulting residue (30.6 g) was subjected to column chromatography on silica gel eluting with petroleum–acetone (5:1–1:1) to afford seven fractions (I–VII). Fraction I was repeatedly subjected to column chromatography on silica gel with petroleum–acetone (20:1) to give compound **2** (18 mg). Repeated chromatography of fraction II on RP-18 silica gel with MeOH–$H_2O$ (8:2) and petroleum–EtOAc (7:3) afforded compound **1** (30 mg). Fraction III was submitted to column chromatography on RP-18 silica gel with MeOH–$H_2O$ gradient (6:4, 8:2) to afford compounds **3** (50 mg) and **9** (26 mg). Fraction IV was repeatedly chromatographed on Sephadex LH-20 with MeOH and RP-18 silica gel with MeOH–$H_2O$ gradient (1:1, 6:4, 7:3) to yield compounds **6** (25 mg) and **11** (93 mg). Fraction V was submitted to repeated column chromatography on RP-18 silica gel with MeOH–$H_2O$ gradient (1:1, 6:4) and silica gel with $CHCl_3$–MeOH gradient (9:1, 8:2) to afford **4** (32 mg), **5** (8 mg). Fraction VI was repeatedly subjected to column chromatography on Sephadex LH-20 with MeOH and RP-18 silica gel with MeOH–$H_2O$ gradient (3:7, 6:4) to give compounds **7** (24 mg), **8** (46 mg), and **10** (35 mg).

The compounds were identified using NMR and mass spectrometry by comparison with reported spectroscopic data in the literature and determined as 5-hydroxymellein (**1**) [6], 5-methylmellein (**2**) [7], 5-carboxymellein (**3**) [7, 8], hymatoxin C (**4**) [9], hymatoxin D (**5**) [9], halorosellinic acid (**6**) [8], cerebroside C (**7**) [10], (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino)octadecane-1,3,4-triol (**8**) [11], cerevisterol (**9**) [12], adenosine (**10**) [13], and succinic acid (**11**) [14]. Compounds **2–9** were isolated from the fungal genus *Xylaria* for the first time.

The primary insecticidal activities of these compounds were tested by the conventional leaf disk method against the third instar larvae of *Plutella xylostella*. The test compounds were dissolved in acetone (including five drops DMSO) at concentrations of 5 mg/mL. Leaf disks of *Brassica oleracea* L. (1.6 cm diameter) were dipped in the test solutions, and the control discs were in acetone (including five drops DMSO) for 3 s. All the leaf disks were dried before being presented to the insect. Test insects of the same size and health were used after starvation for 4 h. For each compound, 60 larvae (20 larvae per group) were used. The number of dead larvae was recorded at 24, 48, and 72 h after treatment. The corrected mortality (CM) was calculated. The results (Table 1) indicated that all the compounds exhibited weak insecticidal activity against *Plutella xylostella*.

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TABLE 1. Insecticidal Activity of Compounds **1–11** against Third Instar Larvae of *Plutella xylostella*

| Compounds | Corrected mortality, % | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| 5-Hydoxylmellein (**1**) | 0 ab | 3.25 b | 5.21 ab |
| 5-Methylmellein (**2**) | 1.75 ab | 15.48 ab | 18.98 ab |
| 5-Carboxylmellein (**3**) | 0 b | 1.39 b | 4.35 ab |
| Hymatoxin C (**4**) | 0 a | 0 a | 3.30 a |
| Hymatoxin D (**5**) | 0 a | 0 a | 7.65 b |
| Halorosellinic acid (**6**) | 11.59 a | 24.60 a | 31.55 b |
| Cerebroside C (**7**) | 3.33 b | 8.17 bb | 12.71 b |
| (2*S*,3*S*,4*R*,2′*R*)-2-(2′-Hydroxytetracosanoylamino)-octadecane-1,3,4-triol (**8**) | 4.55 a | 6.67 a | 17.07 ab |
| Cerevisterol (**9**) | 0 b | 1.52 ab | 5.69 b |
| Adenosine (**10**) | 0 a | 0 ab | 0.14 a |
| Succinic acid (**11**) | 11.36 a | 20.76 ab | 32.36 a |
| Control | 0 a | 0 a | 0 b |

Values in the same column followed by same letter (s) are not significantly different at 5% level of probability (DNMRT).

**5-Methylmellein (1)**. $C_{11}H_{12}O_3$, mp 122–123°C, colorless crystals. ESI-MS, *m/z* 215 $[M + Na]^+$. $^1H$ NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 10.99 (1H, s, 8-OH), 7.29 (1H, d, J = 7.4, H-6), 6.82 (1H, d, J = 7.4, H-7), 4.69 (1H, m, H-3), 2.96 (1H, d, J = 16.4, H-4$\alpha$), 2.74 (1H, dd, J = 16.4, 12.6, H-4$\beta$), 2.20 (3H, s, 5-$CH_3$), 1.59 (3H, d, J = 6.5, 3-$CH_3$). $^{13}C$ NMR (125 MHz, $CDCl_3$, δ, ppm): 170.7 (C-1), 161.0 (C-8), 138.3 (C-6), 137.4 (C-4a), 125.3 (C-8a), 116.1 (C-7), 108.5 (C-5), 75.8 (C-3), 32.3 (C-4), 21.3 (3-$CH_3$), 18.4 (5-$CH_3$).

**5-Hydroxymellein (2)**. $C_{10}H_{10}O_4$, mp 231–233°C, colorless crystals. ESI-MS, *m/z* 217 $[M + Na]^+$. $^1H$ NMR (500 MHz, $C_5D_5N$, δ, ppm, J/Hz): 11.13 (1H, s, 5-OH), 7.36 (1H, d, J = 8.8, H-6), 6.98 (1H, d, J = 8.8, H-7), 4.62 (1H, m, H-3), 3.37 (1H, d, J = 16.8, H-4$\alpha$), 2.72 (1H, dd, J = 16.8, 11.5, H-4$\beta$), 1.36 (3H, d, J = 6.1, 3-$CH_3$). $^{13}C$ NMR (125 MHz, $C_5D_5N$, δ, ppm): 171.5 (C-1), 156.5 (C-8), 147.9 (C-5), 126.3 (C-4a), 125.6 (C-6), 116.9 (C-7), 109.9 (C-8a), 77.3 (C-3), 29.9 (C-4), 21.6 (3-$CH_3$).

**5-Carboxymellein (3)**. $C_{11}H_{10}O_5$, mp 251–252°C, colorless crystals. ESI-MS, *m/z* 245 $[M + Na]^+$. $^1H$ NMR (500 MHz, $C_5D_5N$, δ, ppm, J/Hz): 8.46 (1H, d, J = 8.8, H-6), 7.07 (1H, d, J = 8.8, H-7), 4.60 (1H, m, H-3), 4.14 (1H, dd, J = 17.7, 2.7, H-4$\alpha$), 3.10 (1H, dd, J = 17.7, 11.9, H-4$\beta$), 1.33 (3H, d, J = 6.2, 3-$CH_3$). $^{13}C$ NMR (125 MHz, $C_5D_5N$, δ, ppm): 171.4 (C-1), 169.5 (5-COOH), 166.2 (C-8), 144.7 (C-4a), 140.1 (C-6), 122.1 (C-8a), 116.9 (C-7), 110.4 (C-5), 76.7 (C-3), 33.9 (C-4), 21.5 (3-$CH_3$).

**Hymatoxin C (4)**. $C_{20}H_{32}O_6S$, white amorphous powder. HR-ESI-MS, *m/z* 399.1842 $[M - H]^-$. $^1H$ NMR (500 MHz, $C_5D_5N$, δ, ppm, J/Hz): 5.36 (1H, br.s, H-7), 4.54 (2H, t, J = 6.8, H-16), 4.54 (2H, t, J = 6.8, H-16), 2.82 (1H, t, J = 13.6, H-$6_{ax}$), 2.43 (1H, br.d, J = 12.5, H-$3_{eq}$), 2.29 (1H, t, J = 13.6, H-$6_{eq}$), 1.88 (2H, br.s, H-14), 1.83 (1H, br.d, J = 12.4, H-$1_{eq}$), 1.70 (2H, t, J = 7.5, H-15), 1.53 (1H, br.d, J = 10.1, H-9), 1.50 (1H, m, H-$11_{eq}$), 1.38 (1H, m, H-$12_{eq}$), 1.34 (3H, s, H-18), 1.17 (1H, m, H-$12_{ax}$), 1.08 (1H, m, H-$1_{ax}$), 1.06 (1H, m, H-$3_{ax}$), 0.94 (3H, s, H-20), 0.73 (3H, s, H-17). $^{13}C$ NMR (125 MHz, $C_5D_5N$, δ, ppm): 181.7 (C-19), 136.8 (C-8), 123.4 (C-7), 66.2 (C-16), 53.3 (C-5, 9), 49.2 (C-14), 45.9 (C-15), 45.5 (C-4), 41.8 (C-1), 40.6 (C-3), 38.8 (C-12), 37.7 (C-10), 34.8 (C-13), 31.2 (C-18), 26.6 (C-6), 23.5 (C-17), 22.8 (C-11), 21.9 (C-2), 16.2 (C-20).

**Hymatoxin D (5)**. $C_{20}H_{30}O_6S$, white amorphous powder. HR-ESI-MS, *m/z* 397.4010 $[M - H]^-$. $^1H$ NMR (500 MHz, $CD_3OD$, δ, ppm, J/Hz): 6.10 (1H, d, J = 9.9, H-6), 5.92 (1H, d, J = 9.9, H-7), 5.30 (1H, br.s, H-14), 4.10 (1H, m, H-16a), 4.04 (1H, m, H-16b), 2.18 (1H, br.s, H-5), 1.96 (1H, m, H-9), 1.70 (2H, m, H-15), 1.57 (1H, m, H-$11_{eq}$), 1.51 (2H, m, H-12), 1.42 (1H, m, H-$11_{ax}$), 1.29 (3H, s, H-18), 1.03 (3H, s, H-17), 0.67 (3H, s, H-20). $^{13}C$ NMR (125 MHz, $CD_3OD$, δ, ppm): 181.4 (C-19), 137.5 (C-8), 134.7 (C-14), 129.5 (C-6), 129.2 (C-7), 66.8 (C-16), 57.0 (C-5), 51.0 (C-9), 48.0 (C-4), 44.7 (C-10), 43.9 (C-15), 39.1 (C-3, 12), 35.7 (C-1), 34.7 (C-13), 29.2 (C-18), 28.5 (C-17), 21.5 (C-2), 20.4 (C-11), 12.8 (C-20).

**Halorosellinic Acid (6)**. $C_{25}H_{36}O_6$, white amorphous powder. HR-ESI-MS, *m/z*: 431.2456 $[M - H]^-$, 863.4943 $[2M - H]^-$. $^1H$ NMR (500 MHz, $C_5D_5N$, δ, ppm, J/Hz): 7.77 (1H, d, J = 8.8, H-18), 6.61 (1H, d, J = 7.6, H-8), 5.71 (1H, br.s, H-13), 5.10 (1H, dd, J = 8.7, 5.1, H-17), 3.85 (1H, t, J = 5.6, H-16), 3.54 (1H, m, H-10), 3.44 (1H, m, H-6), 2.89 (1H, m, H-15), 2.75 (1H, br.d, J = 14.4, H-9$\beta$), 2.49 (1H, m, H-2), 2.48 (1H, m, H-5$\alpha$), 2.31 (1H, br.d, J = 15.3, H-12$\beta$), 2.29 (3H, s, H-24), 2.23 (1H, m, H-9$\alpha$), 2.08 (1H, m, H-3), 1.89 (1H, m, H-5$\beta$), 1.85 (1H, br.d, J = 15.3, H-12$\alpha$), 1.75 (1H, m, H-4$\beta$), 1.70 (1H, m, H-1$\beta$), 1.64 (1H, m, H-4$\alpha$), 1.57 (1H, m, H-1$\alpha$), 1.50 (3H, d, J = 6.8, H-23), 1.12 (3H, d, J = 7.1, H-20), 0.99 (3H, s, H-22).

$^{13}$C NMR (125 MHz, $C_5D_5N$, δ, ppm): 173.7 (C-21), 171.9 (C-25), 150.6 (C-14), 142.7 (C-18), 140.6 (C-7), 134.8 (C-8), 132.1 (C-19), 122.4 (C-13), 77.5 (C-16), 70.6 (C-17), 50.3 (C-10), 47.4 (C-2, 12), 47.0 (C-11), 42.4 (C-6), 39.7 (C-3), 39.2 (C-1), 35.9 (C-15), 35.4 (C-4), 29.1 (C-5), 28.1 (C-9), 25.9 (C-22), 16.9 (C-23), 16.2 (C-20), 14.9 (C-24).

**Cerebroside C (7)**. $C_{43}H_{79}NO_9$, white amorphous powder. HR-ESI-MS, *m/z* 754.5560 $[M + H]^+$. $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 5.81 (1H, m, H-5), 5.69 (1H, m, H-4′), 5.43 (2H, m, H-4, 3′), 5.07 (1H, m, H-8), 4.55 (1H, m, H-2′), 4.37 (1H, d, J = 7.2, H-1″), 4.03 (2H, m, H-1a, 3), 3.85 (2H, m, H-2, 6″a), 3.51 (1H, m, H-1b), 3.36 (1H, m, H-6″b), 2.00 (2H, m, H-6, 7), 1.93 (2H, t, J = 7.1, H-10), 1.57 (3H, s, H-19), 0.87 (6H, t, J = 7.1, H-18, 18′). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 175.0 (C-1′), 136.4 (C-9), 134.7 (C-5), 134.6 (C-4′), 128.9 (C-4), 127.1 (C-3′), 123.6 (C-8), 103.4 (C-1″), 77.1 (C-5″), 76.6 (C-3″), 73.7 (C-2″), 73.4 (d, C-2′), 72.5 (C-3), 69.9 (C-4″), 69.4 (C-1), 61.6 (C-6″), 53.9 (C-2), 40.2 (C-10), 32.9 (C-6), 32.3 (C-7, 16), 30.2–29.8 (C-12 to C-15, C-5′ to C-16′), 28.6 (C-11), 23.1 (C-17, 17′), 16.4 (C-19), 14.5 (C-18, 18′).

**(2*S*,3*S*,4*R*,2′*R*)-2-(2′-Hydroxytetracosanoylamino)-octadecane-1,3,4-triol (8)**. $C_{42}H_{85}NO_5$, white amorphous powder. HR-ESI-MS, *m/z* 684.6535 $[M + H]^+$. $^1$H NMR (500 MHz, $C_5D_5N$, δ, ppm, J/Hz): 8.54 (1H, d, J = 8.8, NH), 5.08 (1H, m, H-2), 4.60 (1H, m, H-2′), 4.49 (1H, dd, J = 10.8, 6.4, H-1a), 4.40 (1H, dd, J = 10.8, 4.8, H-1b), 4.33 (1H, m, H-3), 4.26 (1H, m, H-4), 2.24, 2.01 (2H, m, H-3′), 1.91 (2H, m, H-5), 1.68 (2H, m, H-6), 1.31–1.25 (60H, H-5′ to H-23′), 0.86 (6H, t, J = 5.4, H-18, 24′). $^{13}$C NMR (125 MHz, $C_5D_5N$, δ, ppm): 176.1 (C-1′), 77.7 (C-3), 73.9 (C-4), 73.4 (C-2′), 62.9 (C-1), 53.9 (C-2), 36.6 (C-3′), 35.0 (C-5), 30.5–33.0 (C-5′–23′, 7-17), 27.5 (C-6), 26.7 (C-4′), 15.1 (C-18, C-24′).

**Cerevisterol (9)**. $C_{28}H_{46}O_3$, mp 219–221°C, colorless crystals. EI-MS, *m/z* (%): 430 $[M]^+$ (13), 412 (100), 394 (54), 379 (72), 269 (46), 251 (47), 107 (35). $^1$H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 5.71 (1H, dd, J = 5.0, 2.4, H-7), 5.22 (1H, dd, J = 15.2, 7.4, H-23), 5.14 (1H, dd, J = 15.2, 8.1, H-22), 4.81 (1H, m, H-3), 4.30 (1H, br.s, H-6), 1.51 (3H, s, H-19), 1.02 (3H, d, J = 6.6, H-21), 0.92 (3H, d, J = 6.8, H-28), 0.82 (3H, d, J = 6.6, H-27), 0.81 (3H, d, J = 6.6, H-26), 0.66 (3H, s, H-18). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 140.1 (C-8), 134.7 (C-22), 130.6 (C-23), 119.1 (C-7), 75.3 (C-5), 72.8 (C-6), 66.1 (C-3), 54.6 (C-17), 53.8 (C-14), 42.3 (C-9, 13), 41.6 (C-24), 40.5 (C-4), 39.4 (C-20), 38.4 (C-12), 36.6 (C-10), 32.4 (C-2), 31.8 (C-25), 31.2 (C-1), 27.0 (C-16), 22.0 (C-15), 21.5 (C-11), 19.9 (C-21), 18.7 (C-26), 18.4 (C-27), 17.4 (C-19), 16.3 (C-28) 11.0 (C-18).

**Adenosine (10)**. $C_{10}H_{13}N_5O_4$, mp 233–235°C, colorless crystals. EI-MS, *m/z* (%) 267 $[M]^+$ (5), 237 (6), 178 (26), 164 (61), 148 (10), 135 (100), 119 (8), 108 (28), 81 (9), 73 (12). $^1$H NMR (500 MHz, $C_5D_5N$, δ, ppm, J/Hz): 8.77 (1H, s, H-8), 8.55 (1H, s, H-2), 6.68 (1H, d, J = 5.8, H-1′), 5.34 (1H, t, J = 5.4, H-2′), 5.07 (1H, t, J = 5.4, H-3′), 4.82 (1H, d, J = 2.8, H-4′), 4.33 (1H, dd, J = 12.6, 2.9, H-5′a), 4.23 (1H, dd, J = 12.6, 2.6, H-5′b). $^{13}$C NMR (125 MHz, $C_5D_5N$, δ, ppm): 157.6 (C-6), 153.9 (C-2), 149.8 (C-4), 141.5 (C-8), 121.2 (C-5), 91.0 (C-1′), 88.1 (C-4′), 76.2 (C-2′), 72.8 (C-3′), 63.5 (C-5′).

**Succinic Acid (11)**. $C_4H_6O_4$, mp 182–184°C, colorless crystals. $^1$H NMR (500 MHz, $CD_3OD$, δ, ppm): 2.60 (4H, s, H-2, 3). $^{13}$C NMR (125 MHz, $CD_3OD$, δ, ppm): 176.7 (C-1, 4), 30.3 (C-2, 3).

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