SUPPLEMENTARY MATERIAL

Aspergillolide, a new 12-membered macrolide from sea cucumber-derived fungus

Aspergillus sp. S-3-75

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A new 12-membered macrolide, aspergillolide (1), along with nine known compounds (2–10), were isolated from the fungus Aspergillus sp. S-3-75 associated with the sea cucumber Holothuria nobilis Selenka. The structure and absolute stereochemistry of 1 were elucidated on the basis of extensive spectroscopic methods and single crystal X-ray diffraction analysis.

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1. Physical and chemical data of compounds 1-10

Aspergillolide (1): colorless crystal, IR (KBr) \( \nu_{\text{max}} \): 3338, 2945, 1722, 1446, 1362, 1338, 1254, 1188, 1151, 1088, 1053, 1033, 1020, 920, 800. \(^1\)H NMR and \(^{13}\)C NMR data see Table S1. ESI-MS: \( m/z \) 231.2 [M+H]\(^+\), 253.2 [M + Na]\(^+\), HR-ESI-MS: \( m/z \) 231.1590 ([M + H]\(^+\), calcd. for C\(_{12}\)H\(_{23}\)O\(_4\), 231.1591).

X-ray crystal data of 1. C\(_{12}\)H\(_{22}\)O\(_4\), \( M_r = 230.29 \), \( D_c = 1.193 \text{ g/cm}^3 \), monoclinic, space group \( P 1 21 1 \), \( a = 13.9813 \) (5) Å, \( b = 7.7234 \) (3) Å, \( c = 12.1999 \) (4) Å, \( \alpha = 90^\circ \), \( \beta = 103.238 \) (2)°, \( \gamma = 90^\circ \), \( V = 1282.38(8) \) Å\(^3\), \( T = 301.48 \) K, \( Z = 4 \), \( \mu (\text{Cu } K\alpha) = 0.720 \) mm\(^{-1}\), \( F(000) = 504 \), crystal size: 0.35 \( \times \) 0.3 \( \times \) 0.25 mm\(^3\), A total of 10634 reflections were collected on a Bruker APEX-II CCD detector employing graphite monochromated \( \text{Cu } K\alpha \) radiation (\( \lambda = 1.54178 \) Å), 4499 independent reflections \( (R_{int} = 0.0534) \). The final \( R_1 \) value was 0.0826 ((\( I > 2\sigma(I) \))). The final \( wR_2 \) value was 0.2411 ((\( I > 2\sigma(I) \))). The final \( R_1 \) value was 0.1161 ((\( I > 2\sigma(I) \))) (all data). The final \( wR_2 \) value was 0.2992 ((\( I > 2\sigma(I) \))) (all data). The goodness-of-fit on \( F^2 \) was 0.988. The absolute structure parameter was 0.11(15).

2, 4-Dihydroxy-6-methylacetophenone (2): white solid, C\(_9\)H\(_{10}\)O\(_3\). \(^1\)H NMR (500 MHz, CD\(_3\)OD) \( \delta \) 6.19 (d, \( J = 2.1 \) Hz, H-5), 6.13 (d, \( J = 2.1 \) Hz, H-3), 2.55 (s, 3H, H-8), 2.39 (s, 3H, CD\(_3\)-6). \(^{13}\)C NMR (125 MHz, CD\(_3\)OD) \( \delta \) 204.8 (s, C-7), 162.9 (s, C-4), 161.9 (s, C-2), 141.2 (s, C-6), 116.8 (s, C-1), 110.7 (d, C-5), 100.3 (d, C-3), 31.5 (q, C-8), 21.9 (q, CD\(_3\)-6). (+)-ESI-MS \( m/z \) 167.2 [M+H]\(^+\), (-)-ESI-MS \( m/z \) 165.2 [M-H].
Pannorin (3): white solid, C₁₄H₁₀O₅. ¹H NMR (500 MHz, DMSO-d₆) δ 12.13 (s, 4-OH), 10.01 (s, 8-OH), 9.86 (s, 10-OH), 7.19 (s, H-6), 6.55 (d, J = 2.2 Hz, H-7), 6.52 (d, J = 2.2 Hz, H-9), 5.51 (s, H-3), 2.65 (s, CH₃-5). ¹³C NMR (125MHz, DMSO-d₆) δ 169.6 (s, C-4), 161.2 (s, C-2), 158.9 (s, C-8), 156.6 (s, C-10), 154.6 (s, C-10b), 138.1 (s, C-6a), 132.4 (s, C-5), 124.1 (d, C-6), 107.6 (s, C-4a), 106.5 (s, C-10a), 102.7 (s, C-9), 100.9 (d, C-7), 88.9 (d, C-3), 23.5 (q, CH₃-5). (+)-ESI-MS m/z 258.9 [M+H]⁺, (-)-ESI-MS m/z 256.9 [M-H]⁻.

2-Hydroxy-4-(3-hydroxy-5-methylphenoxy)-6-methylbenzoic acid (4): white solid, C₁₅H₁₄O₅. ¹H NMR (500 MHz, CD₃OD) δ 6.47 (s, H-6'), 6.35 (s, H-4'), 6.32 (s, H-5), 6.28 (s, H-2'), 6.20 (s, H-3), 2.52 (s, 3H, CH₃-6), 2.26 (s, 3H, CH₃-5'). ¹³C NMR (125 MHz, CD₃OD) δ 175.1 (s, C-7), 166.2 (s, C-2), 163.4 (s, C-4), 159.9 (s, C-1'), 157.5 (s, C-3'), 145.3 (s, C-6), 142.1 (s, C-5'), 113.5 (d, C-6'), 113.2 (d, C-5), 113.0 (d, C-4'), 109.5 (s, C-1), 105.7 (d, C-2), 103.7 (d, C-3), 24.1 (q, CH₃-6), 21.50 (q, CH₃-5'). (+)-ESI-MS m/z 274.9 [M+H]⁺, (-)-ESI-MS m/z 272.9 [M-H]⁻.

3,3'-Dihydroxy-5, 5'-dimethyldiphenyl ether (5): white solid, C₁₄H₁₄O₄. ¹H NMR (500 MHz, CD₃OD) δ 6.36, 6.27 (br s, H-4 and H-6), 6.20 (t, J = 2.1 Hz, H-2), 2.21 (s, CH₃-5). ¹³C NMR (125 MHz, CD₃OD) δ 159.6, 159.5 (s, C-1 and C-3), 141.6 (s, C-5), 112.0, 111.8 (d, C-4 and C-6), 104.2 (d, C-2), 21.5 (q, CH₃-5). (+)-ESI-MS m/z 231.0 [M+H]⁺, (-)-ESI-MS m/z 229.0 [M-H]⁻.
Aloesone (6): yellow solid, C_{13}H_{12}O_{4}. \textsuperscript{1}H NMR (500 MHz, DMSO-d\textsubscript{6}) δ 6.61 (br s, H-6), 6.59 (br s, H-8), 6.03 (s, H-3), 3.84 (s, 2H, H\textsubscript{-}9), 2.65 (s, 3H, CH\textsubscript{3}-5), 2.21 (s, 3H, H\textsubscript{-}11). \textsuperscript{13}C NMR (125 MHz, DMSO-d\textsubscript{6}) δ 202.7 (s, C-10), 178.1 (s, C-4), 161.3 (s, C-7), 160.5 (s, C-2), 159.2 (s, C-1a), 141.5 (s, C-5), 116.8 (d, C-6), 114.3 (s, C-4a), 112.8 (d, C-3), 100.5 (d, C-8), 47.4 (t, C-9), 29.8 (q, C-11), 22.4 (q, CH\textsubscript{3}-5). (+)-ESI-MS m/z 232.8 [M+H]\textsuperscript{+}, (-)-ESI-MS m/z 230.9 [M-H].

Aloesol (7): yellow solid, C_{13}H_{14}O_{4}. \textsuperscript{1}H NMR (500 MHz, CD\textsubscript{3}OD) δ 6.65 (s, H-8), 6.63 (s, H-6), 6.06 (s, H-3), 4.19 (dq, J = 12.3, 6.2 Hz, H-10), 2.71 (dd, J = 14.4, 5.1 Hz, H-9a), 2.71 (s, 3H, CH\textsubscript{3}-5), 2.65 (dd, J = 14.4, 7.7 Hz, H-9b), 1.27 (d, J = 6.2 Hz, 3H, H-11). \textsuperscript{13}C NMR (125 MHz, CD\textsubscript{3}OD) δ 182.0 (s, C-4), 167.1 (s, C-1a), 163.2 (s, C-7), 161.5 (s, C-2), 143.6 (s, C-5), 118.1 (d, C-3), 115.8 (s, C-4a), 112.5 (d, C-6), 101.7 (d, C-8), 66.4 (d, C-10), 44.2 (t, C-9), 23.5 (q, C-11), 23.1 (q, CH\textsubscript{3}-5). (+)-ESI-MS m/z 234.8 [M+H]\textsuperscript{+}, (-)-ESI-MS m/z 232.9 [M-H].

Acremolin (8): white solid, C_{11}H_{13}O_{2}N\textsubscript{5}. \textsuperscript{1}H NMR (500 MHz, DMSO-d\textsubscript{6}) δ 8.12 (s, C-8), 7.35 (s, H-1\textsuperscript{'}), 3.56 (s, 3H, C-10), 2.87 (m, H-3\textsuperscript{'}), 1.25 (d, J = 6.8 Hz, 6H, H-4\textsuperscript{'} and H-5\textsuperscript{'}). \textsuperscript{13}C NMR (125 MHz, DMSO-d\textsubscript{6}) δ 152.8 (s, C-6), 148.0 (s, C-2\textsuperscript{'}), 142.3 (s, C-2), 141.6 (s, C-4), 140.6 (d, C-8), 109.2 (s, C-5), 103.1 (d, C-1\textsuperscript{'}), 28.9 (q, C-10), 27.6 (d, C-3\textsuperscript{'}), 22.1 (q, 2C, C-4\textsuperscript{'} and C-5\textsuperscript{'}). (+)-ESI-MS m/z 231.7 [M+H]\textsuperscript{+}, (-)-ESI-MS m/z 229.9 [M-H].
Cyclo-(L-Trp-L-phenylalanyl) (9): white solid, C_{20}H_{19}N_{3}O_{2}. ^{1}H NMR (500 MHz, DMSO-\textit{d}_{6}): \delta 10.89 (s, H-1), 7.91 (d, J = 1.8 Hz, H-12), 7.71 (d, J = 2.0 Hz, H-15), 7.49 (d, J = 7.9 Hz, H-4), 7.33 (d, J = 8.1 Hz, H-7), 7.17 (m, H-21), 7.16 (2H, m, H-19 and H-23), 7.07 (t-like, J = 7.5 Hz, H-6), 6.98 (t-like, J = 7.5 Hz, H-5), 6.96 (d, J = 2.0 Hz, H-2), 6.71 (2H, dd, J = 7.4, 1.3 Hz, H-20 and H-22), 3.98 (m, H-11), 3.86 (m, H-14), 2.81 (dd, J = 14.4, 4.3 Hz, H-10), 2.53 (dd, J = 14.4, 5.8 Hz, H-10), 2.47 (dd, J = 13.4, 4.7 Hz, H-17), 1.86 (dd, J = 13.4, 7.1 Hz, H-17). ^{13}C NMR (125 MHz, DMSO-\textit{d}_{6}): \delta 166.8 (s, C-16), 166.2 (s, C-13), 136.5 (s, C-18), 136.1 (s, C-9), 129.7 (2C, d, C-20 and C-22), 128.0 (2C, d, C-19 and C-23), 127.5 (s, C-8), 126.4 (d, C-21), 124.4 (d, C-6), 120.9 (d, C-2), 118.8 (d, C-5), 118.4 (d, C-4), 111.3 (d, C-7), 108.8 (s, C-3), 55.6 (d, C-14), 55.3 (d, C-11), 40.0 (t, C-17), 29.7 (t, C-10). (+)-ESI-MS m/z 334.0 [M+H]^+; 667.3 [2M+H]^+, (-)-ESI-MS m/z 332.0 [M-H].

Cyclo-(L-Trp-L-Leu) (10): white solid, C_{17}H_{22}N_{3}O_{2}. ^{1}H NMR (500 MHz, CD_{3}OD) \delta 7.59 (d, J = 8.0 Hz, H-7), 7.32 (d, J = 8.1 Hz, H-4), 7.07 (t-like, J = 7.4 Hz, H-6), 7.06 (s, H-2), 6.99 (t-like, J = 7.4 Hz, H-5), 4.26 (t, J = 3.4 Hz, H-11), 3.57 (dd, J = 9.8, 4.1 Hz, H-14), 3.48 (dd, J = 14.7, 3.4 Hz, H-10), 3.12 (dd, J = 14.7, 4.4 Hz, H-10), 1.15 (dd, J = 9.4, 5.0 Hz, H-17), 0.68 (ddd, J = 13.7, 9.6, 4.3 Hz, H-17), 0.60, 0.47 (each 3H, d, J = 6.6 Hz, H-19 and H-20), -0.15 (m, H-18). ^{13}C NMR (125 MHz, CD_{3}OD) \delta 170.58 (s, C-14), 169.9 (s, C-11), 137.8 (s, C-9), 129.3 (s, C-8), 125.9 (d, C-2), 122.5 (d, C-6), 120.1 (d, C-5), 120.0 (d, C-4), 112.3 (d, C-7), 109.4 (s, C-3), 57.5 (d, C-12), 54.2 (d, C-11), 45.02 (t, C-10), 30.6 (t, C-17), 24.6 (d, C-18), 23.21, 21.32 (q, C-19 and C-20). (+)-ESI-MS m/z 300.0 [M+H]^+; 599.2 [2M+H]^+, (-)-ESI-MS m/z 298.0 [M-H].
Table S1. The NMR data of 1 (500 MHz and 125 MHz, CD$_3$OD)

| Site | $\delta_C$  | $\delta_H$                      | H2BC (H to C) | HMBC (H to C) |
|------|-------------|---------------------------------|---------------|---------------|
| 1    | 176.1 s     |                                 |               |               |
| 2    | 32.6 t      | a 2.44 (ddd, $J = 14.1, 8.0, 2.6$ Hz) | C-3           | C-1, 3, 4     |
|      |             | b 2.36 (ddd, $J = 14.1, 10.7, 2.6$ Hz) | C-3           | C-1, 4        |
| 3    | 33.3 t      | a 1.88 (ddddd, $J = 14.4, 10.7, 6.2, 2.6$ Hz) | C-2, 4       | C-1, 2, 4, 5  |
|      |             | b 1.78 (ddddd, $J = 14.4, 8.0, 4.7, 2.6$ Hz) | C-2, 4       | C-1, 2, 4, 5  |
| 4    | 72.5 d      | 3.71 (quint, $J = 6.2$ Hz)       | C-3, 5        | C-2, 3(w), 5(w) |
| 5    | 31.0 t      | a 1.52 (m, overlapped)           | C-4           | C-3           |
|      |             | b 1.40 (m)                       | C-5, 6        | C-4, 7        |
| 6    | 32.3 t      | a 1.68 (m)                       | C-5, 7        | C-4, 5, 7, 8  |
|      |             | b 1.54 (m, overlapped)           | C-5, 7        | C-4, 5, 7     |
| 7    | 69.8 d      | 3.77 (dqq, $J = 8.9, 4.4$ Hz)    | C-6, 8        | C-5(w), 6(w), 8 |
| 8    | 33.7 t      | a 1.51 (m, overlapped)           | C-7, 9        | C-7, 9        |
|      |             | b 1.44 (m)                       | C-7, 9        | C-7, 9        |
| 9    | 21.2 t      | a 1.53 (m, overlapped)           | C-10          | C-8           |
|      |             | b 1.25 (m)                       | C-8           | C-7(w), 8, 10, 11 |
| 10   | 35.0 t      | 1.65 (2H, m)                     | C-9, C-11     | C-8, 11       |
| 11   | 71.7 d      | 4.96 (sext, $J = 6.2$ Hz)        | C-10, 12      | C-1, 9, 10    |
| 12   | 21.2 q      | 1.23 (d, $J = 6.2$Hz)            | C-11          | C-10, 11      |
2. Splenocyte proliferation assay

Spleens were also obtained from the Balb/c mice, and single-cell suspensions were prepared. In particular, cell debris and clumps were removed after erythrocyte lysis, and mononuclear cells were washed and re-suspended in RPMI 1640 media supplemented with 10% foetal bovine serum and a 1% penicillin-streptomycin solution. Spleen cells at a density of $4 \times 10^5$/well were cultured in triplicate for 48 h with 1 $\mu$g/ml ConA, samples (final concentration 0.1-10 $\mu$M) or complete medium in flatbottomed 96-well plates. The cells were then pulsed with 20 $\mu$l CCK8/well for 4 h and the plates read OD values at 450 nm.

![Figure S2](image.png)

**Figure S2.** Cytotoxicity on splenocytes (a) and inhibition on Con A-induced splenocyte proliferation (b) of compounds 1–9.

3. Spectroscopes of aspergillolide (1)

![Figure S3](image.png)

**Figure S3.** $^1$H NMR spectrum of aspergillolide (1) in CD$_3$OD.
Figure S4. $^{13}$C NMR (DEPT-135) spectrum of aspergilloside (1) in CD$_3$OD.

Figure S5. HSQC spectrum of aspergilloside (1) in CD$_3$OD.
Figure S6. CT-HSQC spectrum of aspergillolide (1) in CD$_3$OD.

Figure S7. H2BC spectrum of aspergillolide (1) in CD$_3$OD (No. 1).
Figure S8. H2BC spectrum of aspergillolide (1) in CD$_3$OD (No. 2).

Figure S9. HMBC spectrum of aspergillolide (1) in CD$_3$OD.
Figure S10. CT-HMBC spectrum of aspergillolide (1) in CD$_3$OD.

Figure S11. (+/-)-ESI-MS spectra of aspergillolide (1).
Figure S12. (+)-HRESIMS spectrum of aspergillolide (1)

Figure S13. IR spectrum of aspergillolide (1).