Substituted l-tryptophan-l-phenyllactic acid conjugates produced by an endophytic fungus Aspergillus aculeatus using an OSMAC approach†

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The endophytic fungus Aspergillus aculeatus isolated from leaves of the papaya plant Carica papaya was fermented on solid rice medium, yielding a new l-tryptophan-l-phenyllactic acid conjugate (1) and thirteen known compounds (11, 14–25). In addition, an OSMAC approach was employed by adding eight different sodium or ammonium salts to the rice medium. Addition of 3.5% NaNO₃ caused a significant change of the metabolite pattern of the fungus as indicated by HPLC analysis. Subsequent isolation yielded several new substituted l-tryptophan-l-phenyllactic acid conjugates (1–10) in addition to three known compounds (11–13), among which compounds 2–10, 12–13 were not detected in the rice control culture. All structures were unambiguously elucidated by one and two dimensional NMR spectroscopy and by mass spectrometry. The absolute configuration of the new compounds was determined by Marfey’s reaction and X-ray single crystal diffraction. Compounds 19–22 showed cytotoxicity against the LS178Y mouse lymphoma cell line with IC₅₀ values of 3.4, 1.4, 7.3 and 23.7 μM, respectively.

Introduction

Endophytic fungi thrive widely in different healthy tissues of living plants, and have significant influence on the growth of their hosts.1,2 Endophytes also comprise a large reservoir of structurally diverse secondary metabolites including alkaloids, steroids, terpenoids, xanthones, peptides and quinones, which exhibit a variety of biological activities including anticancer, antibacterial, antifungal, anti-inflammatory and antidepressant activity.3–5 For example, 14-membered macrolides isolated from the endophytic fungus Pestalotiopsis microspora showed significant cytotoxicity against the murine lymphoma cell line LS178Y while fusaric acid derivatives produced by the fungal endophyte Fusarium oxysporum showed significant phytotoxicity to leaves of barley.6–8 However, the high rediscovery rate of known compounds from fungi is a severe obstacle for the search for new drug leads.6 One of the approaches to increase the diversity of metabolites from fungi involves the application of the OSMAC (One Strain Many Compounds) method which is based on systematic variations of the cultivation parameters (media type and composition, pH value, temperature etc.).9–11 For instance, the fermentation of the fungus Gymnascella danka-liensis on solid rice medium following addition of 3.5% NaBr led to the isolation of ten brominated tyrosine-derived alkaloids, which showed cytotoxicity against the LS178Y mouse lymphoma cell line.10,11 In addition, cultivation of Fusarium tricinctum on fruit and vegetable juice-supplemented solid rice media that led to the production of the new fusarielins K and L.12

The fungus Aspergillus aculeatus has been reported to produce several bioactive secondary metabolites, such as secalconic acids D and F, aculeatusquinone B and D, aculeacins A–G and aspergillusol A.13–17 In this study, A. aculeatus was isolated from leaves of Carica papaya collected in Awka in Nigeria. The fungus was fermented on solid rice medium, yielding a new l-tryptophan-l-phenyllactic acid conjugate (1) together with thirteen known compounds including N-[(2S)-2-hydroxy-1-oxo-3-
Results and discussion

Compound 1 had the molecular formula C_{22}H_{24}O_{4}N_{2} as established by HRESIMS exhibiting 12 degrees of unsaturation. The \[^{13}\text{C}\] NMR spectrum of 1 (Table 1) included 22 signals, corresponding to two methyls, two methylenes, two aliphatic methines and ten aromatic methines as well as six quaternary carbons. The \[^{1}\text{H}\] NMR spectrum of 1 (Table 2) showed the presence of a monosubstituted benzene ring and an indole moiety as indicated by signals between \(\delta_{\text{H}} 6.74\) and \(\delta_{\text{H}} 8.82\), two –CH\(_2\)–CH– units and two methyl groups (\(\delta_{\text{H}} 3.70\) and 3.82). These data were similar to those of the co-isolated known compound \(N^1[(2S)-2-hydroxy-1-\text{oxo-3-phenylpropyl}]\)-tryptophan methyl ester (11). However, the detection of an additional methyl substituent at \(\delta_{\text{C}} 32.7\) and \(\delta_{\text{H}} 3.70\) and the HMBC correlation from the protons of this methyl group to C-10 (\(\delta_{\text{C}} 138.5\)) and C-12 (\(\delta_{\text{C}} 129.0\)) indicated its attachment to N-11. Detailed analysis of the 2D NMR spectra of 1 (Fig. 2) revealed that its remaining substructure was identical to that of 11. The absolute configuration of 1 was determined by X-ray single crystal analysis as 2S, 2'S (Fig. 3), being identical to that of 11. Thus, the structure of 1, for which the trivial name aculeatine A is proposed, was elucidated as shown in Fig. 1.

Compound 2 possessed the molecular formula C\(_{22}\)H\(_{24}\)O\(_{4}\)N\(_{2}\) as determined by HRESIMS, which indicated the loss of a methyl group compared to 1. This was confirmed by the absence of the \[^{1}\text{H}\] and \[^{13}\text{C}\] signals of the methoxy group attached to C-1 in the NMR spectra of 2 (Tables 1 and 2). The structure of 2 was confirmed by detailed analysis of the 2D NMR spectra. Its absolute configuration was determined as 2S, 2’S by X-ray single crystal analysis (Fig. 3).

The molecular formula of compound 3 was determined as C\(_{27}\)H\(_{32}\)O\(_{4}\)N\(_{2}\) by HRESIMS. The \[^{13}\text{C}\] NMR spectrum of 3 exhibited five additional carbons compared to 1. The \[^{1}\text{H}\] NMR spectrum of 3 was similar to that of compound 1 except for the observation of two singlet methyls at \(\delta_{\text{H}} 1.66\) (Me-17 and 18) and signals of a terminal double bond at \(\delta_{\text{H}} 6.23\) (H-15), 5.11 and 4.98 (H-16) as well as the absence of the singlet aromatic proton at C-12. The COSY correlations between H-15 and H-16 together with the HMBC correlations from Me-17 and 18 to C-15 (\(\delta_{\text{C}} 133.9\)), and C-14 (\(\delta_{\text{C}} 142.2\)), C-14 (\(\delta_{\text{C}} 41.9\)) and C-15 (\(\delta_{\text{C}} 149.1\)) and from Me-13 (\(\delta_{\text{C}} 3.72\)) to C-12 and C-10 (\(\delta_{\text{C}} 139.0\)) indicated the attachment of a 1,1-dimethylprop-2-en-1-yl side chain at C-12. Detailed analysis of the 2D NMR spectra of 3 (Fig. 2) revealed that its remaining substructure was identical to that of 1. Compound 4 had the same molecular formula as 3. The \[^{1}\text{H}\] and \[^{13}\text{C}\] NMR data were also similar to those of 3. The appearance of a broad triplet proton at \(\delta_{\text{H}} 5.11\) (H-15) and one methylene group at \(\delta_{\text{H}} 3.48\) and 3.44, H\(_2\)-14 together with two olefinic methyl groups (\(\delta_{\text{C}} 1.74\) and 1.82, Me-17 and 18) in the \[^{1}\text{H}\] NMR spectrum of 4 suggested the existence of a 3-methyl-but-2-en-1-yl group at C-12, which was confirmed by the COSY correlations between H-15 and H-14 together with the HMBC correlations from Me-17 and 18 to C-15 (\(\delta_{\text{C}} 122.4\)) and C-16 (\(\delta_{\text{C}} 133.9\)) and from H\(_2\)-14 to C-4 (\(\delta_{\text{C}} 106.0\)) and C-12 (\(\delta_{\text{C}} 138.8\)) (Fig. 2).

Compound 5 was isolated as white powder. Its molecular formula was established as C\(_{26}\)H\(_{32}\)O\(_{4}\)N\(_{2}\) by HRESIMS. The \[^{1}\text{H}\] and \[^{13}\text{C}\] NMR spectra of 5 were similar to those of 4 except for

| Table 1 \[^{13}\text{C}\] NMR data of compounds 1–5 (CD\(_{3}\)OD, 150 MHz) |
|---|---|---|---|---|---|
| No. | 1 | 2 | 3 | 4 | 5 |
| 1 | 173.4, C | 174.8, C | 173.9, C | 173.8, C | 173.5, C |
| 2 | 53.7, CH | 53.7, CH | 55.3, C | 54.3, C | 53.6, CH |
| 3 | 28.4, CH\(_2\) | 28.4, CH\(_2\) | 29.2, CH\(_2\) | 28.0, CH\(_2\) | 28.5, CH\(_2\) |
| 4 | 109.2, C | 109.5, C | 107.5, C | 106.0, C | 109.4, C |
| 5 | 129.1, C | 129.4, C | 130.3, C | 129.1, C | 129.4, C |
| 6 | 119.6, CH | 119.8, CH | 119.2, CH | 119.0, CH | 119.6, CH |
| 7 | 119.9, CH | 119.9, CH | 120.1, CH | 120.0, CH | 120.0, CH |
| 8 | 122.6, CH | 122.5, CH | 122.7, CH | 122.0, CH | 122.5, CH |
| 9 | 110.2, CH | 110.1, CH | 109.7, CH | 109.7, CH | 110.6, CH |
| 10 | 138.5, C | 138.5, C | 139.0, C | 138.4, C | 137.8, C |
| 11 | 129.0, CH | 129.0, CH | 142.2, C | 138.8, C | 127.6, CH |
| 12 | 32.7, CH\(_3\) | 32.7, CH\(_3\) | 33.8, CH\(_3\) | 30.0, CH\(_3\) |
| 13 | | | 41.9, C | 24.8, CH\(_3\) | 44.8, CH\(_3\) |
| 14 | | | 149.1, CH | 124.4, CH | 121.5, CH |
| 15 | | | 112.8, CH\(_2\) | 133.9, C | 137.1, C |
| 16 | | | 30.0, CH\(_2\) | 25.8, CH\(_2\) | 25.8, CH\(_2\) |
| 17 | | | 29.9, CH\(_2\) | 18.1, CH\(_2\) | 18.1, CH\(_2\) |
| 18 | 175.8, C | 175.8, C | 176.0, C | 176.1, C | 175.9, C |
| 19 | 73.4, CH | 73.6, CH | 73.7, CH | 73.7, CH | 73.5, CH |
| 20 | 41.4, CH\(_2\) | 41.5, CH\(_2\) | 41.2, CH\(_2\) | 41.4, CH\(_2\) | 41.6, CH\(_2\) |
| 21 | 138.8, C | 139.0, C | 139.1, C | 138.9, C | 138.9, C |
| 22 | 130.9, CH | 130.9, CH | 130.6, CH | 130.6, CH | 130.9, CH |
| 23 | 129.1, CH | 129.1, CH | 129.0, CH | 129.0, CH | 129.1, CH |
| 24 | 127.5, CH | 127.5, CH | 127.3, CH | 127.4, CH | 127.5, CH |
the appearance of a singlet methine at $\delta_{1H}$ 6.70 (H-12) and the absence of the methyl group attached to N-11. The COSY correlations between H-15 ($\delta_{1H}$ 5.32) and H$_2$-14 ($\delta_{1H}$ 4.66) along with the HMBC correlations from H-14 to C-10 ($\delta_{C}$ 137.8) and C-12 ($\delta_{C}$ 127.6), and from both Me-17 ($\delta_{1H}$ 1.74) and Me-18 ($\delta_{1H}$ 1.83) to C-15 ($\delta_{C}$ 121.5) and C-16 ($\delta_{C}$ 137.1) indicated the presence of a 3-methyl-but-2-en-1-yl residue and its attachment to N-11 of compound 5 (Fig. 4).

The molecular formula of 6 was determined as C$_{25}$H$_{28}$O$_4$N$_2$, indicating the lack of a methyl group compared to 5, which was confirmed by the absence of signals of the methoxy group at C-1 (Table 3). The remaining substructure of 6 was unambiguously elucidated to be identical to that of 5 by detailed analysis of 2D NMR spectra.

Compound 7 possessed the molecular formula C$_{25}$H$_{30}$O$_4$N$_2$ as deduced from the HRESIMS data. The $^1$H and $^{13}$C NMR spectra of 7 were similar to those of 4, revealing the presence of one monosubstituted benzene ring, an indole moiety and a 3-methyl-but-2-en-1-yl side chain. However, the appearance of an aromatic proton signal at $\delta_{1H}$ 6.82 (H-7), which was split to a doublet, suggested that the 3-methyl-but-2-en-1-yl group was substituted on the benzene ring of the tryptophan moiety. The HMBC correlation from H$_2$-14 ($\delta_{1H}$ 3.73) to C-5 ($\delta_{C}$ 126.8), C-6 ($\delta_{C}$ 135.4) and C-7 ($\delta_{C}$ 120.7), and from H-7 to C-5, C-9 ($\delta_{C}$ 108.5) and C-14 ($\delta_{C}$ 33.3) indicated the 3-methyl-but-2-enyl group to be located at C-6. The structure of 7 was determined by analysis of the 2D NMR spectra as shown in Fig. 4.

Compound 8 possessed the molecular formula C$_{26}$H$_{30}$O$_4$N$_2$ as established by HRESIMS, thus lacking a methyl group compared to 7. The $^1$H and $^{13}$C NMR spectra were almost identical to those of 7 except for the absence of signals of the methoxy group at C-1, which was further confirmed by detailed analysis of the 2D NMR data of 8.

Compound 9 had the molecular formula C$_{26}$H$_{32}$O$_3$N$_2$ as determined by HRESIMS, corresponding to 12 degrees of unsaturation. The $^1$H NMR spectra of 9 (Table 4) revealed the presence of a 1,2,4-trisubstituted benzene ring and a monosubstituted benzene ring, suggesting a substituent at C-7 or C-8 of the indole moiety. A 3-hydroxy-3-methylbutyl moiety was established based on the correlation between H$_2$-14 ($\delta_{1H}$ 2.77) and H$_2$-15 ($\delta_{1H}$ 1.80) together with the HMBC correlations from Me-17 ($\delta_{1H}$ 1.24) and Me-18 ($\delta_{1H}$ 1.25) to C-16 ($\delta_{C}$ 71.4) and C-15 ($\delta_{C}$ 47.8). In addition, the HMBC correlation from H$_2$-14 to C-6 ($\delta_{C}$ 118.9), C-7 ($\delta_{C}$ 134.4) and C-8 ($\delta_{C}$ 123.5), from H$_2$-14 ($\delta_{1H}$ 7.36) to C-4 ($\delta_{C}$ 109.4), C-8, C-10 ($\delta_{C}$ 137.1) and C-14 ($\delta_{C}$ 32.0), and from H$_2$-9 ($\delta_{1H}$ 7.02) to C-6, C-10 and C-14 indicated the 3-hydroxy-3-methylbutyl moiety to be attached at C-7 (Fig. 4).

Compound 10 shared the same molecular formula as 9. The $^1$H NMR data of 10 resembled those of compound 9 except for the chemical shifts of the aromatic protons of the indole moiety. The HMBC correlation from H$_2$-14 ($\delta_{1H}$ 2.78) to C-7 ($\delta_{C}$ 121.0), C-8 ($\delta_{C}$ 137.7) and C-9 ($\delta_{C}$ 109.4), from H-7 ($\delta_{1H}$ 6.91) to C-5 ($\delta_{C}$ 127.7), C-9 and C-14 ($\delta_{C}$ 32.3), and from H-9 ($\delta_{1H}$ 7.11) to C-5, C-7 and C-14 confirmed that the 3-hydroxy-3-methylbutyl moiety was located at the C-8 position. The remaining substructure of 10 was the same as that of 9 as confirmed by detailed analysis of 2D NMR spectra of 10 (Fig. 4).

Compounds 3–10 share the same core structure with compounds 1 and 2, for which the absolute configuration had been assigned through X-ray analysis. Hence it is concluded on biogenetic terms that the absolute configuration of the former compounds is also 2$S$, 2$'$S. Compounds 11 and 12 were determined to have 2$S$ absolute configuration by Marfey’s reaction. Based on the close biogenetic similarity, the absolute configuration at C-2’ of the latter two compounds is assumed to be S.
A plausible biosynthetic pathway of the substituted L-tryptophan-L-phenyllactic acid conjugates obtained from A. aculeatus is proposed to start from L-tryptophan and phenylpyruvate. The important intermediate 12 is suggested to be formed by a condensation reaction between L-tryptophan and L-phenyllactic acid. The indole metabolites isolated in this study could be produced by further methylation of 12 and prenylation of the indole nucleus of tryptophan as shown in Fig. 5.

Fig. 1 Structures of compounds 1–25 isolated from A. aculeatus.
All isolated compounds (1–25) were evaluated for their cytotoxicity against the L5178Y mouse lymphoma cell line. Secalonic acids D and F (19 and 20), asperdichrome (21) and RF 3192C (22) showed cytotoxicity with IC\textsubscript{50} values of 3.4, 1.4, 7.3 and 23.7 μM, respectively, whereas the remaining compounds proved to be inactive when assayed at a dose of 10 μg mL\textsuperscript{-1}.

The results of the OSMAC experiments indicated that substituted l-tryptophan-l-phenyllactic acid conjugates were induced by nitrate but not by sodium in the medium. In the absence of nitrogen sources favoured by the fungus including ammonium and glutamine, fungi are able to use secondary nitrogen sources such as nitrate, purines, urea, amines and amides etc., which is commonly known as nitrogen metabolite repression (NMR). In fungi, the activity of nitrogen regulators for derepression of NMR genes, which also affect secondary metabolites formation, is regulated by the intracellular nitrogen status and extracellular nitrogen availability. For example, the main GATA transcriptional regulator of nitrogen
Table 3 13C NMR data of compounds 6–10 (CD3OD, 150 MHz)

| No. | 6a | 7 | 8 | 9 | 10 |
|-----|----|---|---|---|----|
| 1   | 176.4, C | 173.7, C | 175.0, C | 175.8, C | 175.6, C |
| 2   | 54.3, CH | 54.7, CH | 54.4, CH | 54.3, CH | 54.1, CH |
| 3   | 28.5, CH2 | 30.3, CH2 | 30.4, CH2 | 28.3, CH2 | 28.4, CH2 |
| 4   | 110.0, C | 110.1, C | 110.4, C | 109.4, C | 109.7, C |
| 5   | 129.6, C | 126.8, C | 127.0, C | 129.8, C | 127.7, C |
| 6   | 119.8, CH | 135.4, C | 135.5, C | 118.9, CH | 119.8, CH |
| 7   | 119.5, CH | 120.7, CH | 120.7, C | 134.4, C | 121.0, CH |
| 8   | 122.0, CH | 122.7, CH | 122.6, CH | 123.5, CH | 137.7, C |
| 10  | 110.1, CH | 108.5, CH | 108.4, CH | 110.0, CH | 109.4, CH |
| 11  | 137.4, C | 139.3, C | 139.3, C | 137.1, C | 138.8, C |
| 12  | 127.3, CH | 129.4, CH | 129.3, CH | 129.1, CH | 128.5, CH |
| 13  | 32.9, CH3 | 32.9, CH3 | 32.7, CH3 | 32.7, CH3 | 32.7, CH3 |
| 14  | 44.5, CH2 | 33.3, CH2 | 33.4, CH2 | 32.0, CH2 | 32.2, CH2 |
| 15  | 121.4, CH | 125.5, CH | 125.5, CH | 47.8, CH2 | 47.8, CH2 |
| 16  | 136.5, C | 132.9, C | 132.9, C | 71.4, C | 71.4, C |
| 17  | 25.5, CH2 | 25.9, CH3 | 25.9, CH3 | 29.3, CH3 | 29.3, CH3 |
| 18  | 17.8, CH2 | 18.2, CH3 | 18.3, CH3 | 29.2, CH3 | 29.2, CH3 |
| 19  | 1’  | 175.4, C | 176.1, C | 176.1, C | 175.9, C | 175.8, C |
| 20  | 2’  | 73.6, CH2 | 73.8, CH3 | 73.8, CH3 | 73.8, CH3 | 73.8, CH3 |
| 21  | 3’  | 41.3, CH2 | 41.7, CH3 | 41.7, CH3 | 41.6, CH3 | 41.4, CH3 |
| 22  | 4’  | 138.9, C | 138.9, C | 139.0, C | 139.1, C | 139.1, C |
| 23  | 5’  | 130.5, CH | 130.6, CH | 130.6, CH | 130.8, CH | 130.8, CH |
| 24  | 6’  | 128.8, CH | 129.1, CH | 129.1, CH | 129.1, CH | 129.1, CH |
| 25  | 7’  | 127.1, CH | 127.4, CH | 127.4, CH | 127.4, CH | 127.4, CH |
| OCH3 | 52.7, CH3 |

* Measured at 175 MHz.

Experimental section

General procedures

A Jasco P-2000 polarimeter was used to measure the optical rotation. 1D and 2D NMR spectra were recorded on Bruker Avance DMX 600 or 700 NMR spectrometers. Chemical shifts were referenced to the solvent residual peaks. Mass spectra were recorded with a LC-MS HP1100 Agilent Finnigan LCQ Deca XP.

Table 4 1H NMR data of compounds 6–10 (CD3OD, 600 MHz)

| No. | 6a | 7 | 8 | 9 | 10 |
|-----|----|---|---|---|----|
| 2   | 4.66, m | 4.71, dd (8.7, 5.6) | 4.70, dd (8.9, 4.8) | 4.67, m | 4.67, m |
| 3   | 3.23, dd (14.5, 6.1) | 3.38, dd (15.0, 5.6) | 3.45, dd (15.1, 4.8) | 3.21, dd (14.8, 5.8) | 3.20, dd (14.8, 5.9) |
| 4   | 3.17, dd (14.5, 4.7) | 3.18, dd (15.0, 8.7) | 3.20, dd (15.1, 8.9) | 3.16, dd (14.8, 4.7) | 3.13, dd (14.8, 4.7) |
| 6   | 7.53, d (7.8) | 6.82, d (7.2) | 6.82, d (7.0) | 7.36, s | 7.40, d (8.0) |
| 7   | 7.01, d (7.8, 7.1) | 7.06, dd (6.8, 7.2) | 7.06, dd (8.3, 7.0) | 7.02, d (8.3) | 6.91, d (8.0) |
| 8   | 7.11, d (8.3, 7.1) | 7.16, d (8.6) | 7.15, d (8.3) | 7.20, d (8.3) | 7.11, s |
| 9   | 6.80, s | 6.82, s | 6.84, s | 6.68, s | 6.63, s |
| 12  | 3.70, s | 3.69, s | 3.68, s | 3.68, s | 3.68, s |
| 14  | 4.65, m | 3.73, m | 3.77, dd (16.3, 6.8) | 3.73, dd (16.3, 6.8) | 2.77, m | 2.78, m |
| 15  | 5.32, br t (6.8) | 5.31, br t (6.6) | 5.32, br t (6.8) | 1.80, m | 1.79, m |
| 17  | 1.72, s | 1.76, s | 1.76, s | 1.24, s | 1.26, s |
| 18  | 1.83, s | 1.77, s | 1.77, s | 1.25, s | 1.26, s |
| 2’  | 4.16, dd (8.0, 3.5) | 4.19, dd (7.8, 3.9) | 4.18, dd (8.0, 3.8) | 4.19, dd (7.8, 3.6) | 4.19, dd (7.6, 3.7) |
| 3’  | 2.93, dd (13.9, 3.5) | 2.88, dd (13.9, 3.9) | 2.87, dd (13.9, 3.8) | 2.95, dd (13.6, 3.9) | 2.93, dd (13.9, 3.7) |
| 5’  | 2.63, dd (13.9, 8.0) | 2.62, dd (13.9, 7.8) | 2.58, dd (13.9, 8.0) | 2.65, dd (13.9, 7.8) | 2.66, dd (13.9, 7.6) |
| 6’  | 7.16, d (7.2) | 7.11, d (6.9) | 7.11, d (6.9) | 7.17, d (7.2) | 7.17, d (7.2) |
| 7’  | 7.20, t (7.2) | 7.14, t (6.9) | 7.12, t (6.9) | 7.20, t (7.2) | 7.22, t (7.2) |
| OCH3 | 3.66, s |

* Measured at 700 MHz.
Thermoquest and HRESIMS were measured with a UHR-QTOF maXis 4G (Bruker Daltonics) mass spectrometer. HPLC analysis was performed on a Dionex 3000 RS system coupled with an Ultimate 3000 pump and a photodiode array detector (DAD 300RS). The analytical column (125 × 4 mm) was prefilled with Eurosphere-10 C18 (Knauer, Germany), and the following gradient solvent system was used: 0 min (10% MeOH), 5 min (10% MeOH), 35 min (100% MeOH), and 45 min (100% MeOH).

Semi-preparative HPLC was performed using a Merck Hitachi HPLC System (UV detector L-7400; pump L-7100; Eurosphere-10 C18, 300 × 8 mm, Knauer) with MeOH-H2O as mobile phase and a flow rate of 5.0 mL min⁻¹. Column chromatography was carried out using Merck MN silica gel 60 M (0.04–0.063 mm). TLC plates with silica gel F254 (Merck) were used to monitor and collect fractions under detection at 254 and 366 nm. Distilled and spectral-grade solvents were used for column chromatography and spectroscopic measurements, respectively.

**Fungal material and cultivation**

*A. aculeatus* was isolated from leaves of *Carica papaya* collected in Awka in Nigeria and was identified by DNA amplification, sequencing of ITS region and by comparing with GenBank data (GeneBank accession no. KX137846) following standard procedures.

The fungal strain was grown on solid rice medium (100 g rice and 100 mL distilled water autoclaved) in ten Erlenmeyer flasks (1 L each) at 22 °C under static conditions for 14 days. The OSMAC experiments were performed on rice medium containing either 3.5% NaCl, 3.5% NaBr, 3.5% NaI, 1% NaF, 3.5% NaN3, 3.5% NH4Cl, 3.5% (NH4)2SO4 or 3.5% NH4OAc under static conditions until they reached their stationary phase of growth (16 days except for rice media spiked with 1% NaF or 3.5% NH4OAc where the fungus failed to grow).

**Extraction and isolation**

Fungal cultures were extracted with EtOAc followed by evaporation under reduced pressure. Initial purification of the EtOAc extract (12.5 g) of the fungal culture fermented on rice was performed by partitioning between n-hexane and 90% aqueous MeOH. The 90% aqueous MeOH phase (9.2 g) was centrifuged and then fractionated by vacuum liquid chromatography on reversed-phase silica gel using a gradient elution of H2O–MeOH (10:90 – 0:100) to give 10 fractions (Fr.1 to Fr.10).

Fr.2 (152 mg) was chromatographed on a Sephadex LH-20 column with MeOH followed by further purification using semi-preparative HPLC to give 17 (1.5 mg), 18 (7.0 mg) and 24 (0.5 mg). Compound 19 (30.2 mg) was obtained from Fr.3 (835 mg) by recrystallization. Part of Fr.3 was purified by semi-preparative HPLC to give 20 (20.0 mg). Fr.4 (208 mg) was subjected to a Sephadex LH-20 column with MeOH to give two subfractions (Fr.4-1 and Fr.4-2). Fr.4-1 was further purified by a silica gel column with DCM/MeOH as mobile phase to give 15 (3.8 mg). Compound 1 (13.1 mg) was obtained by recrystallization from Fr.4-2. Fr.5 (160 mg) was separated by a Sephadex LH-20 column followed by purification using semi-preparative HPLC to yield 14 (0.7 mg) and 21 (8.2 mg). Compound 11 (3.2 mg), 22 (1.2 mg) and 25 (2.4 mg) were obtained from Fr.6 (54 mg) by semi-preparative HPLC. Fr.7 (101 mg) was subjected to a Sephadex LH-20 column with MeOH as mobile phase and then purified by semi-preparative HPLC to give 23 (2.0 mg) and 16 (0.8 mg).

The fungal cultures from the OSMAC experiments that were grown on rice medium containing different salts were extracted with EtOAc (2 × 500 mL) followed by solvent evaporation under reduced pressure. The obtained crude extracts were analyzed by HPLC. The EtOAc extracts (28.6 g) of fungal cultures that had been grown on rice medium (30 flasks) after adding 3.5% NaN3 were dissolved in MeOH and then subjected to vacuum filtering. The obtained MeOH solution was evaporated under reduced pressure and fractionated by vacuum liquid chromatography on silica gel using a gradient elution of n-hexane–EtOAc to give 24 fractions (Fr.N1 to Fr.N24). Fr.N19 (221 mg) was separated by a Sephadex LH-20 column with MeOH as mobile phase followed by semi-preparative HPLC to give 3 (3.5 mg) and 4 (15.0 mg). Fr.N22 (465 mg) was separated by
a Sephadex LH-20 column with MeOH as mobile phase to give 3 fractions. Fr.N22-2 (54 mg) was further purified by semi-preparative HPLC to give 1 (10.1 mg), 2 (10.4 mg), 11 (3.5 mg), 12 (3.4 mg) and 13 (2.5 mg). Fr.N22-3 (380 mg) was further purified by a silica gel column with DCM–MeOH as mobile phase followed by semi-preparative HPLC to give 5 (2.4 mg), 6 (0.9 mg), 7 (4.8 mg), 8 (4.7 mg), 9 (1.2 mg) and 10 (1.2 mg).

Aculetine A (1), white needle crystals; [α]D20 +23 (c 0.25, MeOH); UV (MeOH): λmax 221, 288 nm; HRESIMS m/z 381.1810 [M + H]+ (calcd 381.1809 for C22H25O3N2); 1H and 13C NMR data see Tables 1 and 2.

Aculetine B (2), colorless needle crystals; [α]D20 +3 (c 0.40, MeOH); UV (MeOH): λmax 227, 288 nm; HRESIMS m/z 367.1653 [M + H]+ (calcd 367.1652 for C22H23O3N2); 1H and 13C NMR data see Tables 1 and 2.

Aculetine C (3), white amorphous powder; [α]D20 −54 (c 0.70, MeOH); UV (MeOH): λmax 228, 287 nm; HRESIMS m/z 449.2439 [M + H]+ (calcd 449.2435 for C22H13O3N2); 1H and 13C NMR data see Tables 1 and 2.

Aculetine D (4), white amorphous solid; [α]D20 −47 (c 1.7, MeOH); UV (MeOH): λmax 233, 286 nm; HRESIMS m/z 424.2345 [M + H]+ (calcd 424.2345 for C22H13O3N2); 1H and 13C NMR data see Tables 1 and 2.

Aculetine E (5), white amorphous powder; [α]D20 −2 (c 0.48, MeOH); UV (MeOH): λmax 228, 287 nm; HRESIMS m/z 421.2122 [M + H]+ (calcd 421.2122 for C22H22O3N2); 1H and 13C NMR data see Tables 1 and 2.

Aculetine F (6), white amorphous solid; [α]D20 −11 (c 0.20, MeOH); UV (MeOH): λmax 228, 288 nm; HRESIMS m/z 421.2122 [M + H]+ (calcd 421.2122 for C22H22O3N2); 1H and 13C NMR data see Tables 1 and 2.

Aculetine G (7), white amorphous powder; [α]D20 −24 (c 0.20, MeOH); UV (MeOH): λmax 225, 291 nm; HRESIMS m/z 435.2282 [M + H]+ (calcd 435.2278 for C22H13O3N2) and m/z 471.2252 [M + Na]+ (calcd 471.2254 for C22H12O3N2Na); 1H and 13C NMR data see Tables 1 and 2.

Aculetine H (8), white amorphous powder; [α]D20 −41 (c 0.94, MeOH); UV (MeOH): λmax 226, 291 nm; HRESIMS m/z 435.2282 [M + H]+ (calcd 435.2278 for C22H13O3N2) and m/z 477.2109 [M + Na]+ (calcd 477.2109 for C22H12O3N2Na); 1H and 13C NMR data see Tables 3 and 4.

Aculetine I (9), white amorphous solid; [α]D20 −35 (c 0.24, MeOH); UV (MeOH): λmax 225, 292 nm; HRESIMS m/z 453.2383 [M + H]+ (calcd 453.2384 for C22H13O3N2); 1H and 13C NMR data see Tables 3 and 4.

Aculetine J (10), light yellow amorphous solid; [α]D20 −19 (c 0.24, MeOH); UV (MeOH): λmax 226, 287 nm; HRESIMS m/z 453.2380 [M + H]+ (calcd 453.2384 for C22H13O3N2) and m/z 475.2201 [M + Na]+ (calcd 475.2203 for C22H12O3N2Na); 1H and 13C NMR data see Tables 3 and 4.

X-ray crystallographic analysis of compounds 1 and 2

**Crystallization conditions.** Suitable single crystals of 1 and 2 were obtained by slow evaporation from methanol solution and selected under a polarized light microscope. **Data collection:** compounds 1 and 2 were measured on a Bruker Kappa APEX2 CCD diffractometer with micro focus tube using Cu-Kα radiation (λ = 1.54178 Å). APEX2 was used for data collection, SAINT for cell refinement and data reduction, and SADABS for experimental absorption correction. SHIELXL-2017 was used for the structure solution by intrinsic phasing. SHIELXL-2017 was used for refinement by full-matrix least-squares on F². The hydrogen atoms were positioned geometrically (with C–H = 0.95 Å for aromatic CH, 1.00 Å for tertiary CH, 0.99 Å for CH2 and 0.98 Å for CH3). The refinement was carried out using riding models (AFIX 43, 13, 23, 137, respectively), with Uiso(H) = 1.2Ueq(CH, CH2) and 1.5Ueq(CH3). The hydrogen atoms in the hydroxy and amine groups were refined with Uiso(H) = 1.5Ueq(O/N). The hydrogen atoms in the solvent methanol molecule in 2 were refined with Uiso(H) = 1.5Ueq(O).

The absolute structures of compound 1 and 2 were determined using anomalous dispersion from Cu–Kα radiation, resulting in Flack parameters of −0.14(10) (1) and 0.003(2) (2) using Parsons quotient method. Due to a rather high Flack parameter for 1, its absolute structure was determined using likelihood methods. DIAMOND was used for the drawing of all graphs. PLATON for Windows was used for the analyses of hydrogen bonds and CH–π interactions. The structural data for 2 has been deposited in the Cambridge Crystallographic Data Center (CCDC no. 1589955). Crystals of 1 did not diffract beyond θ = 44.9° (cf. desired 67.7°) for Cu–Kα radiation, resulting in only 1538 total (1484 observed with I > 2σ(I)) reflections versus 263 parameters for anisotropic refinement. Therefore the cif did not meet the requirements for publication and refinement data of compound 1 is only given in the ESL.

**Crystal data of 1.** C22H22N2O4, M = 380.43, orthorhombic system, space group P212121, a = 11.8099(7) Å, b = 11.8099(7) Å, c = 27.4081(17) Å, V = 1936.7(2) Å³, Z = 4, Dcalc = 1.305 g cm⁻³, crystal size 0.12 × 0.03 × 0.03 mm³, μ(Cu-Kα) = 0.73 mm⁻¹, 3.2° < θ < 44.9°, N = 321, N = 1538 (Rint = 0.051), R1 = 0.022, wR2 = 0.053, S = 1.12, Flack parameter = −0.14(10), Hooft parameter = −0.09(8), probability for correct absolute structure P2 = 1.000.

**Crystal data of 2.** C21H22N2O4·CH2O, M = 398.45, monoclinic system, space group C2, a = 23.522(2) Å, b = 5.994(5) Å, c = 15.843(13) Å, V = 2063.3(2) Å³, Z = 4, Dcalc = 1.283 g cm⁻³, crystal size 0.20 × 0.10 × 0.05 mm³, μ(Cu-Kα) = 0.75 mm⁻¹, 5.9° < θ < 67.5°, N = 3100, N = 3146 (Rint = 0.027), R1 = 0.025, wR2 = 0.067, S = 1.09, Flack parameter = 0.00(3), Hooft parameter = 0.00(3), probability for correct absolute structure P2 = 1.000.

**Marfey’s reaction for compounds 11 and 12**

Compounds 11 and 12 (0.5 mg) were hydrolyzed with 2 mL 6 M HCl containing 0.4% β-mercaptoethanol at 110 °C for 24 h. The hydrolysate was evaporated to dryness and treated separately with 4 M NaOH at room temperature for 4 h. 4 M HCl was used to adjust the pH to 4. The above resulting solutions were evaporated until complete elimination of HCl and then resuspended in 50 μL H2O. To 25 μL of each resulting solutions was added 50 μL FDA (1% 1-fluoro-2-4-dinitrophenyl-5-s-alanine amide in acetone) and 10 μL NaHCO3. The reaction tubes were covered with an aluminum paper and heated over a hot plate at
40 °C for 1 h. After cooling to room temperature, 5 μL of 2 M HCl was added and then evaporated to dryness. The residue was dissolved in 500 μL MeOH. α-Tryptophan and α-tryptophan were treated separately with FDAA in the same manner. The analysis of FDAA derivatives were carried out using HPLC and LC-MS by comparison of the retention time and molecular weight.

Cytotoxicity assay

Cytotoxicity was tested against the L5178Y mouse lymphoma cell line (European Collection of Authenticated Cell Cultures, Catalogue no. 87111908) using the MTT method as described before.11 Kahalalide F was used as positive control with an IC50 value of 4.3 μM.

Conflicts of interest

There are no conflicts to declare.

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