A New Cyclic Hexapeptide and a New Isocoumarin Derivative from the Marine Sponge-Associated Fungus Aspergillus similanensis KUFA 0013

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Abstract: A new isocoumarin derivative, similanpyrone C (1), a new cyclohexapeptide, similanamide (2), and a new pyripyropene derivative, named pyripyropene T (3) were isolated from the ethyl acetate extract of the culture of the marine sponge-associated fungus Aspergillus similanensis KUFA 0013. The structures of the compounds were established based on 1D and 2D NMR spectral analysis, and in the case of compound 2 the stereochemistry of its amino acid constituents was determined by chiral HPLC analysis of the hydrolysate by co-injection with the D and L amino acids standards. Compounds 2 and 3 were evaluated for their in vitro growth inhibitory activity against MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A373 (melanoma) cell lines,
as well as antibacterial activity against reference strains and the environmental multidrug-resistant isolates (MRS and VRE). Only compound 2 exhibited weak activity against the three cancer cell lines, and neither of them showed antibacterial activity.

**Keywords:** Aspergillus similanensis; cyclic hexapeptide; similanamide; isocoumarin; similanpyrone C; pyripyropene T

1. Introduction

In recent years, there has been an increasing interest in marine-derived fungi as a target source of bioactive marine natural products because many consider them among the world’s greatest untapped resources for new biodiversity as well as chemodiversity [1–3]. Moreover, through established culture methods, the compounds can be produced in quantity needed for medicinal chemistry development, clinical trials and even marketing. Among the marine fungal strains investigated, the fungi of the genus Aspergillus are the most prolific source of bioactive secondary metabolites, including sterols [4], cerebrosides [5], sesquiterpenoids [6,7], sesterterpenoids [8,9], diterpenoids [10], meroterpenoids [11], anthraquinone derivatives [12,13], nucleoside derivatives [14], indole alkaloids [15–17], prenylated indole alkaloids [18–21], quinazolinone alkaloids [22,23], pyrrolidine alkaloids [8], and cyclic peptides [24–28].

In our ongoing search for new natural products with antibacterial and anticancer activities produced by the marine-derived fungi of the genera Neosartorya and Aspergillus, we have recently reported the isolation of new isocoumarins similanpyrones A and B, a new chevalone (chevalone E), and a new natural product pyripyrone S; besides the previously reported chevalone B and C, a meroterpenoid S14-95, and pyripyropene E, from the crude ethyl acetate extract of the undescribed marine sponge-associated fungus Aspergillus similanensis KUFA 0013 [29]. Reexamination of the fractions remaining from the previous study of this fungus led to the isolation of a new 8-hydroxy-3-methylisocoumarin derivative, which we have named similanpyrone C (1), a new cyclic hexapeptide, similanamide (2), and a new pyripyropene analog, pyripyropene T (3) (Figure 1). Hydrolysis of compound 2, followed by HPLC analysis of its hydrolysate using a chiral column, led to the elucidation of its amino acid constituents. Compounds 2 and 3 were evaluated for their antibacterial activity as well their cytotoxicity against three human cancer cell lines.
2. Results and Discussion

 Compound 1 was isolated as pale yellow viscous mass, and its molecular formula C$_{19}$H$_{20}$O$_6$ was established on the basis of the (+)-HRESIMS $m/z$ 345.1342 [M + H]$^+$, indicating ten degrees of unsaturation. The IR spectrum showed absorption bands for hydroxyl (3443 cm$^{-1}$), ketone carbonyl (1730 cm$^{-1}$), conjugated lactone carbonyl (1683 cm$^{-1}$), olefin (1647 cm$^{-1}$) and aromatic (1625, 1572 cm$^{-1}$) groups. The $^{13}$C NMR (Supplementary Information, Figure S3), DEPTs and HSQC spectra (Table 1, Supplementary Information, Figure S4) revealed the presence of one ketone carbonyl ($\delta_C$ 205.3), one conjugated ester carbonyl ($\delta_C$ 166.8), six quaternary sp$^2$ ($\delta_C$ 160.0, 158.3, 153.4, 136.7, 110.0 and 99.6), two methine sp$^2$ ($\delta_C$ 104.4 and 103.0), one ketal ($\delta_C$ 102.9), two sp$^3$ methine ($\delta_C$ 67.2 and 33.9), two sp$^3$ methylene ($\delta_C$ 48.3 and 47.2), and three methyl ($\delta_C$ 21.6, 19.4 and 15.9) carbons. The $^1$H NMR spectrum (Supplementary Information, Figure S1) revealed, besides a singlet of the hydrogen bonded hydroxyl proton at $\delta_H$ 11.35, one doublet at $\delta_H$ 6.13 ($J = 0.9$ Hz) and one singlet at $\delta_H$ 6.26, two multiplets at $\delta_H$ 4.15 and $\delta_H$ 1.98, two double double doublets at $\delta_H$ 2.48 ($J = 14.7, 2.9, 1.9$ Hz) and $\delta_H$ 2.28 ($J = 14.7, 11.3, 0.7$ Hz), two double double doublets at $\delta_H$ 2.81 ($J = 16.8, 5.6$ Hz) and $\delta_H$ 2.55 ($J = 14.0, 1.9$), two broad double double doublets at $\delta_H$ 2.81 ($J = 14.0$ Hz) and $\delta_H$ 2.57 ($J = 16.8$ Hz), one methyl singlet at $\delta_H$ 2.24, and two methyl double double doublets at $\delta_H$ 1.23 ($J = 6.2$ Hz) and $\delta_H$ 1.21 ($J = 6.2$ Hz).
Table 1. $^1$H and $^{13}$C NMR (CDCl$_3$, 500.13 MHz and 125.8 MHz) and HMBC assignment for 1.

| Position | $\delta_C$, Type | $\delta_H$, (J in Hz) | COSY | HMBC | NOESY |
|----------|------------------|-----------------------|------|------|-------|
| 1        | 166.8, CO        | -                     |      |      |       |
| 3        | 153.4, C         | -                     |      |      |       |
| 4        | 104.4, CH        | 6.13, d (0.9)         | CH$_3$-3 | C-3, 5, 8a | CH$_3$-3 |
| 4a       | 136.7, C         | -                     |      |      |       |
| 5        | 103.0, CH        | 6.26, s               | C-4, 6, 7, 8a |      |       |
| 6        | 158.3, C         | -                     |      |      |       |
| 7        | 110.0, C         | -                     |      |      |       |
| 8        | 160.0, C         | -                     |      |      |       |
| 8a       | 99.6, C          | -                     |      |      |       |
| 9α       | 23.6, CH$_2$     | 2.81, dd (16.8, 5.6)  | H-9β, H-10 | C-6, 7, 10, 11 | H-9β, H-10, CH$_3$-10 |
| 9β       | 2.57, brd (16.8) | H-9α, H-10            | C-7, 10, 11 | H-9α, H-10, CH$_3$-10 |
| 10       | 33.9, CH         | 1.98, m               | H-9α, H-9β, CH$_3$-10 | H-9α, H-9β, CH$_3$-10 |
| 11       | 102.9, C         | -                     |      |      |       |
| 12α      | 47.2, CH$_2$     | 2.55, dd (14.0, 1.9)  | H-12β | C-10, 11, 13 | H-12β |
| 12β      | 2.81, brd (14.0) | H-12α                 | C-10, 11, 13 | H-12α |
| 13       | 205.3, CO        | -                     |      |      |       |
| 14α      | 48.3, CH$_2$     | 2.28, ddd (14.7, 11.3, 0.7) | H-14β, H-15 | C-13, 15 | H-14β, CH$_3$-15 |
| 14β      | 2.48, ddd (14.7, 2.9, 1.9) | H-14α, H-15 | C-13 | H-14β, CH$_3$-15 |
| 15       | 67.2, CH         | 4.15, m               | H-14α, H-14β, CH$_3$-15 | H-14β, CH$_3$-15 |
| CH$_3$-3 | 19.4, CH$_3$     | 2.24, s               | H-4  | C-3, 4 |       |
| CH$_3$-10| 15.9, CH$_3$     | 1.23, d (6.2)         | H-10 | C-9, 10, 11 | H-9β, H-10, H-12α, 2β |
| CH$_3$-15| 21.6, CH$_3$     | 1.21, d (6.2)         | H-15 | C-14, 15 | H-14α, H-14β, H-15 |
| OH-8     | -                | 11.35, s              | C-7, 8, 8a |       |       |
Analysis of the $^1$H, $^{13}$C NMR, HSQC and HMBC spectra (Table 1) revealed the presence of a 6,7-disubstituted 3-methyl-1H-isochromen-1-one nucleus, similar to that of similanpyrone B [29]. Thus, another portion of the molecule consisted of one ketone ($\delta_C 205.3$), one ketal ($\delta_C 102.9$), one methine ($\delta_H 1.98$, m; $\delta_C 33.9$), one oxymethine ($\delta_H 4.15$, m; $\delta_C 67.2$), three methylene ($\delta_H 2.81$, dd, $J = 16.8, 5.6$ Hz and $\delta_H$ 2.57, brd, $J = 16.8$ Hz, $\delta_C 23.6$; $\delta_H$ 2.81, brd, $J = 14.0$ Hz and 2.55, dd, $J = 14.0$, 1.9 Hz; $\delta_C$ 47.2; $\delta_H$ 2.48, ddd, $J = 14.7$, 2.9, 1.9 Hz and 2.28, ddd, $J = 14.7$, 11.3, 0.7 Hz, $\delta_C$ 48.3), two methyl ($\delta_H$ 1.21, d, $J = 6.2$ Hz; $\delta_C$ 21.6 and $\delta_H$ 1.23, d, $J = 6.2$ Hz, $\delta_C$ 15.9) groups. That this portion was 2,10-dimethyl-1,7-dioxaspiro[5.5]undec-8-en-4-one was evidenced by the COSY correlations (Table 1, Supplementary Information, Figure S2) of H$_2$-9 ($\delta_H$ 2.81, dd, $J = 16.8$, 5.6 Hz and 2.57, brd, $J = 16.8$ Hz) to H-10 ($\delta_H$ 1.98, m), of H-10 to CH$_3$-10 ($\delta_H$ 1.23, d, $J = 6.2$ Hz), of H$_2$-14 ($\delta_H$ 2.48, ddd, $J = 14.7$, 2.9, 1.9 Hz and 2.28, ddd, $J = 14.7$, 11.3, 0.7 Hz) to H-15 ($\delta_H$ 4.15, m), and of H-15 to CH$_3$-15 ($\delta_H$ 1.21, d, $J = 6.2$ Hz), as well as by the HMBC cross peaks (Table 1, Supplementary Information, Figure S5) of H$_2$-9 to C-10 and C-11 ($\delta_C 102.9$), of H$_2$-12 ($\delta_H$ 2.81, brd, $J = 14.0$ Hz and 2.55, dd, $J = 14.0$, 1.9 Hz) to C-10, 11, 13 ($\delta_C 205.3$), and of H-14 to C-13 and 15, respectively (Figure 2). That the 1,7-dioxaspiro[5.5]undec-8-en-4-one ring system was fused with the 3-methyl-1H-isochromen-1-one nucleus, through C-8 and C-9 of the methylidihydropyran ring of the former and C-6 and C-7 of the latter, was supported by the HMBC correlations of H$_2$-9 to C-6, 7 (Table 1, Supplementary Information, Figure S5). Literature search revealed that 1 is a new compound, and in compliance with our previous work, we have therefore named it similanpyrone C. Since 1 was isolated as viscous mass, it was not possible to obtain suitable crystals for the X-ray analysis. Consequently, the absolute configuration of C-10, C-11 and C-15 is still undetermined.

![Figure 2. Key HMBC correlations of compound 1.](image-url)

In an attempt to determine the stereochemistry of C-10 and C-15, we have sorted out the coupling constants of both H$_2$-9 and H$_2$-14. The fact that H$_2$-9 appeared as a double doublet at $\delta_H$ 2.81 with a germinal coupling of 16.8 Hz, and a small coupling at $\delta_H$ 5.6 Hz, typical of axial-equatorial coupling, and a broad doublet at $\delta_H$ 2.57 with a germinal coupling constant of 16.8 Hz, we concluded that CH$_3$-10 was in the $\beta$-axial position of the half-chair conformation of the tetrahydropyran ring. This was corroborated by the correlations of H-10, which was in $\alpha$ equatorial position, to both H-9$\beta$ ($\delta_H$ 2.57, brd, $J = 16.8$ Hz) and H-9$\alpha$ ($\delta_H$ 2.81, dd, $J = 16.8$, 5.6 Hz) in the NOESY spectrum (Table 1, Supplementary Information, Figure S6). On the contrary, one of H-14 appeared as a double double doublet at $\delta_H$ 2.28, with a germinal coupling of 14.7 Hz and a diaxial coupling of 11.3 Hz, as well as a small coupling (long range) of 0.7 Hz, while another appeared also as a double double doublet at...
δH 2.48, with a germinal coupling of 14.7 Hz and an axial-equatorial coupling of 2.9 Hz, as well as a small coupling (long range) of 1.9 Hz. These patterns of couplings revealed that CH3-15 was in α equatorial position of the chair conformation of the tetrahydro-4H-pyran-4-one ring. This analysis was corroborated by the NOESY spectrum (Table 1, Supplementary Information, Figure S6), which exhibited a correlation only between H-15 and H-14β (δH 2.48, ddd, J = 14.7, 2.9, 1.9 Hz) and not between H-15 and H-14α (δH 2.28, ddd, J = 14.7, 11.3, 0.7 Hz). This assignment was also supported by the higher chemical shift value of H-14β than that of H-14α since the former is under the anisotropic deshielding of the C-13 carbonyl group. Based on the same reasoning, we assigned the broad doublet at δH 2.81 (J = 14.0 Hz) as H-12β, and the double doublet at δH 2.55 (J = 14.0, 1.9 Hz) as H-12α. Consequently, the relative configuration of C-10 and C-15 was tentatively assigned as 10S* and 15R*. The relative configuration of C-11 was tentatively assigned as 11S* based on the fact that the NOESY spectrum (Table 1, Supplementary Information, Figure S6) did not exhibit any correlation between H-10 and H-15. According to the molecular model, when the configuration of C-11 is S*, the substituents on C-11 are arranged in a way that H-10 and H-15 are pointing toward the opposite directions. On the contrary, the R* configuration of C-11 would have H-10 and H-15 close enough to give a strong NOESY correlation.

Similanpyrone C (I) can be assumed to be derived from the acetate-malonate pathway (Scheme 1). Cyclization and enolization of the pentaketide (I) leads to the formation of 6,8-dihydroxy-3-methylisocoumarin (II), which, after Claisen condensation with the tetraketide (III), gives rise to IV. Enolization of the side chain, together with a formation of the hemiketal by the phenolic hydroxyl group on C-6 of the isocoumarin nucleus and the ketone carbonyl of the side chain, leads to the formation of a hemiketal V. Formation of the ketal and methylation by SAM in the side chain finally gives rise to similanpyrone C (I).

Compound 2 was isolated as pale yellow viscous mass, and its molecular formula C34H3zN6O6 was established on the basis of the (+)-HRESIMS m/z 641.4053 [M + H]+, indicating twelve degrees of unsaturation. The IR spectrum showed absorption bands for amine (3335 cm−1), carbonyl (1682, 1644 cm−1) and aromatic (1594, 1519 cm−1). The 13C NMR (Supplementary Information, Figure S8), DEPTs and HSQC spectra (Table 2, Supplementary Information, Figure S10) revealed the presence of six amide carbonyls (δc 174.3, 174.2, 170.7, 170.2, 169.3, 168.9), two quaternary sp3 (δc 137.0, 122.7), four methine sp2 (δc 131.7, 127.1, 123.9, 123.4), eight methine sp3 (δc 65.1, 61.4, 59.3, 50.9, 47.9, 29.9, 25.5, 24.4), six methylene sp3 (δc 52.5, 37.8, 36.2, 28.1, 27.4, 24.5) and eight methyl (δc 37.9, 23.3, 23.2, 22.1, 21.7, 19.8, 18.4, 16.2) carbons. The 1H NMR spectrum (Table 2, Supplementary Information, Figure S7) revealed, besides four NH signals at δH 7.43, d (J = 7.4 Hz), 7.64, d (J = 9.8 Hz), 8.02, d (J = 7.9 Hz) and 9.41, brs, the signals of the aromatic protons of the 1,2-disubstituted benzene ring at δH 7.20, dd (J = 7.7, 1.5 Hz), 7.13, ddd (J = 7.9, 7.9, 1.0 Hz), 7.47, ddd, (J = 7.9, 7.9, 1.6 Hz) and 8.29, d (J = 8.3 Hz). That the 1,2-disubstituted benzene ring belonged to the anthranilic acid residue was corroborated by the HMBC correlations of the NH signal at δH 9.41, brs to the carbon signal at δC 123.9 (C-6), and of the double doublet at δH 7.20 (J = 7.7, 1.5 Hz, H-3) to the carbons at δC 131.7 (C-5), 137.0 (C-7) and 170.2 (CO-1) (Table 2, Figure 3, Supplementary Information, Figure S11). The anthranilic acid residue was linked to the valine residue, through the amino group of the former and the carboxyl group of the latter, since the HMBC spectrum (Supplementary Information, Figure S11) showed correlations of the NH signal at δH 9.41, brs to the
carbonyl carbon at $\delta^C 170.7$ (C-8), of the methine proton at $\delta^H 4.32$ dd, $J = 7.4$, 3.3 Hz (H-9) to the methine carbon at $\delta^C 29.9$ (C-10), the methyl carbon at $\delta^C 16.2$ (C-12) and C-8, and of the NH signal at $\delta^H 7.43$, d ($J = 7.5$ Hz) to C-9 ($\delta^C 59.3$) and C-10 (Table 2, Figure 3). The presence of the leucine residue was supported by the coupling system from CH-14 ($\delta^H 4.57$, m; $\delta^C 50.9$) through CH$_3$-17 ($\delta^H 0.97$, d, $J = 6.5$ Hz; $\delta^C 23.3$) and CH$_3$-18 ($\delta^H 0.88$, d, $J = 6.4$ Hz; $\delta^C 21.1$), and of NH at $\delta^H 8.02$ d ($J = 7.9$ Hz) to H-14, as observed in the COSY spectrum (Table 2, Supplementary Information, Figure S9), as well as by the HMBC correlations of the NH signal at $\delta^H 8.02$ d ($J = 7.9$ Hz) to C-14 (Table 2, Figure 3). That the valine residue was linked to the leucine residue was supported by the HMBC cross peak between the NH signal of the former ($\delta^H 7.43$, d, $J = 7.5$ Hz) to the signal of the carbonyl carbon ($\delta^C 174.2$, C-13) of the latter. In turn, the leucine residue was linked to the alanine residue, as evidenced by the HMBC cross peaks of the NH signal of the former to the carbonyl carbon signal ($\delta^C 174.3$, C-19) of the latter, and of the proton signal at $\delta^H 4.82$, dd, $J = 9.7$, 7.3 Hz (H-20) to C-19 and the methyl carbon at $\delta^C 18.4$ (C-21), as well as by the COSY cross peaks of H-20 to CH$_3$-21 ($\delta^H 1.29$, d, $J = 7.3$ Hz), and of H-20 to NH at $\delta^H 7.64$, d ($J = 7.9$ Hz). The presence of the $N$-methyl leucine moiety was evidenced by the coupling system from H-23 ($\delta^H 3.49$, dd, $J = 9.0$, 4.7 Hz) through CH$_3$-26 ($\delta^H 0.97$d, $J = 6.5$; $\delta^H 23.2$) and CH$_3$-27 ($\delta^H 0.99$, d, $J = 6.5$; $\delta^H 22.1$), as observed in the COSY spectrum (Table 2, Supplementary Information, Figure S9), as well as by the HMBC correlations of H-23 to C-22 ($\delta^C 169.3$) and CH$_3$-28 ($\delta^C 37.9$). Finally, the presence of the pipecolic acid residue was supported by the coupling system from CH-30 ($\delta^H 3.71$, dd, $J = 11.3$, 2.5; $\delta^C 61.4$) through CH$_2$-34 ($\delta^H 3.16$, dd, $J = 13.2$, 2.3; 4.14, dd, $J = 14.4$, 2.4; $\delta^C 52.5$), as observed in the COSY spectrum (Table 2, Supplementary Information, Figure S9). Since both H-30 and CH$_3$-28 gave HMBC cross peaks to C-29 ($\delta^C 168.9$), the pipecolic acid residue was linked to the $N$-methyl leucine residue through the carboxyl group of the former and a nitrogen atom of the latter. Since 2 presents twelve degrees of unsaturation, the nitrogen atom of the piperidine ring of the pipecolic acid residue was linked to the carbonyl of the anthranilic acid residue. The proposed structure was supported by the NOESY correlations which showed cross peaks of NH at $\delta^H 9.41$, brs to H-9, CH$_3$-12, of NH at $\delta^H 7.43$ (d, $J = 7.5$ Hz) to H-9, CH$_3$-11 ($\delta^H 1.06$, d, $J = 6.9$ Hz), CH$_3$-12, H-14, of NH at $\delta^H 8.02$ (d, $J = 7.9$ Hz) to H-14, H-15 ($\delta^H 2.02$, m; 1.77, m), CH$_3$-17, of NH at $\delta^H 7.64$ (d, $J = 7.9$ Hz) to CH$_3$-21, H-23, CH$_3$-28, of H-3 to H-34$\beta$ ($\delta^H 4.14$, dd, $J = 14.4$, 2.4 Hz), and of H-30 to CH$_3$-28 (Table 2, Supplementary Information, Figure S12). Combining this information, it was possible to conclude that 2 was cyclo (anthranilic acid-Val-Leu-Ala-$N$-methyl-Leu-pipecolic acid).
Scheme 1. Proposed biogenesis of similanpyrone C (1).
Table 2. $^1$H and $^{13}$C NMR (CDCl$_3$, 500.13 MHz and 125.8 MHz) and HMBC assignment for 2.

| Position | $\delta_C$ (Type) | $\delta_H$ ($J$ in Hz) | COSY | HMBC | NOESY |
|----------|------------------|------------------------|------|------|-------|
| Anthranilic acid |
| 1 | 170.2, CO | - | - | - | - |
| 2 | 122.7, C | - | - | - | - |
| 3 | 127.1, CH | 7.20, dd (7.7, 1.5) | H-4 | C-1, 5, 7 | H-34 |
| 4 | 123.4, CH | 7.13, ddd (7.9, 7.9, 1.0) | H-3, 5 | C-2, 6 | |
| 5 | 131.7, CH | 7.47, ddd (7.9, 7.9, 1.6) | H-4, 6 | C-3, 7 | |
| 6 | 123.9, CH | 8.29, d (8.3) | H-5 | C-2, 4 | H-12 |
| 7 | 137.0, C | - | - | - | - |
| NH | - | 9.41, brs | C-6, 7, 8 | NH (Val), H-9, 12 | |
| Val |
| 8 | 170.7, CO | - | - | - | - |
| 9 | 59.3, CH | 4.32, dd (7.4, 3.3) | H-10, NH | C-8, 10, 11, 12 | H-10, 11 |
| 10 | 29.9, CH | 2.68, m | H-9, 11, 12 | - | H-9, 11, 12 |
| 11 | 19.8, CH$_3$ | 1.06, d (6.9) | H-10 | C-9, 10, 12 | |
| 12 | 16.2, CH$_3$ | 0.94, d (7.0) | H-10 | C-9, 10, 11 | |
| NH | - | 7.43, d (7.5) | H-9 | C-9, 10, 13 | H-9, 11, 12, 14 |
| Leu |
| 13 | 174.2, CO | - | - | - | - |
| 14 | 50.9, CH | 4.57, m | H-15, NH | - | H-15, 18 |
| 15 | 36.2, CH$_2$ | 2.02, m; 1.77, m | H-14, 16 | - | - |
| 16 | 24.4, CH | 1.77, m | H-15, 17, 18 | - | - |
| 17 | 23.3, CH$_3$ | 0.97, d (6.5) | H-16 | C-15, 16, 18 | |
| 18 | 21.7, CH$_3$ | 0.88, d (6.4) | H-16 | C-15, 16, 17 | |
| NH | - | 8.02, d (7.9) | H-14 | C-13, 19 | NH (Ala), H-14, 15, 17 |
| Ala |
| 19 | 174.3, CO | - | - | - | - |
| 20 | 47.9, CH | 4.82, dd (9.7, 7.3) | H-21, NH | C-19, 21 | H-21 |
| 21 | 18.4, CH$_3$ | 1.29, d (7.3) | H-20 | C-19, 20 | |
| NH | - | 7.64, d (7.9) | H-20 | C-22 | C-21, 23, 28 |
Table 2. Cont.

|      |      |      |      |      |      |
|------|------|------|------|------|------|
| 22   | 169.3, CO | -   |      |      |      |
| 23   | 65.1, CH  | 3.49, dd (9.0, 4.7) | H-24 |      | C-22, 24, 28, 29 |
| 24   | 37.8, CH₂ | 1.95, m; 2.20, m   | H-23, 25 |      |      |
| 25   | 25.5, CH  | 1.65, m           | H-24, 26, 27 |      |      |
| 26   | 23.2, CH₃ | 0.97, d (6.5)      | H-25 |      | C-24, 25, 27 |
| 27   | 22.1, CH₃ | 0.99, d (6.5)      | H-25 |      | C-24, 25, 26 |
| 28   | 37.9, CH₃ | 3.20, s           |      | C-23, 29 | 23, 30, 32, 34α |
| 29   | 168.9, CO | -   |      |      |      |
| 30   | 61.4, CH  | 3.71, dd (11.3, 2.5) | H-31 |      | C-1, 29 | H-34α |
| 31   | 28.1, CH₂ | 2.05, m             | H-30, 32 |      |      |
| 32   | 24.5, CH₂ | 2.07, m             | H-31, 33 |      |      |
| 33   | 27.4, CH₂ | 1.56, m             | H-32, 34 |      |      |
| 34α  | 52.5, CH₂ | 3.16, dd (13.2, 2.3) | H-33 |      |      |
| 34β  | 4.14, dd (14.4, 2.4) |      | H-34β |      |      |

N-Me Leu

Pipolic acid
The $^1$H and $^{13}$C NMR data of compound 2 resembled those of PF1171C, a cyclic hexapeptide isolated from extracts of the unidentified ascomycete OK-128 fermented with okara by Kai et al. [30], and later by a total synthesis by Masuda et al. [31]; however, its value of optical rotation was quite different from that of PF1171C [30,31]. Moreover, PF1171C was reported as white solid (mp 138–140 °C) [31], while compound 2 is pale yellow viscous mass. This observation suggested that compound 2 and PF1171C could be diastereomers.

The stereochemistry of the amino acid residues of compound 2 was determined by chiral HPLC analysis of its acidic hydrolysate, using appropriate D and L amino acids standards, according to the previously described method [32]. The enantioseparations of the standard amino acids were successfully performed with the Chirobiotic T column under reversed-phase elution conditions [33]. Actually, the teicoplanin selector of this column has several characteristic features that make it suitable for amino acid analysis [33–35]. The elution order of the enantiomers of all the standard amino acids was confirmed by injecting the solutions of the racemic or enantiomeric mixtures and then each enantiomer separately at a flow rate of 1 mL/min (Supplementary Information, Figure S18). As predicted, the D enantiomer was always more strongly retained than the corresponding L enantiomer on the Chirobiotic T column [33]. Based on mix HPLC analyses of the acidic hydrolysate with standard amino acids (co-injection) (Supplementary Information, Figure S19 and Table S1), compound 2 was elucidated unambiguously as cyclo (anthranilic acid-L-Val-D-Leu-L-Ala-N-methyl-L-Leu-D-pipecolic acid). Although the amino acid sequence of compound 2 is the same as that of PF1171C, the stereochemistry of its amino acid constituents is different from that of the amino acids constituent of PF1171C. While PF1171C contains D-Ala, L-Leu, D-Val, and L-pipecolic acid, compound 2 contains L-Ala, D-Leu, L-Val and D-pipecolic acid. Thus compound 2 is a new compound, and we have named it similanamide.

It is interesting to note that Kai et al. [30] has firstly assigned the stereochemistry of the amino acid constituents of PF1171C using the reversed phase HPLC analysis of the Marfey derivatives of the amino acids. Since the retention times for the Marfey derivatives of D-Ala (19.4 min) and L-Ala...
(20.0 min) were too close for resolution, they had wrongly assigned D-Ala for L-Ala. However, in our chiral HPLC analysis using the Chirobiotic T column under reversed-phase elution conditions, not only the retention time of L-Ala (7.16 min) was very different from that of D-Ala (9.36 min), but also the retention times of the D/L pair of other amino acid constituents (Supplementary Information, Table S1).

So far, only few cyclohexapeptides have been reported from marine-derived microorganisms. Wu et al. [36] have isolated two cyclohexapeptides, nocardiomides A and B, from the culture broth of the marine-derived actinomycete Nocardiosis sp. CNX037, isolated from sediment. Cai et al. [37] have isolated two anti-Mycobacterium tuberculosis cyclohexapeptides from a Streptomyces hygroscopicus Strain, while Song et al. [38] reported isolation of three new cyclopeptides, desotamides B–D, from the deep South China Sea-derived Streptomyces scopoliridis SCSIO ZJ46. To the best of our knowledge, compound 2 is the first cyclopeptide containing D-pipelicolic acid residue ever isolated from marine fungi.

Compound 3 was isolated as pale yellow viscous mass, and its molecular formula C_{29}H_{33}NO_{8} was established on the basis of (+)-HRESIMS m/z 524.2287 [M + H]^+ indicating fourteen degrees of unsaturation. The IR spectrum showed absorption bands for hydroxyl (3418 cm\(^{-1}\)), ester carbonyl (1732 cm\(^{-1}\)), conjugated ester carbonyl (1667 cm\(^{-1}\)), olefin (1643 cm\(^{-1}\)), aromatic (1557, 1507 cm\(^{-1}\)). The \(^{13}\)C NMR (Supplementary Information, Figure S15), DEPTs and HSQC spectra (Table 3, Supplementary Information, Figure S16) revealed the presence of two ester carbonyl (\(\delta_C 170.1, 169.8\)), one conjugated carbonyl (\(\delta_C 161.6\)), five quaternary sp\(^3\) (\(\delta_C 160.2, 156.8, 146.1, 126.9, 100.3\)), six methine sp\(^2\) (\(\delta_C 151.3, 146.5, 132.8, 123.9, 109.4, 98.7\)), one oxyquaternary sp\(^3\) (\(\delta_C 85.9\)), two oxymethine sp\(^3\) (\(\delta_C 75.7, 72.8\)) and one oxymethylene sp\(^3\) (\(\delta_C 64.4\)), two quaternary sp\(^3\) (\(\delta_C 40.3, 38.4\)), one methine sp\(^3\) (\(\delta_C 40.7\)), three methylene sp\(^3\) (\(\delta_C 35.2, 27.3, 22.9\)), and five methyl (\(\delta_C 23.8, 20.8, 20.5, 20.1, 12.7\)) carbons. The general feature of \(^{13}\)C and \(^1\)H spectra of compound 3 closely resembled those of pyripyropene S, previously isolated from the same fungus [29]. Analysis of the \(^1\)H (Supplementary Information, Figure S13), \(^{13}\)C, HSQC and HMBC spectra (Table 3, Supplementary Information, Figure S17) revealed the presence of the ring system comprising of the decahydonaphthalene fused, on C-5 and C-6, with the 2H,5H-pyran[4,3-b]pyran-5-one, which connected to the pyridine ring through C-6′ of the former and C-3″ of the latter, similar to pyripyropene S [29]. However, there were only two acetoxyl groups (\(\delta_C 170.1, \text{CO}; \delta_C 20.5, \text{CH}_3; \delta_H 2.00, \text{s} \text{and} \delta_C 169.8, \text{CO}; \delta_C 20.8, \text{CH}_3; \delta_H 2.00, \text{s} \) in compound 3. That the acetoxyl groups were on C-1 and C-11 was supported by the fact that the chemical shift values of H-1 (\(\delta_H 4.64, \text{t}, J = 8.5 \text{Hz}\)) and H-11 (\(\delta_H 3.75, \text{s}\)) were very similar to those of pyripyropene S, whereas the chemical shift of H-7 (\(\delta_H 3.85, \text{dd}, J = 10.6, 4.2 \text{Hz}\)) was nearly 1.4 ppm less than that of pyripyropene S. On the other hand, the \(^{13}\)C chemical shift values of C-6 (\(\delta_C 85.9\)) and C-8 (\(\delta_C 27.4\)) of compound 3 were 2.00 and 3.00 ppm, respectively, higher than those of the corresponding carbons in pyripyropene S, while the \(^{13}\)C chemical shift value of C-7 (\(\delta_C 75.5\)) of compound 3 was 2.00 ppm lower than that of C-7 of pyripyropene S. Since H-7 appeared as a double doublet with coupling constants of 10.6 and 4.2 Hz, the position of the hydroxyl group on C-7 was β. Thus, compound 3 is 7-deacetylpyripyropene S. In order to prove the stereochemistry of compound 3, the NOESY experiment was carried out. As the NOESY spectrum (Table 3) clearly exhibited correlations of CH\(_3\)-15 to CH\(_3\)-12, but not to H-1 and H-9; of CH\(_3\)-12 to CH\(_3\)-14 and CH\(_3\)-15, and of CH\(_3\)-14 to CH\(_3\)-12, but not to H-7 (Table 3,
Supplementary Information, Figure S18), the stereochemistry of compound 3 is the same as that of pyripyropene S [29], i.e., 1S*, 4R*, 6S*, 7S*, 9R*, 10R*. Since it is a new compound we have named it pyripyropene T.

Table 3. $^1$H and $^{13}$C NMR (DMSO, 300.13 MHz and 75.47 MHz) and HMBC assignment for 3.

| Position | $\delta_{C}$, Type | $\delta_{H}$, ($J$ in Hz) | COSY | HMBC | NOESY |
|----------|--------------------|--------------------------|-------|-------|-------|
| 1        | 72.8, CH           | 4.64, t (8.5)            | H-2   |       |       |
| 2        | 22.9, CH$_2$      | 1.79, m                  | H-1, 3|       |       |
| 3        | 35.2, CH$_2$      | 1.98, m                  | H-2   |       |       |
| 4        | 38.4, C           | -                        |       |       |       |
| 5        | 146.1, C          | -                        |       |       |       |
| 6        | 85.9, C           | -                        |       |       |       |
| 7        | 75.7, CH          | 3.85, dd (10.6, 4.2)     |       | H-8   |       |
| 8        | 27.3, CH$_2$      | 1.70, m                  | H-7   |       |       |
| 9        | 40.7, CH          | 1.48, m                  | H-8   |       |       |
| 10       | 40.3, C           | -                        |       |       |       |
| 11       | 64.4, CH$_2$      | 3.75, s                  |       | C-1, 9| H$_2$,15|
| 12       | 23.8, CH$_3$      | 1.19, s                  |       | C-3, 4, 5| H$_2$,14, 15|
| 13       | 109.4, CH         | 6.16, s                  |       | C-4, 6, 2$^\prime$, 4$^\prime$| |
| 14       | 20.1, CH$_3$      | 1.45, s                  |       | C-5, 6, 7| H$_3$,12|
| 15       | 12.7, CH$_3$      | 0.84, s                  |       | C-1, 9, 10, 11| H$_2$,11, H$_3$,12|
| 2$^\prime$ | 161.6, C         | -                        |       |       |       |
| 3$^\prime$ | 100.3, C         | -                        |       |       |       |
| 4$^\prime$ | 160.2, C         | -                        |       |       |       |
| 5$^\prime$ | 98.7, CH         | 7.11, s                  |       | C-3$^\prime$, 4$^\prime$, 6$^\prime$, 3$^\prime$| |
| 6$^\prime$ | 156.8, C         | -                        |       |       |       |
| 2$^\prime$ | 146.5, CH        | 9.0, d (1.7)             |       | C-3$^\prime$, 6$^\prime$| H-5$^\prime$|
| 3$^\prime$ | 126.9, C         | -                        |       |       |       |
| 4$^\prime$ | 132.8, CH        | 8.25, dt (8.7, 2.2)      |       | H-2$^\prime$, 5$^\prime$| H-5$^\prime$, 5$^\prime$|
| 5$^\prime$ | 123.9, CH        | 7.54, dd (7.9, 4.8)      |       | H-4$^\prime$, 6$^\prime$| C-3$^\prime$| H-4$^\prime$, 6$^\prime$|
| 6$^\prime$ | 151.3, CH        | 8.68, dd (4.8, 1.5)      |       | H-2$^\prime$, 5$^\prime$| C-2$^\prime$, 4$^\prime$| H-5$^\prime$|
| OAc-1    | 170.1, CO         | -                        |       | CO (Ac) |       |
|          | 20.5, CH$_3$     | 2.00, s                  | CO (Ac)|       |       |
| OAc-11   | 169.8, CO         | -                        |       | CO (Ac)|       |

Since compound 1 was isolated in a very small amount, only compounds 2 and 3 were evaluated for their cytotoxicity and antibacterial activity. Compounds 2 exhibited weak in vitro growth inhibitory activity, by Sulforhodamine B (SRB) assay [39], against the MCF-7 (breast adenocarcinoma, GI$_{50}$ = 125 ± 0), NCI-H460 (non-small cell lung cancer, GI$_{50}$ = 117.50 ± 3.55) and A373 (melanoma, GI$_{50}$ = 115 ± 7.07) cell lines. Compounds 2 and 3 were also tested for their antibacterial activity against four reference strains (Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa), as well as the environmental multidrug-resistant isolates, according to the previously described method [40], and neither of them showed activity (MIC values higher than 256 µg/mL).
3. Experimental Section

3.1. General Procedure

Melting points were determined on a Bock monoscope and are uncorrected. Optical rotations were determined on an ADP410 Polarimeter (Bellingham + Stanley Ltd., Tunbridge Wells, Kent, UK). Infrared spectra were recorded in a KBr microplate in a FTIR spectrometer Nicolet iS10 from Thermo Scientific (Waltham, MA, USA) with Smart OMNI-Transmission accessory (Software 188 OMNIC 8.3). ¹H and ¹³C-NMR spectra were recorded at ambient temperature on a Bruker AMC instrument (Bruker Biosciences Corporation, Billerica, MA, USA) operating at 500.13 and 125.8 MHz or at 300.13 and 75.4 MHz, respectively. High-resolution mass spectra were measured with a Waters Xevo QToF mass spectrometer (Waters Corporations, Milford, MA, USA) coupled to a Waters Aquity UPLC system. A Merck (Darmstadt, Germany) silica gel GF254 was used for preparative TLC, and a Merck Si gel 60 (0.2–0.5 mm) was used for analytical chromatography.

3.2. Extraction and Isolation

Isolation and identification of the fungus as well as fractionation of the crude extract of the culture of A. similanensis KUFA0013 have been previously described by us [29]. Frs 185–196 were combined (654 mg) and purified by TLC (Si gel, CHCl₃:Me₂CO:HCO₂H, 97:3:0.1) to give 7.4 mg of 1. Frs 310–327 were combined (1.19 g), applied on a Sephadex H-20 column (10 g) and eluted MeOH, wherein ten sfrs of 1 mL were collected. Sfrs 1–7 were combined and purified by TLC (Si gel, CHCl₃:Me₂CO:HCO₂H, 19:1:0.01) to give 108 mg of 2. Frs 336–345 were combined (165 mg) and purified by TLC (Si gel, CHCl₃:Me₂CO:HCO₂H, 17:3:0.01) to give additional 60 mg of 2. Frs 354–398 were combined (1.15 g), applied on a Sephadex LH-20 column (10 g) and eluted with MeOH, wherein thirty four sfrs of 1 mL were collected. Sfrs 7–15 were combined (150 mg) and purified by TLC (Si gel, CHCl₃:Me₂CO:HCO₂H, 4:1:0.01) to give 67 mg of pyripyropene S [29]. Frs 435–443 were combined (377 mg), applied on a Sephadex LH-20 column (10 g) and eluted with a mixture of 1:1 v/v of CHCl₃: MeOH, wherein fourteen sfrs of 1 mL were collected. Sfrs 8–11 (62 mg) were combined and purified by TLC (Si gel, CHCl₃:Me₂CO:HCO₂H, 19:1:0.01) to give 35 mg of 3.

3.2.1. Similanpyrone C (1)

Pale yellow viscous mass; [α]D²⁰ = −80.0 (c 0.01, CHCl₃); λmax (log ε) 239 (4.57), 245(4.59), 332 (2.10) nm; IR (KBr) νmax 3443, 2923, 2852, 1730, 1683, 1647, 1625, 1572, 1508, 1457, 1429, 1352, 1251 cm⁻¹; ¹H and ¹³C NMR (Table 1); HRESIMS m/z 345.1342 (M + H)⁺ (calculated for C₁₉H₂₁O₆, 345.1338).

3.2.2. Similanamide (2)

Pale yellow viscous mass; [α]D²⁰ = +30.3 (CHCl₃, c 0.03), IR (KBr) νmax 3335, 3054, 2958, 2870, 1682, 1644, 1594, 1519, 1449, 1292 cm⁻¹; ¹H and ¹³C NMR (Table 2); HRESIMS m/z 641.4053 (calculated for C₃₄H₅₅N₆O₆, 641.4027).
3.2.3. Pyripyropene T (3)

Pale yellow viscous mass; [α]D$^20 = +106$ (CHCl$_3$, c 0.03), IR (KBr) $\nu_{\text{max}}$ 3418, 2949, 1732, 1667, 1643, 1557, 1507, 1480, 1246, 1028 cm$^{-1}$; $^1$H and $^{13}$C NMR (Table 3); HRESIMS $m/z$ 524.2287 (M + H)$^+$, calcd for C$_{29}$H$_{34}$NO$_8$, 524.2284.

3.3. Amino Acids Analysis of Acidic Hydrolysate of Compound 2

3.3.1. Acid Hydrolysis

The stereochemistry of the amino acids was determined by analysis of the acidic hydrolysate from compound 2. Compound 2 (5.0 mg) was dissolved in 6 N HCl (5 mL) and heated at 110 °C, in a furnace, for 24 h in a sealed glass tube. After cooling to room temperature, the solution was dried under N$_2$ for 24 h, reconstituted in methanol for HPLC-MS (200 µL), filtered through a 4 mm PTFE Syringe Filter F2504-4 of 0.2 µm pore size (Thermo Scientific, Mumbai, India), and then analyzed by HPLC equipped with a chiral column.

3.3.2. Chiral HPLC Analysis

The HPLC system consisted of Shimadzu LC-20AD pump, equipped with a Shimadzu DGV-20A5 degasser, a Rheodyne 7725i injector fitted with a 20 µL loop, and a SPD-M20A DAD detector (Kyoto, Japan). Data acquisition was performed using Shimadzu LCMS Lab Solutions software, version 3.50 SP2. The chiral column used in this study was Chirobiotic T (15 cm × 4.6 mm I.D., particle size 5 µm) manufactured by ASTEC (Whippany, NJ, USA). The mobile phase composition was MeOH:H$_2$O:CH$_3$CO$_2$H (70:30:0.02, v/v/v), all were LC-MS grade solvents obtained from Sigma-Aldrich Co (St. Louis, MO, USA). The flow rate was 0.5 mL/min and the UV detection wavelength was 210 nm. Analyses were performed at room temperature in an isocratic mode.

All standards of racemic amino acids and pure amino acid enantiomers were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). The elution order of the enantiomers of all the standards amino acids was confirmed by injecting the solutions of the racemic or enantiomeric mixtures, and then each enantiomer separately (or only L-amino acid in the case of N-methyl leucine) at a flow rate of 1 mL/min or 0.5 mL/min. Working solutions of single enantiomeric amino acids were prepared by dissolution in MeOH at the concentration of 1 mg/mL (10 µL sample injection), while the enantiomeric mixtures were prepared by mixing equal aliquots of each enantiomer (20 µL sample injection). Mix HPLC analyses of the acidic hydrolysate with standard amino acids (co-injection) confirmed the stereochemistry of the amino acids of compound 2.

4. Conclusions

Following our first report of the isolation of new isocoumarin derivatives and merotepenoids from the ethyl acetate crude extract of the culture of the undescribed marine sponge-associated fungus Aspergillus similanensis KUFA 0013, we have reexamined its remaining column fractions and have isolated a new isocoumarin derivative containing an unusual 1,7-dioxaspiro-undecenone moiety, together with a new cyclohexapeptide and a new pyripyropene analog. Although several cyclopeptides
have been reported from many fungi of the genus *Aspergillus*, this is the first report of isolation of cyclohexapeptide from the marine-derived fungus. The fact that these new cyclohexapeptide and pyripyropene analog did not exhibit relevant antibacterial and the *in vitro* growth inhibitory activities on human cancer cell lines does not mean that they are void of other interesting biological activities. In order to prove this hypothesis, it is necessary to explore their potential in a broader biological or pharmacological assay system.

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**Author Contributions**

Chadaporn Prompanya performed isolation, purification and structure elucidation of some compounds; Carla Fernandes and Sara Cravo performed HPLC analysis of amino acids by chiral column; Tida Dethoup isolated, identified, cultured the fungi, and prepared the crude extract; Artur M.S. Silva provided 1D and 2D NMR spectra. Madalena M.M. Pinto, and Anake Kijjoa conceived, designed the research, elucidated the structure of the compounds and wrote the paper.

**Conflicts of Interest**

The authors declare no conflict of interest.

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