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Novel terpenoids of the fungus *Aspergillus insuetus* isolated from the Mediterranean sponge *Psammocinia* sp. collected along the coast of Israel

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ABSTRACT

Three novel meroterpenoids, insuetolides A–C (**1**–**3**) and four drimane sesquiterpenes, the new (*E*)-6-(4′-hydroxy-2′-butenoyl)-strobilactone A (**4**) and the known 2α, 9α, 11-trihydroxy-6-oxodrim-7-ene (**5**), strobilactone A (**6**) and (*E*,*E*)-6-(6′,7′-dihydroxy-2′,4′-octadienoyl)-strobilactone A (**7**), were isolated from the EtOAc extract of the culture medium of the marine-derived fungus *Aspergillus insuetus* (OY-207), which was isolated from the Mediterranean sponge *Psammocinia* sp. The structures of the compounds were determined by spectroscopic methods. Insuetolides A-C reveal a new carbon skeleton derived from the cyclization of farnesyl and 3, 5-dimethylorsellinic acid. Compounds **1**, **6**, and **7** exhibited anti-fungal activity towards *Neurospora crassa* with MIC values of 140, 242, and 162 μM, respectively; and compounds **3**, **4**, and **7** exhibited mild cytotoxicity towards MOLT-4 human leukemia cells.

1. Introduction

Fungi are prolific producers of diverse types of natural products including metabolites of mixed polyketide and terpene biosynthesis such as the meroterpenoids. Meroterpenoids derived from farnesyl diphosphate and the tetraketide, orsellinic acid or its derivatives, are the most abundant group of meroterpenoids found in fungi.[1] The first member of this group, andibenin, was isolated from the terrestrial fungus *Aspergillus variecolor*.[2] Biosynthetic studies using various labeled precursors revealed that andibenin is derived from mixed polyketide-terpene biosynthesis.[3] Subsequently, it was found that a large number of fungal metabolites with a wide range of structural diversity are derived from this mixed biosynthetic route.[1] Recently, several meroterpenoids were isolated from marine-derived *Aspergillus* spp. For example, tropolactones A–D were isolated from an *Aspergillus* sp. derived from an unidentified sponge[4]; asperdemin was isolated from *A. versicolor* collected in Sakhalin Bay[5]; and terretonins E and F were isolated from *Aspergillus insuetus*, which was in turn isolated from the Mediterranean sponge *Petrosia ficifornis*.[6]

As part of our ongoing research on the chemistry and chemical ecology of marine-derived fungi[7,8] we have isolated a new structural group of meroterpenoids, insuetolides A-C (**1**–**3**) along with one new, **4**, and three known, **5**–**7**, drimane sesquiterpenes (see Fig. 1) from a marine-derived *A. insuetus* (OY-207), which was isolated from the Mediterranean sponge *Psammocinia* sp. collected using SCUBA diving offshore of Sdot-Yam, Israel. Insuetolides A–C (**1**–**3**) are thought to arise from the same drimane-type precursor leading to andibenin, via an additional oxidation step prior to the last step of condensation to the pentacyclic intermediate.

2. Results and discussion

The fungal isolate OY-207 was cultured from the sponge sample with the use of fungicide-amended media.[8] Based on amplicons obtained with the 5.8S ribosomal RNA gene internal transcribed spacers (ITS) primers (1 and 4[9]) and β-tubulin[10] gene primers, the strain was identified as *A. insuetus*. The fungus was mass-cultivated in Potato Dextrose Broth in stationary flasks for 20 days at 25 °C in the dark. The culture medium was extracted with ethyl acetate. The ethyl acetate extract was chromatographed using a reversed-phase open column, Sephadex LH-20 and finally a reversed-phase HPLC column to afford seven natural products.

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2.1. Insuetolides

Insuetolide A (**1**) was isolated as a transparent glassy material. Its molecular formula, $C_{25}H_{32}O_6$, was deduced from a HRESIMS protonated cluster ion at *m*/*z* 429.2279, suggesting 10° of unsaturation. In the IR spectrum three carbonyl stretching bands were evident: 1780 (saturated γ-lactone), 1717 (saturated ketone) and 1686 (unsaturated lactone) cm^{-1}. The ^1H NMR spectrum measured in $CDCl_3$ (see Table 1) revealed two vinyl protons of a conjugated double bond (δ_H 5.82 d and 5.91 d), isolated oxymethylene protons (δ_H 3.98 d and 4.32 d), a methine α to a carbonyl (δ_H 3.11 dd), eight resolved protons between 2.50 and 1.45 ppm, and five singlet methyl signals between 1.43 and 1.12 ppm. The ^{13}C NMR of **1** contained 25 carbon signals (see Table 1) assigned to a ketone, a conjugated carboxyl, a saturated carboxyl, two carbons of a conjugated double bond, three quaternary and one methylene oxy-carbons, and three quaternary, three methine, five methylene and five methyl carbons in the aliphatic region. Interpretation of the COSY spectrum allowed the assignment of six isolated spin systems (see Fig. 2). HSQC assigned the carbons to fragments a–f and to the methyl groups (see Table 1). The six short fragments (a–f) could be assembled through the HMBC correlations to the entire

Table 1
NMR Data of Insuetolide A (**1**) in $CDCl_3$[a]

Position		δ_C, mult.	δ_H, mult. *J* (Hz)	HMBC[b]	NOE
1		149.5, CH	5.82, d, 12.7	2, 15	2, 9, 15
2		119.2, CH	5.91, d, 12.7	1, 15	1,
3		166.7, qC		1, 2, 13	
4		84.0, qC		5, 6β, 13, 14	
5		40.9, CH	2.48, dd, 12.2, 7.3	1, 6α, 7α, 13, 14, 15	6α, 11α, 13, 1′α, 1′β
6	α	21.3, CH_2	1.97, dddd, 13.3, 9.7, 9.1, 7.3	5, 7α, 7β	5, 6β 7α, 13
	β		1.31, ddd, 13.3, 12.2, 9.8		6α, 7α, 7β, 14
7	α	32.5, CH_2	1.85, m	6α, 12*exo*	6α, 6β, 12*exo*
	β		1.78, m		6β 12*endo*, 15
8		75.8, qC		6β, 7α, 11α, 11β, 12*endo*, 12*exo*	
9		50.6, CH	1.40, m	1, 5, 7α, 11α, 11β, 12*endo*, 12*exo*, 15	1, 12*endo*, 15
10		44.2, qC		1, 2, 5, 6α, 11α, 15	
11	α	33.7, CH_2	1.15, m	9, 9′	5
	β		1.40, m		9′
12	*exo*[c]	54.2, CH_2	2.09, d, 14.3	7α, 7β, 9, 6′α, 6′β, 10′	7α, 12*endo*, 6′β, 7′, 10′
	endo		1.48, dd, 14.3, 1.3		9, 7β, 12*exo*, 10′
13		29.8, CH_3	1.37, s	14	5, 6α
14		22.3, CH_3	1.43, s	5, 13	6β, 15
15		25.0, CH_3	1.26, s	1, 5, 9, 11α	1, 7β, 9, 14
1′ α		70.7, CH_2	4.33, d, 9.8		1′β, 9′
β			3.98, d, 9.8		1′α, 7′
2′		83.5, qC		11α, 1′α, 1′β, 6′β, 7′, 9′	
3′		48.1, qC		11α, 11β, 1′α, 1′β, 7′, 9′	
4′		214.4, qC		11α, 12*endo*, 6′α, 6′β, 9′, 10′	
5′		45.7, qC		11β, 12*endo*, 12*exo*, 6′α, 6′β, 10′	
6′	α	33.9, CH_2	1.79, ddd, 14.8, 8.2, 1.3	12*endo*, 12*exo*, 7′, 10′	12*endo*, 6′β7′, 9′, 10′
	β		2.20, dd, 14.8, 11.4		6′α12*exo*, 7′, 10′
7′		44.4, CH	3.11, dd, 11.4, 8.2	1′α, 6′α, 6′β	12*exo*, 1′β, 6′α, 6′β
8′		176.0, qC		1′α, 6′α 6′β, 7′	
9′		19.0, CH_3	1.15, s	11β	11β, 1′α, 6′α
10′		23.7, CH_3	1.12, s	12*exo*, 6′β	12*endo*, 12*exo*, 6′α, 6′β

[a] 400 MHz for ^1H and 100 MHz for ^{13}C.
[b] From numbered H to C in row.
[c] *exo* to ketone at position 4′.

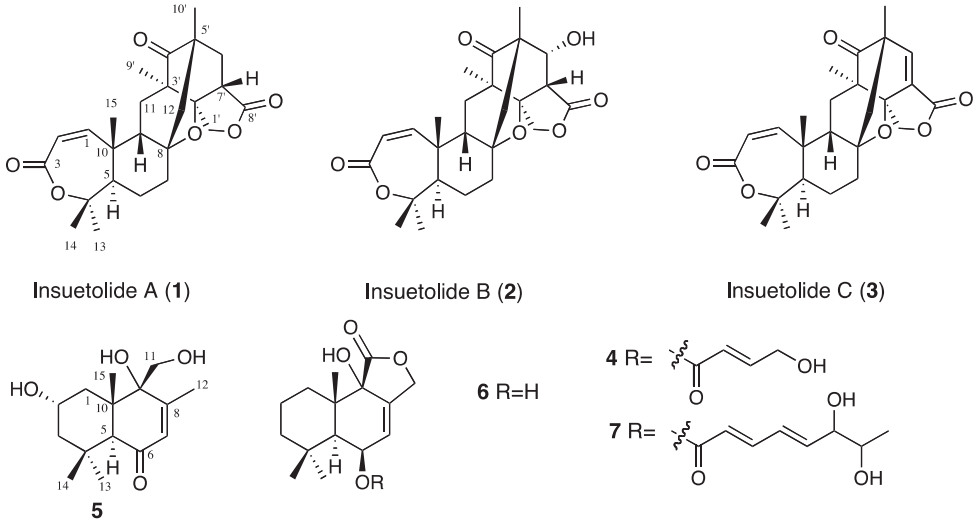


Figure 1. Metabolites of *A. insuetus* (OY-207).

planar structure. The vinyl protons (H-1 and H-2) presented HMBC correlations with the unsaturated carboxyl (δ_C 166.7, C-3) and a quaternary carbon (δ_C 44.2, C-10). The latter quaternary carbon presented additional correlations with the methine proton of fragment b (δ_H 2.48 dd, H-5) and the singlet methyl that resonate at δ_H 1.26 (Me-15). The latter methyl group presented HMBC correlations with the vinyl carbon (C-1) and the methine carbon (δ_C 40.9, C-5), which presented correlations with two additional methyl groups (δ_H 1.37 s, Me-13 and δ_H 1.43, Me-14). H-5, Me-13 and Me-14 presented HMBC correlations with a quaternary oxy-carbon (δ_C 84.0, C-4), establishing the connectivity of carbons 3-2-1-10-5-4 and the substitution of C-4 by Me-13 and Me-14 and C-10 by Me-15. Finally, ring A was established through the ^4J HMBC correlation of Me-13 with C-3. Fragment b (C-5 through C-7) could be extended through the HMBC correlations of H-6 (δ_H 1.31) and H-7 (δ_H 1.85) with a quaternary oxy-carbon (δ_C 75.8, C-8), and the closure of the six-membered ring (ring B) was established through the ^3J correlations of H-1, H-5, H-7α (δ_H 1.85) and Me-15 with the methine carbon (δ_C 50.6, C-9) of fragment c (C-9 and C-11). The HMBC correlations of H-11ax (δ_H 1.15) with C-10, of H-12*endo* (δ_H 2.09) with C-7 and of H-11ax, H-11eq (δ_H 1.40), H-12*endo* and H-12*exo* (δ_H 1.48) with C-8 and C-9, further support this assignment and establish the connectivity of C-12 to C-8. The above discussion establishes the structure of the sesquiterpene part of the molecule as a drimane skeleton with an oxygenated ring A.

The tetraketide moiety was linked to C-11 of the drimane skeleton through the correlation of a singlet methyl (δ_H 1.15, Me-9′) with C-11 (δ_C 33.7), and through those of H-11α with a quaternary oxygenated carbon (δ_C 83.5, C-2′, ^3J), a quaternary carbon (δ_C 48.1, C-3′, ^2J) and the ketone carbon (δ_C 214.4, C-4′, ^3J) and of H-11β with C-2′ (^2J), a quaternary carbon (δ_H 45.7, C-5′, ^4J) and with the carbon of Me-9′ (δ_C 19.0, ^3J). The chemical shifts of the carbon and protons of Me-9′ suggested that it is situated on a quaternary carbon rather than an oxygenated quaternary carbon, establishing C-3′ as the connecting point with C-11, and the sequence 2′-3′-4′. C-4′ presented an additional ^3J correlations with the singlet methyl (δ_H 1.12, Me-10′), the methylene protons of fragment e (δ_H 2.20 and 1.79, H-6′β and H-6′α) and with H-11α. C-5′ presented ^2J correlations with H-12*endo* and H-12*exo*, H-6′β and H-6′α and with Me-10′. These HMBC correlations established the sequence of carbons 4′-5′-6′-7′ and C-5′ as the site of the second linkage between the drimane skeleton (C-12) and the tetraketide moiety, and as the point of connection of Me-10′. Ring D closure was suggested based on the HMBC correlations of C-2′ with H-6′β (^3J) and H-7′ (^2J) and of C-3′ with H-7′ (^3J). C-8′ was attached to C-7′ through the correlations of H-6′β and H-6′α (^3J) and H-7′ (^2J) with C-8′. Finally, the oximethine-1′ was attached to the quaternary oxygenated C-2′ through the ^2J HMBC correlations of H-1′β and H-1′α with C-2′ and the ^3J correlations of C-3′ with H-1′α and H-1′β and of C-7′ with H-1′α. The lactone ring E was established based on the ^3J correlation of C-8′ with H-1′b. There was no direct evidence for the closure

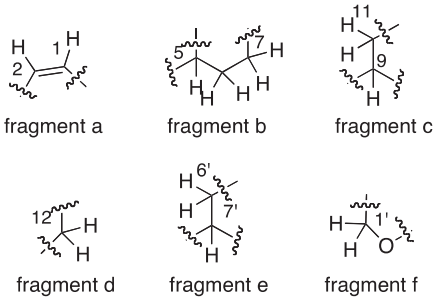


Figure 2. Fragments established by COSY correlations.

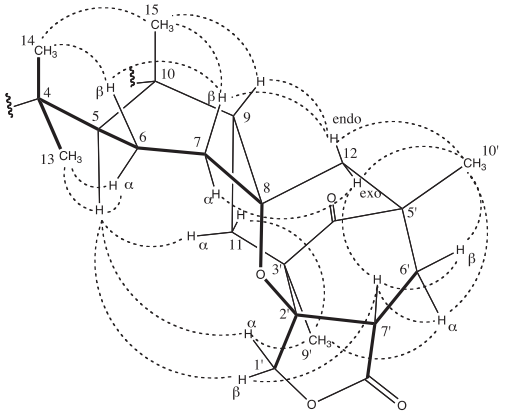


Figure 3. Selected NOEs confirming the relative stereochemistry of **1**.

of ring C, but the 10° of unsaturation and six oxygen atoms dictated by the high-resolution mass measurement, suggested that the two unpaired oxygenated carbons, C-8 and C-2′ must be linked, one to the other, through an ether bridge. This established the structure of insuetolide A as planar **1** (Fig. 1).

NOE correlations obtained from a 2D and a 1D NOE experiments (see Fig. 3) allowed the assignment of the relative configuration of all chiral centers in **1**. Me-14 showed strong NOE with Me-15 and H-6β (δ_H 1.31 bearing three large coupling constants), confirming their 1, 3-diaxial relationships. Me-13 showed NOE′s with H-5 ($J_{5\text{-}6\beta}$ = 12.2 Hz) and H-6α thus establishing Me-13 and H-6α as equatorial and H-5 as axial and α-oriented. H-6β exhibited NOEs with H-7α and H-7β and H-7β presented NOE with Me-15, designating a twist-boat conformation of the six-membered ring B. H-7α presented NOE with H-12*exo* and H-7β presented NOE with H-12*endo*, thus confirming that methylene-12 is β-oriented and equatorial to ring B. H-9 displayed a NOE with axial Me-15 and H-12*endo*, suggesting that it was β-oriented and equatorial establishing methylene-11 as α-oriented and axial to ring B. This was confirmed by the NOE of the α-oriented H-5 with H-11α (δ_H 1.15), which is axial in the twisted boat conformation of ring C. H-11β exhibited a NOE with Me-9′ (α-oriented and equatorial to ring C and axial to ring D) which in turn presented NOE with H-1′α confirming the *cis* fusion of rings C and D. Me-9′ exhibited an additional NOE with H-6′α (δ_H 1.79), and a 1–4 diaxial relationship, which confirmed the twisted boat conformation of ring D forced by the methylene (C-12) bridge between C-8 and C-5′. H-12*exo* exhibited NOEs with H-6′ (δ_H 2.20) and H-7′, thus confirming their β orientation and the α orientation of the *cis* fused ring E. These findings establish the relative stereochemistry of insuetolide A as presented in structure **1**.

Insuetolide B (**2**), a transparent glassy material, presented a molecular ion of *m/z* 443.2069 in the negative ESIMS measurement. The molecular formula that was calculated from the high-resolution mass measurements $C_{25}H_{31}O_7$, confirmed 10° of unsaturation in **2**. In the IR spectrum three carbonyl stretching bands were observed: 1770, 1717 and 1685 cm^{-1}. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to that of **1**. When compared with the ^{1}H NMR spectrum of **1**, the spectrum of **2** revealed a few significant changes, the protons of methylene-6′ were substituted by an oxymethine (δ_H 4.22 br d); H-7′ (δ_H 3.32) changed multiplicity from a double doublet to a doublet; a new acidic proton appeared at δ_H 3.33 (br s); and the signals of methyl-9′ and methyl-10′ were shifted downfield also presenting some changes when compared with that of **1**. Methylene-6′ (in **1**) was replaced by oxymethine carbon (δ_C 72.1) in **2**, C-5′ and C-7′ were shifted downfield by ca. 6 ppm, and C-4′ and C-8′ were shifted upfield by ca. 1.5 ppm in **2** relative to **1**, in accordance with the substitution

of C-6′ in **2** by an hydroxyl. This was confirmed by the COSY, HSQC and HMBC data (see Table 2). The chemical shifts of the protons and carbons of the rest of the molecule seemed almost identical with that of **1**. H-6′ exhibited NOEs with H-7′, Me-10′ and H-12*exo*, thus confirming the α-orientation of 6′-OH. The rest of the NOEs presented by insuetolide B (**2**) were similar to that of **1**. On the basis of these arguments structure **2** was assigned to insuetolide B.

Insuetolide C (**3**) was isolated as a glassy material. It exhibited a sodiated molecular cluster ion (HRESIMS) of *m/z* 449.1941 from which the molecular formula $C_{25}H_{30}O_6$ was calculated, with 11° of unsaturation. Its IR spectrum exhibited stretching bands of three carbonyls: 1765, 1719 and 1686 cm^{-1}. Its NMR spectra were similar to those of insuetolides A (**1**) and B (**2**). The ^1H NMR spectrum of **3** in $CDCl_3$ (see Table 2) revealed an additional conjugated vinyl proton (δ_H 7.02 s) and the disappearance of H-7′, when compared with that of **1**. In the ^{13}C NMR spectrum of **3**, a new three-substituted double bond appeared, while methylene-6′ and methine-7′ disappeared compared to the spectrum of **1**. The allocation of the conjugated double bond to position 6′ and 7′ of the meroterpenoid skeleton was confirmed by the HMBC correlation (see Table 2). Insuetolide C (**3**) presented NOEs similar to those of **1** and **2**. On the basis of these arguments structure **3** was assigned to insuetolide C.

The absolute configuration of the insuetolides was established using Riguera's method for secondary alcohols.[11] The (*R*)-MPA ester of insuetolide B (**2**) was prepared, and its ^1H and COSY NMR spectra in CD_3CN were measured, followed by addition of $Ba(ClO_4)_2$ to saturation and measurement of the ^1H and COSY NMR spectra of the MPA ester Ba^{+2} complex. The $\Delta\delta^{Ba}$ values of the protons in the ester vicinity were calculated (see Fig. 4) and the absolute configuration of C-6′ was determined as (*R*). Based on the relative stereochemistry established above for **1**, **2**, and **3**, the absolute stereochemistry of the chiral centers of insuetolide A (**1**) were determined as 5*R*,8*S*,9*S*,10*R*,2′*R*,3′*R*,5′*S*,7′*S*, of insuetolide B (**2**) as 5*R*,8*S*,9*S*,10*R*,2′*R*,3′*R*,5′*S*,6′*R*,7′*S* and those of insuetolide C (**3**) as 5*R*,8*S*,9*S*,10*R*,2′*R*,3′*R*,5′*S*.

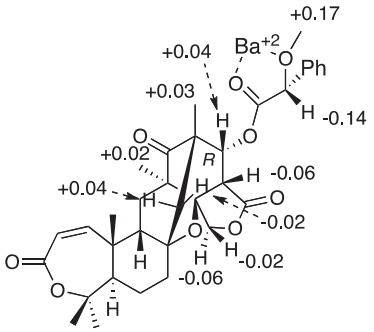


Figure 4. $\Delta\delta^{Ba}$ values of the insuetolide B MPA ester Ba^{+2} complex.

2.2. Drimane sesquiterpenes

The known 2α,9α,11-thrihydroxy-6-oxodrim-7-ene (**5**), strobilactone A (**6**) and (*E*,*E*)-6-(6′,7′-dihydroxy-2′,4′-octadienoyl)-strobilactone A (**7**), were isolated as the major constituents of the ethyl acetate extract of the culture medium. They were found to be identical with compounds previously isolated from two marine-derived strains of *Aspergillus ustus*[12,13] (**5** and **7**) and the edible mushroom *Strobilurus ohshimae*[14] (**6**).

Table 2
NMR Data of Insuetolide B (**2**) and Insuetolide C (**3**) in $CDCl_3$[a]

	Insuetolide B (**2**)			Insuetolide C (**3**)		
Position	δ_C, mult.	δ_H, mult. *J* (Hz)	HMBC[b]	δ_C, mult.	δ_H, mult. *J* (Hz)	HMBC[b]
1	149.5, CH	5.82, d, 12.8	2, 15	149.1, CH	5.84, d, 12.6	2, 15
2	119.3, CH	5.91, d, 12.8	1	119.1, CH	5.93, d, 12.6	1
3	167.0, qC		1, 2, 13	166.5, qC		1, 2, 13
4	84.0, qC		5, 13, 14	83.8, qC		5, 13, 14
5	41.0, CH	2.47, dd, 12.5, 7.8	1, 6β, 13, 14	40.7, CH	2.47, dd, 12.2, 7.2	1, 6α, 7α, 13, 14, 15
6 α	21.3, CH_2	1.98, m	5, 7α	20.8, CH_2	2.02, m	5
β		1.30, m			1.30, m	
7 α	32.8, CH_2	1.81, m	9, 11α, 12*exo*	33.6, CH_2	1.88, dd. 14.8, 9.7	11α, 12*exo*
β		1.78, m			1.70, dt, 14.8, 9.5	
8	75.9, qC		6β, 7α, 11α, 11β, 12*exo*	77.4, qC		7α, 11β, 12*endo*, 12*exo*
9	50.2, CH	1.45, m	1, 5, 6β, 7α, 11α, 11β, 12*exo*, 15	51.4, CH	1.49, m	1, 5, 7α, 11α, 11β, 12*exo*, 15
10	44.1, qC		1, 2, 5, 6α, 11α, 11β, 15	44.0, qC		1, 2, 5, 6α, 6β, 15
11 α	33.9, CH_2	1.14, m	9, 9′	32.5, CH_2	1.08, t, 11.4	9, 9′
β		1.43, m			1.56, m	
12 *exo*[c]	49.2, CH_2	2.00, d, 14.7	10′	49.7, CH_2	2.01, d, 13.7	7β, 9, 10′
endo		1.36, d, 14.7			1.26, d, 13.7	
13	29.9, CH_3	1.37, s	14	29.6, CH_3	1.38, s	14
14	22.4, CH_3	1.43, s	13	22.0, CH_3	1.44, s	13
15	25.1, CH_3	1.25, s	1, 5, 9, 11α	24.8, CH_3	1.27, s	1, 2, 5, 9
1′ α	71.7, CH_2	4.04, d, 10.3	7′	73.8, CH_2	4.56, d, 10.7	9′
β		4.44, d, 10.3			4.27, d, 10.7	
2′	82.5, qC		11α, 11β, 1′α, 1′β, 6′, 7′, 9′	79.8, qC		11α, 11β, 1′α, 1′β, 6′, 9′
3′	48.5, qC		9, 11α, 12*exo*, 1′α, 1′β, 7′, 9′	45.6, qC		11β, 1′α, 1′β, 9′
4′	213.0, qC		12*exo*, 6′, 9′, 10′	211.3, qC		11α, 12*endo*, 6′α, 6′β, 9′, 10′
5′	51.5, qC		11α, 12*endo*, 12*exo*, 6′, 10′	45.7, qC		11α, 12*endo*, 12*exo*, 6′, 9′, 10′
6′	72.1, CH	4.22, br d, 7.0	12*endo*, 12*exo*, 7′, 10′	146.2, CH	7.02 s	12*endo*, 12*exo*, 10′
6′-OH		3.33 br s				
7′	51.8, CH	3.32, d, 7.0	1′β, 6′	135.1, qC		1′α
8′	174.6, qC		1′β, 7′	166.4, qC		1′α1′β, 6′
9′	18.9, CH_3	1.20, s	11β	21.1, CH_3	1.21, s	11β
10′	20.8, CH_3	1.27, s	12*exo*, 6′	23.7, CH_3	1.31, s	12*endo*, 6′

[a] 400 MHz for ^1H and 100 MHz for ^{13}C.
[b] From numbered H to C in row.
[c] *exo* to ketone at position 4′.

Table 3
NMR data of **4** in $CDCl_3$[a]

Position	δ_C, mult.	δ_H, mult. *J* (Hz)	HMBC[b]
1 ax	29.8, CH_2	1.74, m	2ax, 2eq, 3ax, 3eq, 5, 15
eq		2.11, br d, 9.0	
2 ax	17.2, CH_2	1.60, m	3ax, 13
eq		1.74, m	
3 ax	44.3, CH_2	1.31, dt, 4.0, 13.5	1ax, 1eq, 2ax, 13
eq		1.43, br d, 13.5	
4	33.4, qC		2ax, 5, 13, 14
5	44.3, CH	2.03, d, 5.0	1eq, 1ax, 3eq, 15
6	66.0, CH	5.74, br s	5
7	123.2, CH	5.92, br s	5, 12a, 12b
8	134.5, qC		6, 9-OH, 12a, 12b
9	74.2, qC		1ax, 5, 7, 9-OH, 15
9-OH		2.59, br s	
10	37.3, qC		1ax, 1eq, 2ax, 5, 6, 15
11	174.3, qC		9-OH, 12b, 15
12 ax	68.5, CH_2	4.96, dt, 12.2, 2.0	7
eq		4.76, br d, 12.2	
13	24.3, CH_3	1.14, s	3ax, 3eq, 5, 14
14	32.0, CH_3	1.01, s	3ax, 5, 13
15	18.0, CH_3	1.19, s	1ax, 1eq, 5
1′	164.8, qC	6.12, ddd, 16.0, 2.5, 2.0	6, 2′, 3′
2′	119.6, CH	7.04, dt, 16.0, 4.0	3′, 4′
3′	147.3, CH		4′
4′	61.4, CH_2	4.39, br m	2′, 3′, 4′-OH
4′-OH		1.65, m	

[a] 500 MHz for ^1H and 125 MHz for ^{13}C.
[b] From numbered H to C in row.

(*E*)-6-(4′-hydroxy-2′-butenoyl)-strobilactone A (**4**) was isolated as a glassy transparent material. It exhibited a pseudo-molecular cluster ion $[M+Na]^+$ at *m/z* 373.1615, in the HRESIMS, from which a $C_{19}H_{26}O_6$ formula was calculated. The two carbonyl stretching bands which appeared in the IR spectrum, 1772 and 1715 cm^{-1}, suggested a five-membered lactone ring and an unsaturated ester moieties, respectively. Its NMR spectra were similar to those of **6** and **7**. Analysis of the ^1H and ^{13}C NMR spectra, as well as the COSY, HSQC and HMBC experiments of **4**, revealed that its bicyclic terpenoid moiety is identical to that of **7**. The polyketide unsaturated chain differed from the octyl ester of **7**. The structure of the hydroxyl butenoyl moiety was elucidated by interpretation of the 2D NMR data (see Table 3), and its assembly with the terpenoid core was established by the HMBC correlation of H-6 (δ_H 5.74 br s) and C-1′ (δ_C 164.8 s). The absolute stereochemistry of **4** was confirmed by the specific rotation that had the negative sign as the known **6** and **7**. Based on these arguments the structure of compound **4** was established as (*E*)-6-(4′-hydroxy-2′-butenoyl)-strobilactone A.

2.3. Biological activity

The inhibitory activity of the pure compounds was assessed against the fungus *Neurospora crassa*[15] and the MOLT-4 human leukemia cancer cell line.[16] The minimal inhibitory concentration (MIC) values of **1**, **6** and **7** against the fungus *N. crassa* were 140, 242, and 162 μM, respectively. Compounds **3**, **4**, and **7** inhibited the proliferation of a MOLT-4 cell line by 51%, 55% and 72%, respectively, at 50 mg/mL while **1** and **6** were not active at the same concentration.

2.4. Ending remarks

Meroterpenoids are most often isolated from fungi and marine organisms, but bacteria and higher plants produce such mixed biosynthesis products as well. Mixed polyketide-terpenoid-derived fungal natural products constitute the largest class of meroterpenoids, while those derived from orsellinic acid or its mono- and di-methyl derivatives are the most frequently isolated meroterpenoids from fungi, especially *Penicillium* and *Aspergillus* spp. Meroterpenoids derived from 3,5-dimethylorsellinic acid are produced via a common intermediate through the alkylation of 3,5-dimethylorsellinic acid by farnesyl diphosphate.[17] This common precursor yields a large number of secondary metabolites with a wide range of apparent structural diversity through (i) multi oxidation steps, for example andibenins,[2] (ii) backbone rearrangements, for example the andilesins,[18] anditomins[19] and tropolactones,[4] and (iii) backbone fragmentation, such as in austin[20] and the terretonins.[21] Insuetolides A–C exhibit a new skeleton of meroterpenoids closely related to that of the andilesins[18] and andibenins.[2] The difference between the skeletons of the latter two and the skeleton of the insuetolides lies in ring C, a cyclopentane in the former two and a perhydropyran ring in the insuetolides. This suggests that an additional oxidation step occurs in the biosynthesis of the insuetolides prior to the coupling of C-12 to the tetraketide moiety. Based on this assumption we posit a biosynthetic route to the insuetolides, which is summarized in Scheme 1. It involves the biosynthesis of a drimane type sesquiterpene linked through C-11 to C-3′ of the orsellinic acid. The 8, 12-terminal double bond of this common meroterpenoid-intermediate is oxidized to an epoxide, which in turn reacts in a 'penol-oxidation' type coupling reaction with C-5′ (C-12) and C-5′ (C-8 oxy-radical). The rest of the biosynthetic route to the insuetolides is similar to that elucidated for the andilesins.[3]

3. Experimental section

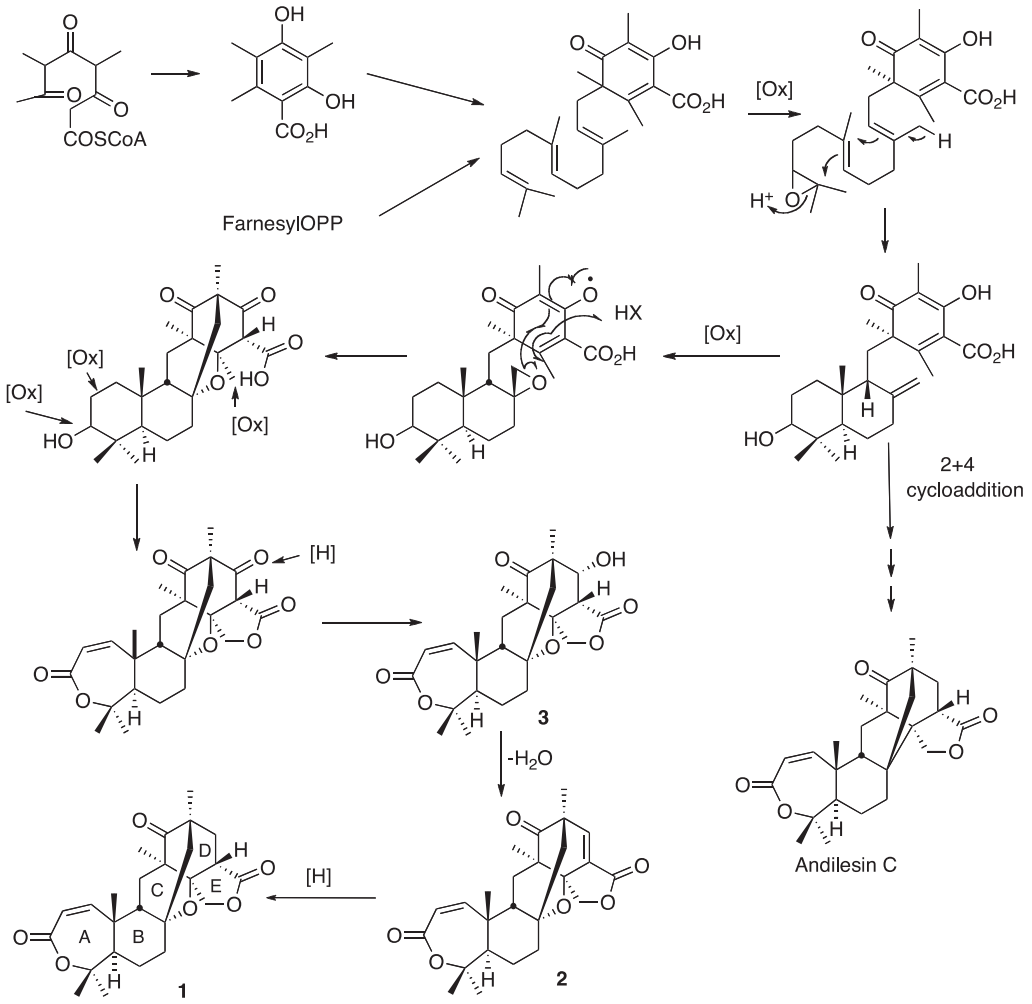
3.1. General experimental procedures

Low- and high-resolution ESI mass spectra were recorded on a Waters Synapt instrument. UV spectra were recorded on an Agilent 8453 spectrophotometer. Optical rotation values were obtained on a Jasco P-1010 polarimeter at the sodium D line (589 nm). IR spectra were recorded on a Bruker Vector 22 spectrometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer at 500.13 MHz for ^1H and 125.76 MHz for ^{13}C and a Bruker Avance 400 Spectrometer at 400.13 MHz for ^1H, 100.62 MHz for ^{13}C. COSY-45, gTOCSY, gROESY, gHSQC, gHMQC and gHMBC spectra were recorded using standard Bruker pulse sequences. HPLC separations were preformed on a Merck Hitachi (model L-6200 intelligent pump, model L-4200 UV–vis) and an Agilent 1100 series HPLC system.

3.2. Biological material

The Mediterranean sponge *Psammocinia* sp. was collected using SCUBA diving at a depth of 2–6 m, approximately 200 m off-shore from Sdot-Yam (32°30.940′N 034°50.64′E), Israel. The sponge samples were sealed underwater in bags with seawater, and brought to the laboratory for fungal culture from this fresh material.

The fungal strain OY-207 was isolated from the sponge sample using fungicide-amended media as described by Paz et al.[8] The strain was identified as *A. insuetus* by amplification and direct sequencing of the fungal RNA with ITS1 and 4 and β-tubulin gene primers followed by phylogentic analysis. The OY-207 strain was maintained on Potato Dextrose Agar. It was mass cultivated in Potato Dextrose Broth in stationary flasks for 20 days at 25 °C in the dark. The mycelium was separated from the growth medium by filtration through cheesecloth. The *N. crassa* (strain *74-OR23-1A*) test fungus was maintained on Vogel's minimal medium supplemented with sucrose.[15]



Scheme 1. Proposed biosynthetic route to the insuetolides.

3.3. Isolation procedure

Extraction of the mycelium-free culture medium (4 L) with EtOAc (2 × 4 L) afforded, after evaporation of the solvent, the crude extract (1.29 g). The crude extract exhibited anti-fungal activity against *N. crassa* at a concentration of 1 mg/mL. The EtOAc-extract (1.29 g) was separated on an open column C-18 reversed-phase material (YMC ODS, 25 g) eluted with solvent of decreasing polarity from water through MeOH (10% steps of MeOH each fraction). Fractions 6 and 9–12 exhibited the strongest anti-fungal activity and were thus farther separated. Fraction 6 (297 mg) was separated on a HPLC column (YMC Pack ODS-A, 20 mm × 250 mm, 10 μm; flow rate 5 mL/min; UV detection; eluent acetonitrile/water, 1:1) to give in fractions 1 and 4 semi-pure compounds that were further purified on the same column (fraction 1, eluted with MeOH/water 7:3), and on a Hibar Lichospher 60 RP-Select B (25 mm × 250 mm, 5 μm; flow rate 5 mL/min; UV detection; eluent MeOH/water, 3:1) to afford **1** (t_R 14.5 min, 1.4 mg, 0.11% yield of crude extract) and **4** (t_R 37.9 min, 2.3 mg, 0.18% yield of crude extract), respectively. The united fractions 9–12 from the initial separation (530 mg) were further separated on a Sephadex LH-20 column ($CHCl_3$/MeOH 7:3) to yield two anti-fungal active fractions, fraction 3 (40 mg) and fraction 4 (30 mg). Fraction 3 was separated on an HPLC column (Phenomenex Gemini-NX, C-18, 21.2 mm × 250 mm, 10 μm; flow rate 5 mL/min; UV detection; eluent MeOH/water 3:2, to obtain three pure compounds: **2** (t_R 53.5 min, 7.2 mg, 0.56% yield of crude extract), **3** (t_R 49.6 min, 10.1 mg, 0.78% yield of crude extract) and **6** (t_R 47.2 min, 7.2 mg, 0.56% yield of crude extract). Fraction 4 (30 mg) was further separated on the same semi-preparative HPLC column eluted with 9:1 MeOH/water, to afford **7** (t_R 11.5 min, 21.2 mg, 1.64% yield of crude extract) and **5** (t_R 27.8 min, 2.2 mg, 0.17% yield of crude extract).

3.3.1. Insuetolide A (1)

Glassy material; $[\alpha]_D^{30}$ −68 (*c* 0.35, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 214 (4.02) nm; IR ($CHCl_3$): 2979, 2939, 1780, 1717, 1686 cm^{-1}; ^1H and ^{13}C NMR (see Table 1), HRESIMS *m/z* 429.2279 $[M+H]^+$, (calcd for $C_{25}H_{33}O_6$, 429.2277).

3.3.2. Insuetolide B (2)

Glassy material; $[\alpha]_D^{26}$ −98 (*c* 0.05, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203 (4.07), 302 (3.32) nm; IR ($CHCl_3$): 2977, 2929, 1780, 1717, 1686 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); HRESIMS *m/z* 443.2069 $[M-H]^-$, (calcd for $C_{25}H_{31}O_7$, 443.2070).

3.3.3. Insuetolide C (3)

Glassy material; $[\alpha]_D^{26}$ −209 (*c* 0.17, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203 (3.77), 248 (2.90) nm; IR ($CHCl_3$): 2980, 2928, 1765, 1719, 1686 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); HRESIMS *m/z* 449.1941 $[M+Na]^+$, (calcd for $C_{25}H_{30}O_6Na$, 449.1940).

3.3.4. (*E*)-6-(4′-hydroxy-2′-butenoyl)-strobilactone A (4)

Glassy material; $[\alpha]_D^{25}$ −212 (*c* 0.15, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 206 (3.99) nm; IR ($CHCl_3$): 2990, 2950, 1772, 1715 cm^{-1};

^{1}H and ^{13}C NMR (see Table 3); HRESIMS *m/z* 373.1615 $[M+Na]^+$, (calcd for $C_{19}H_{26}O_6Na$, 373.1627).

3.3.5. 2α, 9α, 11-Thrihydroxy-6-oxodrim-7-ene (5)

Glassy material; $[\alpha]_D^{25}$ −65 (*c* 0.05, $CHCl_3$); ESIMS *m/z* 269 $[M+H]^+$; Identical in all respect with the published data.[12]

3.3.6. Strobilactone A (6)

Glassy material; $[\alpha]_D^{21}$ −164 (c 0.35, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (3.61) nm; IR ($CHCl_3$): 3680, 3590, 3550, 3019, 2997, 1719 cm^{-1}; HRESIMS *m/z* 289.1413 $[M+Na]^+$, (calcd for $C_{15}H_{22}O_4Na$, 289.1416); Identical in all respect with the published data.[13]

3.3.7. (*E*,*E*)-6-(6′,7′-dihydroxy-2′,4′-octadienoyl)-strobilactone A (7)

Glassy material; $[\alpha]_D^{21}$ −155 (*c* 0.21, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 201 (4.11), 261 (4.41) nm; IR ($CHCl_3$): 3610, 3017, 2982, 2951, 1773, 1704 cm^{-1}; HRESIMS *m/z* 443.2044 $[M+Na]^+$, (calcd for $C_{23}H_{32}O_7Na$, 443.2046); Identical in all respect with the published data.[12]

3.4. Bioassays

The anti-fungal activity of the crude extract, fractions and pure compounds was assessed in liquid medium. The crude extract, fractions or compounds were dissolved at various concentrations in Vogel's minimal medium with sucrose[15] and 1 mL aliquots were distributed into each well of a 24-well tissue culture dish, in duplicates. Conidia of *N. crassa* (10^7) were used to inoculate each well. Following 12 h of incubation at 34 °C, growth was assessed and the MIC determined. The cytotoxicity assay was performed as described in Rotem et al.[16]

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Supplementary data

Supplementary data (1D and 2D NMR data and HR-MS of compounds **1**–**4**, are included.) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.045.

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