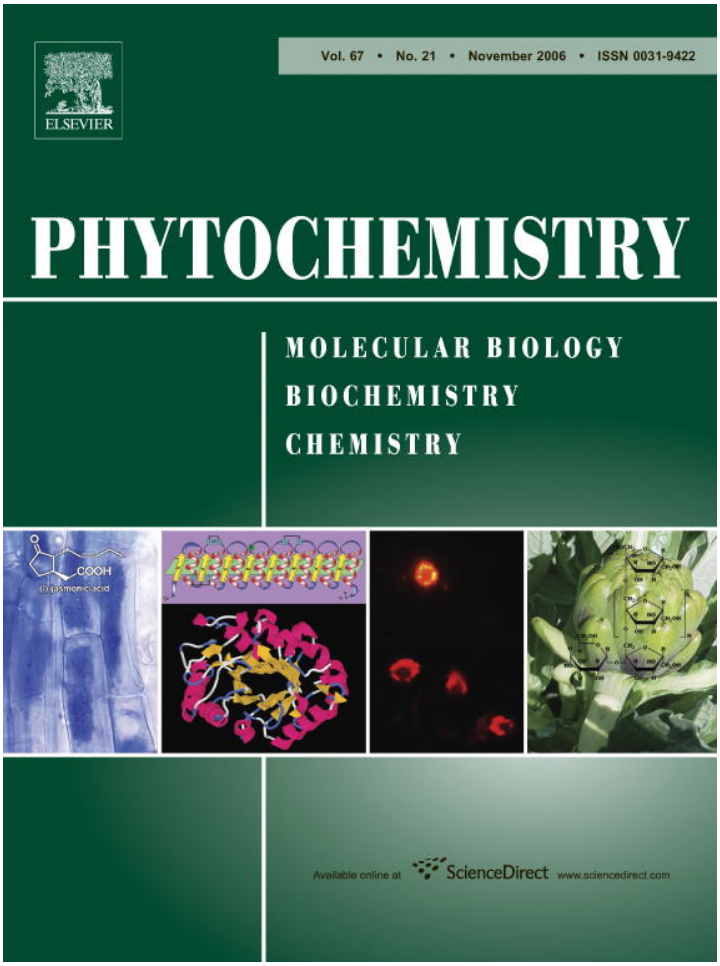

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Phytochemistry 67 (2006) 2403–2410
PHYTOCHEMISTRY
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Antimicrobial metabolites produced by an intertidal *Acremonium furcatum*

Gabriela L. Gallardo [a], Matías Butler [a], Mariana L. Gallo [a], M. Alejandra Rodríguez [b], Marcos N. Eberlin [c], Gabriela M. Cabrera [a,*]

[a] *Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pab. II, 1428 Buenos Aires, Argentina*
[b] *DBBE, FCEN, UBA, Ciudad Universitaria, Pab. II, 1428 Buenos Aires, Argentina*
[c] *Thomson Mass Spectrometry Laboratory, Instituto de Química, Universidade Estadual de Campinas, 13083-970 Campinas, SP, Brazil*

Received 13 January 2006; received in revised form 27 June 2006
Available online 7 September 2006

Abstract

In a screening for antimicrobial metabolites, amides of D-*allo*- and L-isoleucine derivatives were isolated from the culture of a marine strain of *Acremonium furcatum*. Structural elucidation of these compounds was performed by analysis of spectroscopic data and confirmed by synthesis. All of the compounds, natural and synthetic intermediates, were bioassayed against bacteria and phytopathogenic fungi, with many showing remarkable antifungal activities.

Keywords: *Acremonium furcatum*; Marine fungi; Isoleucinol; Isoleucinaldehyde; Isoleucine derivatives; Antifungal activity; *Fusarium virguliforme*; *Colletotrichum truncatum*

1. Introduction

Since marine microorganisms grow in unique and extreme habitats, they may have the capability to produce unique and unusual metabolites. In particular, it is known that marine fungi, native or adapted to the environment, are an important source of bioactive natural products (Schiehser et al., 1986; Fenical and Jensen, 1993; Kobayashi and Ishibashi, 1993; Davidson, 1995; Bringmann et al., 2005). In the course of screening for new bioactive metabolites with potential use in agriculture from fungal cultures of diverse origin (Cabrera et al., 2002; Gallo et al., 2004; Levy et al., 2003), we investigated a strain *Acremonium furcatum* isolated from an intertidal sediment sample. The extract of the culture medium of this fungus produced new metabolites with antimicrobial activity against bacteria and phytopathogenic fungi. This report deals with the fermentation, isolation, structure elucidation, synthesis and biological activity of these compounds, which are amides of the corresponding amino alcohols and amino aldehydes of D-*allo*- and L-isoleucine.

2. Results and discussion

The organic extract of the culture media of *A. furcatum*, isolated from an intertidal sediment sample (collected on the coast of Buenos Aires, Argentina), showed moderate antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Botrytis cynerea*, and a very simple pattern by thin layer chromatography (TLC). Prep. TLC was then employed and two fractions in a 10:1 ratio were obtained. The major and more polar fraction showed, by ^{1}H NMR spectroscopy, three olefinic protons at δ 6.88 (1H, *d*, 11.0 Hz), 6.33 (1H, *ddq*, 15.0, 11.0 and 1.5 Hz) and 6.03 (1H, *dq*, 15.0 and 6.9 Hz), one broad signal at 5.90 ppm, five complex multiplets at 4.00, 3.70, 1.70,

* Corresponding author. Tel./fax: +54 11 4576 3376.
E-mail address: gabyc@qo.fcen.uba.ar (G.M. Cabrera).

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doi:10.1016/j.phytochem.2006.07.028

1.46–1.50 and 1.23 ppm, two methyl protons attached to sp^2 carbons at δ 1.86 (*d*, 6.9 Hz) and 1.95 (*s*), and two set of methyl groups in a 3:1 ratio, the major group at δ 0.93 (*t*, 7.4 Hz) and 0.93 (*d*, 7.0 Hz) and the minor at 0.91 (*t*, 7.0 Hz) and 0.95 (*d*, 6.8 Hz). These data indicated that the fraction was actually a mixture of two compounds **1** and **2**, in an approximately 3:1 ratio, which were very similar in nature. The high field signals resembled those of amino acids and the similarity between both compounds suggested an epimeric mixture.

Exhaustive efforts to separate this mixture using HPLC, employing different stationary and mobile phases, or employing enzymes to selectively acylate one epimer (after knowing the structures), were only partially successful – the major compound **1**, obtained via HPLC separation with a chiral stationary phase, was only of sufficient purity for full characterization. Thus, a provisional structural elucidation of compound **2** was performed on the natural mixture (Che et al., 2002).

Compound **1** has the molecular formula $C_{13}H_{23}NO_2$, as determined from its high-resolution electron ionization mass spectrometry (EIMS). A COSY H,H experiment and irradiation of the olefinic protons allowed unambiguous determination of the presence of a $CH_3CH{=}CH{-}CH{=}C(CH_3)$ moiety and, taking into account the coupling constants, an *E* configuration for the terminal double bond was determined. The COSY spectrum also showed that the methine proton at δ 4.00 (CH) correlated with protons at δ 3.70 (CH_2) and 1.72 (CH).

The ^{13}C NMR spectrum exhibited four olefinic carbons (δ 136.3 *d*, 134.2 *d*, 127.3 *s* and 126.9 *d*) and two methyl groups at 18.6 and 12.7 ppm, corresponding to the above-mentioned olefinic moiety, plus another seven signals, which were all accompanied by a duplicate resonance of lower intensity in the ^{13}C NMR spectrum of the mixture. One of these carbons was a carbonyl at 170.3 ppm, while the other carbons were a CH_2 attached to an oxygen at δ 64.0, a CH linked to a heteroatom at 55.3 ppm and aliphatic carbons at 35.5 (*d*), 26.3 (*t*), 14.8 and 11.3 (*q*). The multiplicities were determined by a distortionless enhancement by polarization transfer (DEPT) experiment.

The methyl group at 1.95 ppm correlated with the carbonyl in a heteronuclear multiple bond correlation (HMBC) experiment, indicating that an acid derivative $CH_3CH{=}CH{-}CH{=}C(CH_3)COX$ was present and attached to the other part of the molecule. The *E* configuration for the second double bond was determined by comparison with the spectroscopic literature data for the corresponding methyl ester, previously synthesized (Ceroni and Séquin, 1982).

A comparison of the remaining high-field proton and carbon signals with the literature data for amino acids (MacDonald et al., 1976) showed that compound **1** contained a substructure similar to the amino acid isoleucine, but in a reduced way, isoleucinol, as 2D experiments revealed. The connectivities observed in the HMBC experiment are shown in Fig. 2. The structure of compound **1** was thus assigned as the 2-methyl-hexa-2,4-dienoic acid, isoleucinol amide.

Similar spectroscopic analysis was performed on the mixture, revealing the presence of a minor component with the same structural features as **1**. The 2D NMR spectroscopic experiments showed that all the correlations in the high field region were merely duplicated and partially overlapped. Furthermore, the available ^{13}C NMR spectroscopic data for amino acids (MacDonald et al., 1976) suggested that **1** and **2** were a 3:1 mixture of C-2 epimers, i.e. D-*allo*-IleOH:L-IleOH or L-*allo*-IleOH:D-IleOH, indicating that the major epimer was either D-*allo* or L-*allo*-IleOH, whereas the minor component was L- or D-IleOH.

The mixture of compounds**1** and **2** was oxidized and hydrolyzed to determine the absolute configuration of each compound, with the resulting amino acids analyzed by HPLC using a chiral column, Chirex (D) Penicillamine, yielding peaks with retention times of 29.9 and 39.9 min, identical to authentic samples of D-*allo*-Ile and L-Ile, respectively. This result thus established the absolute configuration as being 2′*R*, 3′*S* for the major epimer **1**, and 2′*S*, 3′*S* for the minor compound **2**. With this rationale, the structures of **1** and **2** were established as shown in Fig. 1.

The synthesis of compounds **1** and **2** was performed to confirm the structures, to determine the physical constants of compound **2**, and to fundamentally evaluate the biological properties of each individual compound. For this

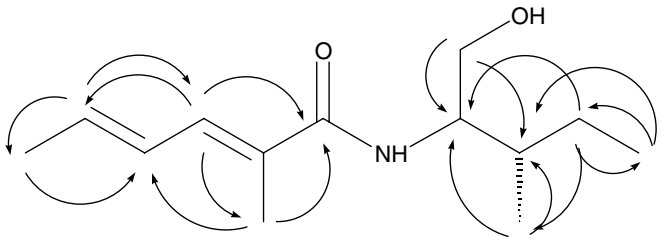
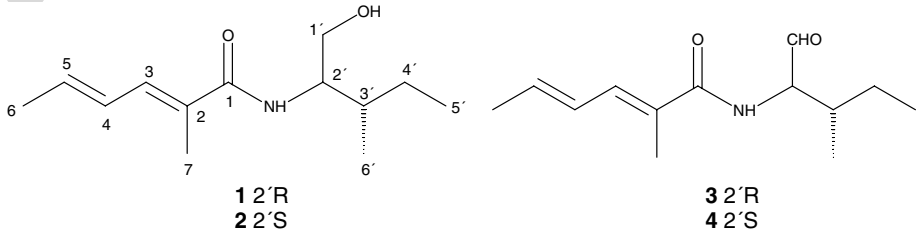


Fig. 2. Observed HMBC correlations.



1 2′R
2 2′S

3 2′R
4 2′S

Fig. 1. Isolated compounds from *Acremonium furcatum*.

purpose, the general methodology of Hamada et al. (1987) was employed. Although some modifications were made to improve yields, in general, they were lower due to simple decomposition of the unsaturated acid subunit. This subunit was synthesized instead according to the procedure of Ceroni and Séquin (1982) as shown in Fig. 3. The spectroscopic data of the synthetic compounds **1** and **2** were in full accordance with those of the natural metabolites.

The minor and less polar fraction isolated from the extract had similar ^{1}H and ^{13}C NMR spectra that of compounds **1** and **2**. It differed, however, by the absence of the CH_2OH moiety and the presence of an aldehydic functionality at δ 200.2 (^{13}C NMR) and 9.73 and 9.67 ppm (*s*, ^{1}H NMR), respectively, suggesting structures **3** and **4** as shown in Fig. 1. An epimeric mixture was also evident, as the signals of the aminoaldehyde unit were duplicated in both spectra. Due to the small amount of this mixture, and its instability, it was not possible to perform any further analysis. The presence of the epimers, D-*allo*- and L-Ile-isoleucinaldehyde, was thus assumed on the basis of the absolute stereochemistry of **1** and **2**. The synthesis of *N*-Boc-L-isoleucinaldehyde **10** (Fig. 4) was made to confirm the assignments and identify unambiguously both epimers. The ^{1}H and ^{13}C NMR spectroscopic data for **10** were in full agreement with those of the minor epimer **4**, confirming its identity.

A time-course of the production of compounds **1** + **2** was carried out in an attempt to explain the reason why both epimers were present in the extract (Fig. 5). Two interesting findings were made. First, the pH of the medium became basic after day 8. Second, the presence of the minor metabolites **3** and **4** were observed on all days, via TLC of the extract, in approximately the same relative proportions to that of the epimers **1** and **2**. Both results thus suggested that the aldehydic precursors of the alcohols epimerize due to a keto–enol equilibrium in the basic media, thereby producing an epimeric mixture of alcohols.

The synthetic compounds **1** and **2**, and the synthetic intermediates **5**–**8** and **10**, were bioassayed against the bacteria *B. subtilis*, *S. aureus*, *E. coli* and the fungi *Fusarium virguliforme* (causal agent of Sudden Death Syndrome in soy bean) *Colletotrichum truncatum* (responsible for anthracnose disease), *Macrophomina phaseolina* (causal agent of charcoal rot in beans), *B. cynerea* and *Aspergillus fumigatus*. The results are shown in Table 2. Interestingly, the L-derivatives **2** and **8** showed moderate antibiotic activity and low, if any, antifungal activity, whilst the D-*allo* derivatives **1** and **7** showed antifungal activity. Nonetheless, the acid **6** and the ester **5** displayed the greatest antifungal activity, in the same order as benomyl, a commercial antifungal product. The natural (2*Z*, 4*E*) isomer of **6** was previously reported as an antifungal metabolite against other fungal strains (Proksa et al., 1992).

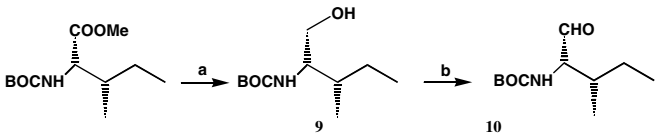


Fig. 4. Synthetic route to compound **10**.

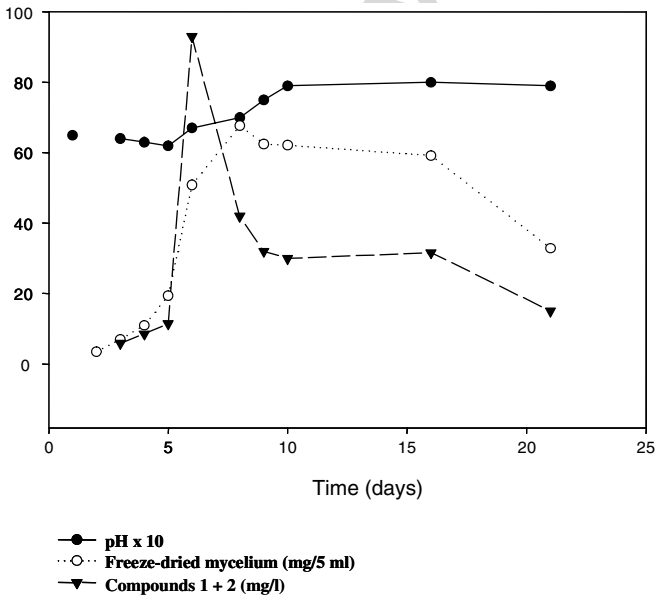
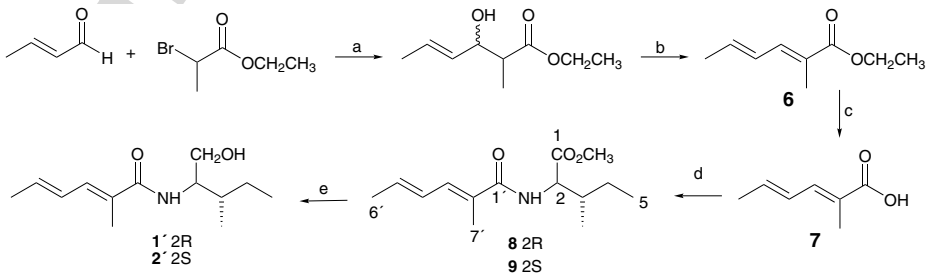


Fig. 5. Time-course production of **1** and **2**.



a. Zn/Bz b. P_2O_5/silicagel c. NaOH/*t*-BuOH d. $\ominus Cl\ H_3\overset{\oplus}{N}CHRCOOMe$, Py, DCC/THF e. $LiBH_4$/EtOH

Fig. 3. Synthetic route to compounds **1** and **2**.

Table 1
NMR spectroscopic data of compounds **1** and **2** ($CDCl_3$), δ in ppm, J in Hz

	Compound **1**		Compound **2**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	170.3		170.1	
2	127.3		126.9	
3	134.2	6.88 *d* (11.0)	134.2	6.88 *d* (11.0)
4	126.9	6.33 *ddq* (15.0, 11.0, 1.5)	126.9	6.33 *ddq* (15.0, 11.0, 1.5)
5	136.3	6.03 *dq* (15.0, 6.9)	136.3	6.03 *dq* (15.0, 6.9)
6	18.6	1.86 *d* (6.9)	18.6	1.86 *d* (6.9)
7	12.7	1.95 *s*	12.7	1.95 *s*
1′	64.0	3.70 *dd* (11.2, 4.1) 3.67 *dd* (11.2, 6.5)	63.4	3.74 *m*[a]
2′	55.3	4.00 *m*	55.9	3.88 *m*
3′	35.5	1.72 *m*	35.7	1.70 *m*
4′	26.3	1.23, 1.46 *m*	25.5	1.23, 1.51 *m*
5′	11.3	0.93 *t* (7.4)	11.3	0.91 *t* (7.0)
6′	14.8	0.93 *d* (7.0)	15.5	0.95 *d* (6.8)
N*H*		5.90 *d* (7.7)		5.92[b]

Assignments based on COSY H,H, HSQC and HMBC experiments. Multiplicities determined by DEPT.
[a] Overlapped.
[b] Partially overlapped.

Several isoleucine derivatives were previously isolated from different microbial strains as C-amides (Kern et al., 1985), esters (Iwamoto et al., 1990) or unknowns (Kawakami et al., 1978). In particular, isoleucinol was reported as a C-terminal amino acid of peptaibols (Jaworski and Bruckner, 2001).

The acid subunit was previously found as an ester of an antifungal compound isolated from *Graphium putredinis* (Kennedy et al., 1998) and the above-mentioned antifungal isomer (2*Z*, 4*E*) was identified as a free acid from culture extracts of *Penicillium vermiculatum* (Proksa et al., 1992). It is noteworthy that, although the strain grew well in the same medium without artificial sea water and also in malt extract agar, the compounds were not produced, even at trace levels, under those conditions. It is known that metabolite profiles expressed by fungi are sometimes dependent on media salinity (Rabaek et al., 1998; Christophersen et al., 1999) although this question deserves still an explanation.

Acremonium is a wide-spread fungal genus, noted for their secondary metabolite content, with around 90 compounds having been reported from fungi of this genus (Abdel-Lateff et al., 2002), including alkaloids (Munday-Finch et al., 1995), terpenoids (Kawashima et al., 1994), aromatic compounds (Toki et al., 1994) and peptides (Sharman et al., 1996). Particularly, several marine *Acremonium* strains have been isolated, including strains of *A. furcatum* (Koh et al., 2002), and antioxidant hydroquinones (Abdel-Lateff et al., 2002), anti-inflammatory oxepines and weak antifungal quinolines (Belofsky et al., 2000) were isolated and identified from marine strains of this genus.

2.1. Concluding remarks

In summary, we have isolated and identified two new antimicrobial amino alcohol derivatives **1** and **2**, which are epimers biosynthesized from the corresponding aldehydes, that were also isolated. A rapid epimerization of the aldehydes could explain these data, as a basic pH was produced in the medium during fermentation. The structure elucidations were confirmed by total synthesis of compounds **1** and **2**, and remarkably, the two synthetic intermediates **5** and **6**, exhibited higher antifungal activity against phytopathogenic fungi, comparable to that of the commercial agent benomyl.

3. Experimental

3.1. General

FTIR spectra were recorded on a Nicolet Magna-IR 550. The UV spectra were recorded on a Hewlett–Packard 8451 A diode array spectrophotometer, whereas optical rotations were acquired on a Perkin–Elmer polarimeter 343. NMR spectra were recorded on a Bruker AM-500 instrument at 500.13 MHz for ^{1}H and at 125.13 MHz for

Table 2
Antimicrobial activities of natural and synthetic compounds

	1	**2**	**5**	**6**	**7**	**8**	**10**	**A**	**B**
Escherichia coli[a]	–	7	–	–	–	7	–	9	nd
Bacillus subtilis[a]	–	9	15	9	–	10	–	10	nd
Staphylococcus aureus[a]	7	–	15	9	–	–	–	8	nd
Fusarium virguliforme[b]	15	10[c]	15 (5 μg)	25 (1 μg)	15	12[c]	–	12	20
Aspergillus fumigatus[b]	15	10[c]	24 (1 μg)	29 (1 μg)	10	–	14	10	20
Botrytis cynerea[b]	15	–	12 (5 μg)	15 (1 μg)	–	–	–	12	28
Colletotrichum truncatum[b]	10	5	10 (10 μg)	12 (1 μg)	8	–	11	nd	25
Macrophomina phaseolina[b]	nd	nd	10 (25μg)	20 (1μg)	nd	nd	nd	nd	22

Diameter of inhibition zone in mm (MIC μg/pt).
nd, not determined.
[a] 50 μg/6 mm disk was used.
[b] 50 μg/spot was used except the concentration shown in parentheses and B: benomyl (25 μg/spot). A: a mixture of natural products **1** and **2**.
[c] Diffuse halos.

^{13}C NMR. EIMS was carried out on a mass spectrometer Trio-2 VG Masslab (Manchester, UK), whereas HR-MS employed a Fisons VG AutoSpec. Some of the HR-MS were run at the Washington University Resource (St. Louis, Missourri) for Biomedical and Bio-organic Mass Spectrometry.

3.2. Fungal strain

The fungus *A. furcatum* (F. & R. Moreau) ex W. Gams, was isolated from an intertidal marine sediment sample collected at Miramar, Province of Buenos Aires, Argentina and classified by Dr. J. E. Wright (PRHIDEB-CONICET, Depto de Biodiversidad y Biología Experimental DBBE, FCEN, UBA) and Dr. M. A. Rodriguez (DBBE, FCEN-UBA). The strain was deposited in the BAFC Culture Collection (FCEN-UBA, CONICET) under the Accession Number BAFC 51375.

3.3. Fermentation

Fermentation was carried out using a medium which consisted of peptone 1%, yeast extract 0.5%, dextrose 1% and artificial sea water (Instant Ocean) 100%. Erlenmeyer flasks (250 ml) containing 75 ml of medium were inoculated with agar slants of the strain. Fermentation was carried out at 25 °C for 20 days under static conditions.

3.4. Extraction and isolation

The fermentation broth was filtered and the filtrate was partitioned with EtOAc. The crude organic extract was subjected to prep. TLC on silica gel (0.25 mm), eluting with EtOAc:CH_2Cl_2 1:1, yielding one band containing compounds **1** and **2** (29 mg/l, R_F 0.5) and another with compounds **3** and **4** (1 mg/l, R_F 0.7). Both bands were detected by UV at 254 nm, and EtOAc was employed to recover the compounds from the adsorbent. Compounds **1** and **2** were separated by HPLC (Chirex (D) Penicillamine column-Phenomenex, 250 × 4.60 mm, $CuSO_4$ 1 mM:MeOH 6:4, 0.7 ml/min, UV 215 nm). A re-purification of the major peak by peak shaving was done employing the same conditions as above to yield pure **1** (1 mg).

3.5. Time-course of production of compounds *1* and *2*

A loopful of the cells of a slant culture of the strain was inoculated in 75 ml of the above liquid medium in a 250 ml Erlenmeyer flask and incubated on a rotary shaker at room temperature for one day to give a seed culture. An aliquot of this culture (1 ml) was transferred quantitatively onto 50 ml of medium in 40 × 125 ml Erlenmeyer flasks. Two of these cultures were filtered every day with the mycelia freeze-dried and the media extracted with EtOAc (20 ml); the aqueous layers were re-extracted with EtOAc (2 × 20 ml). The organic layers were combined and evaporated in vacuo with the amount of compounds **1** + **2** determined by HPLC (Column: YMC-Pack ODS-A, 250 × 4.6 mm, 5 μm; MeOH–H_2O 6:4, 0.5 ml/min, UV 230 nm).

3.6. Physico-chemical properties of compounds

3.6.1. Compound *1*

(2*E*,4*E*)-2-methyl-hexa-2,4-dienoic acid (2′*R*,3′*S*)-isoleucinol amide or ((2*E*,4*E*)-2-methyl-hexa-2,4-dienoic acid [(1′*R*,2′*S*)-1-hydroxymethyl-2-methyl-butyl]-amide). Oil. $[\alpha]_D = -50$ ($CHCl_3$, c0.1). For ^1H NMR and ^{13}C NMR spectra, see Table 1. HREIMS *m/z* $[M]^{+\cdot}$, found 225.17300, calc. for $C_{13}H_{23}NO_2$ 225.17288 ($\Delta m = 0.4$ ppm), $[M+1]^{+\cdot}$, found 226.17656, calc. for $^{13}C^{12}C_{12}H_{23}NO_2$ 226.17623 ($\Delta m = 0.4$ ppm). EIMS 70 eV, *m/z* (rel. int.): 225 $[M]^{+\cdot}$(8), 210 $[M-CH_3]^+$ (3), 207 $[M-H_2O]^{+\cdot}$ (3), 194 $[M-CH_2OH]^{+\cdot}$ (24), 126 (22), 109 $[RCO]^+$ (100), 81 (69).

3.6.2. Compound *2*

(2*E*,4*E*)-2-methyl-hexa-2,4-dienoic acid (2′*S*,3′*S*)-isoleucinol amide or ((2*E*,4*E*)-2-methyl-hexa-2,4-dienoic acid [(1′*R*,2′*S*)-1-hydroxymethyl-2-methyl-butyl]-amide). For ^1H NMR and ^{13}C NMR spectra (in the natural mixture), see Table 1.

3.6.3. Compounds *3* and *4*

(2′*R*, 3′*S*) and (2′*S*,3′*S*) [(2*E*,4*E*)-2-Methyl-hexa-2,4-dienoic acid isoleucinaldehyde] or ((1′*R*,2′*S*) and (1′*S*,2′*S*) (2*E*,4*E*)-2-methyl-hexa-2,4-dienoic acid (1-formyl-2-methyl-butyl)-amide). ^1H NMR (500 MHz, $CDCl_3$): δ 9.73 (*s*, H-$1'_m$), 9.67 (*s*, H-$1'_M$), 6.89 (*m*, H-3), 6.34 (*m*, H-4), 6.06 (*m*, H-5), 5.85 (N*H*), 3.73 (*m*, H-2′), 1.96 (*s*, H-7), 1.87 (*d*, $J = 6.4$ Hz, H-6), 1.30–40 (*m*, H-4′), 0.94 (*t*, $J = 6.9$ Hz, H-$5'_M$), 0.94 (*d*, $J = 6.6$ Hz, H-$6'_M$), 0.99 (*d*, $J = 7.0$ Hz, H-$6'_m$), 0.97 (overlapped, H-$5'_m$). ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.2 (*d*, C-1′), 169.5 (*s*, C-1_M), 169.2 (*s*, C-1_m), 136.5 (*d*, C-5), 134.4 (*d*, C-3), 127.0 (C-2,4), 62.0 (*d*, C-$2'_M$), 63.1 (*d*, C-$2'_m$), 36.5 (*d*, C-$3'_m$), 35.5 (*d*, C-$3'_M$), 26.4 (*t*, C-$4'_M$), 25.7 (*t*, C-$4'_m$), 18.8 (*q*, C-6), 15.6 (*q*, C-$6'_m$), 14.8 (*q*, C-$6'_M$), 12.8 (*q*, C-7), 11.9 (*q*, C-5′). M: major epimer **3** (2′*R*,3′*S*), m: minor epimer **4** (2′*S*,3′*S*).

3.7. Absolute configuration determination

3.7.1. Oxidation of compounds *1* and *2* to isoleucine

A solution of 5 mg of a sample of compounds **1** and **2** in acetone was oxidized with Jones reagent at ambient temperature (Harding et al., 1975). The product mixture was then subjected to TLC (CH_2Cl_2–MeOH 85:15) yielding the corresponding dicarbonylated intermediates (3-methyl-2-(2-oxo-propionylamino)-pentanoic acid). ^{1}HNMR (500 MHz, CD_3OD-$CDCl_3$ 1:1): δ 4.51 (*d*, $J = 4.0$ Hz, H-α_M), 4.40 (*d*, $J = 4.8$ Hz, H-α_m), 2.47 (C*H*$_3$CO), 0.95 (*t*, $J = 6.9$ Hz, H-5_M), 0.92 (*d*, $J = 6.6$ Hz, H-6_M); M: major epimer, m: minor epimer. The intermediates were hydrolyzed with 6 N HCl at 110 °C for 16 h, with the product neutralized and directly used for HPLC analysis.

The absolute configuration determination of the amino-acids, derived from **1** and **2,** was done by HPLC (Chirex (D) Penicillamine column-Phenomenex, 250 × 4.60 mm, $CuSO_4$ 2 mM: MeOH 85:15, 0.7 ml/min, UV 215 nm) by comparison with authentic standards of amino acids. RT values: L-*allo*-Ile 25.6 min, L-Ile 29.9 min, D-*allo*-Ile 39.9 min, D-Ile 49.5 min. Sample from **1** and **2**: 29.9 and 39.9 min. Coinjections confirmed the identities.

3.8. Synthesis of compounds *1* and *2*

3.8.1. Synthesis of (2E,4E)-2-methyl-hexa-2,4-dienoic acid ethyl ester (*5*)

This compound was prepared according to the previous reported procedure for the corresponding methyl ester (Ceroni and Séquin, 1982). Instead of the use of Sicapent drying agent, P_2O_5 on Silicagel Kieselgel 60 G-Merck (1:2) was employed. *Compound* **5**: Oil. UV (CH_2Cl_2)λ_{max} nm (log ε): 236 (3.4). FTIR (KBr) ν_{max} cm^{-1}: 2981 (CH), 2931 (CH), 1740 (CO), 1455, 1376, 1241, 1177, 1099, 950. ^{1}H NMR ($CDCl_3$): δ 7.15 (*br d*, $J = 11.4$ Hz, H-3), 6.36 (*ddq*, $J = 15.0$, 11.4 and 1.6 Hz, H-4), 6.08 (*dq*, $J = 15.0$ and 6.8 Hz, H-5), 4.20 (*q*, $J = 7.0$ Hz, CH_2CH$_3$), 1.92 (*br s*, H-7), 1.86 (*br d*, $J = 6.8$ Hz, H-6), 1.29 (*t*, $J = 7.0$ Hz, CH$_2$CH_3). ^{13}C NMR ($CDCl_3$): δ 168.4 (*s*, C-1), 138.2 (*d*, C-3), 137.3 (*d*, C-5), 127.3 (*d*, C-4), 124.8 (*s*, C-2), 60.2 (*t*, CH_2CH$_3$), 18.6 (*q*, C-6), 14.1 (*q*, CH$_2$CH_3), 12.3 (*q*, C-7). EIMS 70 eV *m/z* (rel. int.): 155 $[M+1]^+$ (30), 154 $[M]^{+\cdot}$ (21), 127 (21), 126 (8), 99 (45), 81 (50), 55 (100).

3.8.2. Hydrolysis of *5* to (2E,4E)-2-methyl-hexa-2,4-dienoic acid (*6*)

NaOH (4.8 g) was added to a suspension of compound **5** (18 g) in *t*-BuOH (200 ml) and the whole was next heated until reflux began. This was maintained for 1 h, following which compound **6** (10.1 g, 69%) was obtained after purification through dry column flash chromatography on silica gel, eluting with EtOAc:cyclohexane 1:1. Amorphous powder. UV (CH_2Cl_2)λ_{max} (nm) (log ε): 265 (4.3). FTIR (KBr) ν_{max} (cm^{-1}): 2935 (CH), 2656, 2595, 1689 (CO), 1640, 1612, 1441, 1305, 1270, 969, 940. ^{1}H NMR ($CDCl_3$): δ 7.27 (*br d*, $J = 11.4$ Hz, H-3), 6.38 (*ddq*, $J = 15.1$, 11.4 and 1.8 Hz, H-4), 6.15 (*dq*, $J = 15.1$ and 6.7 Hz, H-5), 1.91 (*br s*, H-7), 1.89 (*br d*, $J = 6.7$ Hz, H-6). ^{13}C NMR ($CDCl_3$) : δ 173.9 (*s*, C-1), 140.9 (*d*, C-3), 139.1 (*d*, C-5), 127.4 (*d*, C-4), 123.9 (*s*, C-2), 18.9 (*q*, C-6), 12.1 (*q*, C-7). EIMS 70 eV, *m/z* (rel. int.): 126 $[M]^{+\cdot}$(37), 111 (81), 79 (44), 55 (47), 43 (80), 41 (100).

3.8.3. Synthesis of (2R,3S)-3-methyl-2-[(2′E,4′E)-2′-methylhexa-2′,4′-dienoylamino]- pentanoic acid ethyl ester (*7*)

N-Boc-D-*allo*-Ile methyl ester was prepared according to the procedure of Hamada et al. (1987). Dry EtOAc saturated with HCl (vapor) (5 ml) was added to a solution of *N*-Boc-D-*allo*-Ile methyl ester (340 mg) in dry EtOAc (5 ml). After 5 h of stirring at room temperature, the crude product was concentrated in vacuo, and was directly employed for the next step. It was dissolved in THF (10 ml) together with compound **6** (173 mg) and 1 equiv. of pyridine. The mixture was then cooled in an ice bath and dicyclohexylcarbodiimide (DCC) (300 mg) was added. After 30 min, the reaction was allowed to proceed for 4 h at room temperature. The dicyclohexylurea was removed by filtration (Klausner and Bodanski, 1972). The reaction mixture was evaporated to dryness and the crude product (150 mg) was purified by dry column flash chromatography, eluting fractions with mixtures of cyclohexane–CH_2Cl_2 1:1 to CH_2Cl_2–EtOAc 1:1 of increasing polarity. Pure compound **7** was eluted with CH_2Cl_2. *Compound* **7**: Oil. $[\alpha]_D = -50$ (CH_2Cl_2, c0.49). UV (CH_2Cl_2)λ_{max} (nm) (log ε): 240 (4.7). FTIR (KBr) ν_{max} (cm^{-1}): 3408 (NH), 2968 (CH), 2920 (CH), 2860 (CH), 1763 (CO), 1669, 1520, 1467. ^{1}H NMR ($CDCl_3$): δ 6.88 (*br d*, $J = 11.1$ Hz, H-3′), 6.33 (*ddq*, $J = 14.9$, 11.1 and 1.6 Hz, H-4′), 6.18 (*br d*, $J = 8.7$ Hz, N*H*), 6.04 (*dq*, $J = 14.9$ and 6.9 Hz, H-5′), 4.80 (*dd*, $J = 8.7$ and 4.1 Hz, H-2), 3.75 (*s*, CO_2CH_3), 1.96 (*br s*, H-7′), 1.97 (overlapped *m*, H-3), 1.86 (*br d*, $J = 6.9$ Hz, H-6′), 1.45 and 1.19 (*m*, H-4), 0.96 (*t*, $J = 7.3$ Hz, H-5), 0.90 (*d*, $J = 6.8$ Hz, H-6). ^{13}C NMR ($CDCl_3$): δ 173.1 (*s*, C-1), 169.1 (*s*, C-1′), 136.4 (*d*, C-5′), 134.3 (*d*, C-3′), 127.3 (*s*, C-2′), 127.0 (*s*, C-4′), 55.5 (*d*, C-2), 52.1 (*s*, CO_2CH_3), 38.0 (*d*, C-3), 26.3 (*t*, C-4), 18.7 (*q*, C-6′), 14.7 (*q*, C-6), 12.8 (*q*, C-7′), 11.7 (*q*, C-5). HREIMS *m/z*: $(M)^{+\cdot}$, found 253.16753, calc. for $C_{14}H_{23}NO_3$ 253.16779 ($\Delta m = 1.0$ ppm). EIMS 70 eV, *m/z* (rel. int.): 253 $[M]^{+\cdot}$(24), 238 (15), 144 (17), 130 (37), 128 (7), 109 $[RCO]^+$ (100), 86 (50), 81 (24).

3.8.4. Synthesis of (2S,3S)-3-methyl-2-[(2′E,4′E)-2′-methylhexa-2′,4′-dienoylamino]- pentanoic acid ethyl ester (*8*)

The procedure was similar as above. *N*-Boc-L-Ile methyl ester (400 mg) was employed instead of the D-*allo*-Ile derivative. After purification, compound **8** (200 mg) was obtained. *Compound* **8**: Oil. $[\alpha]_D = +9$ ($CHCl_3$, c2.72). UV (CH_2Cl_2)λ_{max} (nm) (log ε): 244 (4.4). FTIR (KBr) ν_{max} (cm^{-1}): 3320 (NH), 2970 (CH), 2929 (CH), 2860 (CH), 1750 (CO), 1631, 1372, 697. ^{1}H NMR ($CDCl_3$): δ 6.88 (*br d*, $J = 11.1$ Hz, H-3′), 6.33 (*ddq*, $J = 14.9$, 11.1 and 1.6 Hz, H-4′), 6.24 (*br d*, $J = 8.4$ Hz, N*H*), 6.04 (*dq*, $J = 14.9$ and 7.0 Hz, H-5′), 4.68 (*dd*, $J = 8.4$ and 5.0 Hz, H-2), 3.75 (*s*, CO_2CH_3), 1.96 (*br s*, H-7′), 1.94 (*m*, H-3), 1.86 (*br d*, $J = 7.0$ Hz, H-6′), 1.48 and 1.21 (*m*, H-4), 0.94 (*t*, $J = 7.4$ Hz, H-5), 0.92 (*d*, $J = 7.0$ Hz, H-6). ^{13}C NMR ($CDCl_3$): δ 172.8 (*s*, C-1), 168.9 (*s*, C-1′), 136.4 (*d*, C-5′), 134.3 (*d*, C-3′), 127.3 (*s*, C-2′), 127.0 (*d*, C-4′), 56.5 (*d*, C-2), 52.0 (*s*, CO_2CH_3), 38.2 (*d*, C-3), 25.4 (*t*, C-4), 18.8 (*q*, C-6′), 15.4 (*q*, C-6), 12.8 (*q*, C-7′), 11.6 (*q*, C-5). HREIMS *m/z*: $(M)^{+\cdot}$, found 253.16899, calc. for $C_{14}H_{23}NO_3$ 253.16779 ($\Delta m = 4.7$ ppm), $(M+1)^{+\cdot}$ found 254.17115, calc. for $^{13}CC_{13}H_{23}NO_3$ 254.17118 ($\Delta m = 1.1$ ppm). EIMS 70 eV, *m/z* (rel. int.): 253 $[M]^{+\cdot}$(9), 238 (4), 224 (4), 144 (14), 128 (12), 109 $[RCO]^+$ (100), 81 (25).

*3.8.5. Synthesis of compound **1***

$LiBH_4$ (14 mg) and MeOH (0.025 ml) were added to a solution of compound **7** (70 mg) in Et_2O (2 ml). The mixture was heated until reflux began, this being maintained for 15 min, with the resulting reaction mixture poured onto 1 N HCl in an ice-bath. The reaction mixture was next extracted with CH_2Cl_2 (3×25 ml). The crude evaporated product was subjected to dry column flash chromatography on silica gel, eluting pure compound **1** (30 mg) with CH_2Cl_2:EtOAc 70:30.

Compound **1**. Oil. $[\alpha]_D = -45$ ($CHCl_3$, c0.1). UV (CH_2Cl_2)λ_{max} (nm) (log ε): 236 (3.0). FTIR (KBr) ν_{max} (cm^{-1}): 3321 (NH), 2932, 2846, 1742, 1489, 1380, 1193. ^{1}H NMR ($CDCl_3$): δ 6.90 (*br d*, $J = 10.8$ Hz, H-3), 6.33 (*ddq*, $J = 14.8$, 10.8 and 1.6 Hz, H-4), 6.04 (*dq*, $J = 14.8$ and 6.9 Hz, H-5), 5.90 (*br d*, $J = 8.0$ Hz, N*H*), 4.00 (*m*, H-2′), 3.69 (*m*, H-1′), 1.96 (*br s*, H-7), 1.86 (*br d*, $J = 6.9$ Hz, H-6), 1.70 (*m*, H-3′), 1.44 and 1.25 (*m*, H-4′), 0.93 (*t*, $J = 7.3$ Hz, H-5′), 0.93 (*d*, $J = 6.9$ Hz, H-6′). ^{13}C NMR ($CDCl_3$): δ 170.3 (*s*, C-1), 136.3 (*d*, C-5), 134.2 (*d*, C-3), 127.3 (*s*, C-2), 126.9 (*d*, C-4), 64.3 (*t*, C-1′), 55.3 (*d*, C-2′), 35.5 (*d*, C-3′), 26.3 (*t*, C-4′), 18.7 (*q*, C-6), 14.8 (*q*, C-6′), 12.8 (*q*, C-7), 11.3 (*q*, C-5′). HREIMS *m*/*z*: $(M)^{+\cdot}$, found 225.17263, calc. for $C_{14}H_{23}NO_3$ 225.17288 ($\Delta m = 1.1$ ppm). EIMS 70 eV, *m*/*z* (rel. int.): 225 $[M]^{+\cdot}$(8), 194 (22), 109 $[RCO]^+$ (100), 86 (62).

*3.8.6. Synthesis of compound **2***

The procedure was similar as above. Compound **8** (150 mg) was employed instead of **7**. After purification, pure compound **2** (80 mg) was obtained. *Compound* **2**: Oil. $[\alpha]_D = +72$ (CH_2Cl_2, c0.37). UV (CH_2Cl_2)λ_{max} (nm) (log ε): 240 (4.0). FTIR (KBr) ν_{max} (cm^{-1}): 3390 (NH), 2968, 2939, 1662, 1539, 1467, 1388, 1085. ^{1}H NMR ($CDCl_3$): δ 6.88 (*br d*, $J = 10.8$ Hz, H-3), 6.33 (*ddq*, $J = 14.8$, 10.8 and 1.5 Hz, H-4), 6.03 (*dq*, $J = 14.8$ and 6.9 Hz, H-5), 6.00 (overlapped, N*H*), 3.88 (*m*, H-2′), 3.73 (*m*, H-1′), 1.95 (*br s*, H-7), 1.86 (*br d*, $J = 6.9$ Hz, H-6), 1.70 (*m*, H-3′), 1.50 and 1.22 (*m*, H-4′), 0.95 (*d*, $J = 6.8$ Hz, H-6′), 0.91 (*t*, $J = 7.0$ Hz, H-5′). ^{13}C NMR ($CDCl_3$): δ 170.0 (*s*, C-1), 136.4 (*d*, C-5), 134.2 (*d*, C-3), 127.2 (*s*, C-2), 127.0 (*d*, C-4), 63.7 (*t*, C-1′), 56.2 (*d*, C-2′), 35.7 (*d*, C-3′), 25.4 (*t*, C-4′), 18.7 (*q*, C-6), 15.5 (*q*, C-6′), 12.7 (*q*, C-7), 11.6 (*q*, C-5′). HREIMS *m*/*z*: $(M)^{+\cdot}$, found 225.17197, calc. for $C_{14}H_{23}NO_3$ 225.17288 ($\Delta m = 4.0$ ppm). EIMS *m*/*z* (rel. int.): 225 $[M]^{+\cdot}$ (5), 210 (1), 195 (3), 194 (12), 126 (11), 109 $[RCO]^+$ (100), 86 (20), 81 (28).

*3.8.7. Synthesis of N-Boc-L-isoleucinaldehyde (**10**)*

N-Boc-L- isoleucinol **9** (172 mg), prepared from *N*-Boc-L-Ile methyl ester in the same way as above, was oxidized with pyridinium chlorochromate (PCC) according to Corey and Suggs (1975), to yield compound **10** (152 mg). An aliquot of the product (50 mg) was purified by prep. TLC (CH_2Cl_2–EtOAc 95:5, $R_F = 0.6$). *Compound* **10**: Oil. $[\alpha]_D = +71$ (CH_2Cl_2, c0.12). UV (CH_2Cl_2)λ_{max} (nm) (log ε): 234 (3.5). ^{1}H NMR ($CDCl_3$): δ: 9.66 (*s*, H-1), 5.13 (*br d*, $J = 8.0$ Hz, N*H*), 4.29 (*m*, H-2), 1.70 (*m*, H-3), 1.47 (overlapped, H-4), 1.45 (*s*, C(C*H*$_3$)$_3$), 1.27 (*m*, H-4), 0.99 (*d*, $J = 6.9$ Hz, H-6), 0.96 (*t*, $J = 7.3$ Hz, H-5). ^{13}C NMR ($CDCl_3$): δ 200.6 (*d*, C-1), 156.9 (*s*, N*C*OO), 79.6 (*s*, *C*(CH$_3$)$_3$) 64.2 (*d*, C-2), 36.4 (*d*, C-3), 28.3 (*s*, C(*C*H$_3$)$_3$), 25.3 (*t*, C-4), 15.6 (*q*, C-6), 11.9 (*q*, C-5). HREIMS *m*/*z*: $(M)^{+\cdot}$, found 215.15258, calc. for $C_{14}H_{23}NO_3$ 215.15214 ($\Delta m = 2$ ppm). EIMS *m*/*z* (rel. int.): 215 $[M]^{+\cdot}$ (50), 186 (27), 130 (88), 86 (79), 57 (100).

3.8.8. Antibiotic activity

The antibiotic activity was determined by the agar diffusion method using 50 μg of sample/6 mm disk against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Gentamicin, which was used as the positive test compound, showed inhibition halos of 25–30 mm at a conc. level of 25 μg/disk.

3.8.9. Antifungal activity

Direct bioautography on TLC was employed as the method for detecting fungitoxic substances (Homans and Fuchs, 1970). A concentration level of 50 μg/spot of each assayed compound was used. Benomyl, which was used as a control, showed inhibition zones of 20–28 mm at a conc. level of 25 μg/spot. When appropriate, as large inhibitory halos were observed, minimum inhibitory concentration (MIC) was measured by the same method.

Acknowledgements

We thank UMYMFOR (CONICET-FCEN, UBA) for the NMR and LR-MS spectra and Universidad de Buenos Aires, ANPCYT and CONICET for partial financial support.

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