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ORIGINAL ARTICLE

Isolation of the antibiotic methyl (*R*,*E*)-3-(1-hydroxy-4-oxocyclopent-2-en-1-yl)-acrylate EA-2801 from *Trichoderma atroviridae*

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The endophytic *Trichoderma atroviridae* UB-LMA was isolated as a symbiont of *Taxus baccata* and analyzed for its antimicrobial activity. By applying an original approach consisting of solid-state cultivation coupled with solid-phase extraction, a new methyl (*R*,*E*)-3-(1-hydroxy-4-oxocyclopent-2-en-1-yl)-acrylate derivative named EA-2801 (1) was isolated together with the previously reported isonitrin A and dermadin methyl ester. The chemical structure of 1 was determined by NMR and MS. Compound 1 showed antimicrobial activity against a panel of Gram-positive and -negative bacteria.
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INTRODUCTION

Fungi of the genus *Trichoderma* have been studied extensively as a potential source of bioactive secondary metabolites. Over the years, more than 120 *Trichoderma* secondary metabolites have been reported.[1] *Trichoderma* spp. displayed antibiotic activity against bacteria, yeasts and fungi, involving a large panel of chemical scaffolds such as peptaibols,[2] diketopiperazines,[3] polyketides,[4] pyrones[5] and terpenes.[6] The increasing resistance of pathogens to existing antibiotics has prompted intensive efforts towards new and more effective antibiotics.[7,8]

Various cyclopentenone derivatives were isolated from fungal strains of terrestrial and marine origin (Figure 1). These include terrein,[9] trichodenones A–C,[10] pentenomycins I–III,[11] isonitrin D,[12] homothallins I–II,[13] hygrophorones A–G[14] and myrothenones A–B,[15] bromomyrothenone B and botrytinone.[16,17]

In our previous work on *Trichoderma atroviridae UB-LMA* metabolites, a continuous *in situ* solid-phase extraction of the fermentation broth was used. In these conditions, four compounds belonging to the harziane tetracyclic diterpene family were isolated, among which three were new. In addition, a bicyclic potential precursor of harzianes, issued from geranyl-geranyl pyrophosphate, was isolated.[6] In this paper, an original cultivation/extraction approach developed in CNRS–ICSN (Centre National de la Recherche Scientifique–Institut de Chimie des Substances Naturelles) was applied to *T. atroviridae UB-LMA*.[18,19] It consists of agar-supported cultivation coupled with solid-phase extraction with Amberlite XAD-16 (AgSF/SPE).[20]

We have previously demonstrated that this type of cultivation offers a different metabolic pattern compared with classical submerged cultivation.[18]

A new compound methyl (*R*,*E*)-3-(1-hydroxy-4-oxocyclopent-2-en-1-yl)-acrylate derivative named EA-2801 (**1**) was isolated, together with the previously reported isonitrin A,[12] and dermadin methyl ester.[20] Antimicrobial and cytotoxic activities were investigated, showing the efficacy of **1** as a large-spectrum Gram-positive and -negative antibiotic.[21]

RESULTS AND DISCUSSION

The fungal strain *T. atroviridae* UB-LMA was isolated as a symbiotic endophyte from the bark of *Taxus baccata* and has been previously reported.[6] Preliminary investigation using agar diffusion bioassay was carried out on *Acinetobacter baumanii* ATCC 19606. *Acinetobacter* is a Gram-negative pathogen involved in a variety of infections such as pneumonia, meningitis and urinary tract infection.[22] The ethyl acetate extract of the resin from AgSF/SPE cultivation also confirmed the antibiotic activity on *Acinetobacter*. Bioguided fractionation on normal-phase chromatography led to the isolation of three compounds, among which **1** is a new natural compound (Figure 2).

The structures of isonitrin A and methyl-3-(3-isocyano-6-oxabicyclo[3.l.0]hex-2-en-5-y1)-2-propenoate were unambiguously established by comparing the spectroscopic data with the data reported in existing literature.[12,20] Fortunately, suitable crystals for X-ray diffraction were obtained and the structures confirmed (Figure 3).

Compound **1** was isolated as colorless oil. The high-resolution electrospray ionization MS (HR-ESI-MS) analysis gave the molecular formula $C_9H_9O_4$ on the basis of *m/z* 181.0495 $[M\text{-}H]^-$ (calcd 181.0501) that requires 4 degrees of unsaturation. The IR spectrum suggested the presence of a hydroxyl (3392 cm^{-1}) and a carbonyl group (1724 and 1658 cm^{-1}) and the optical rotation was measured as $[\alpha]_D^{20} = +40.9$ (c 0.2, CH_2Cl_2); The NMR data for compound **1** are reported in Table 1. The ^1H NMR spectrum along with heteronuclear single quantum correlation data displayed one singlet methoxy, one

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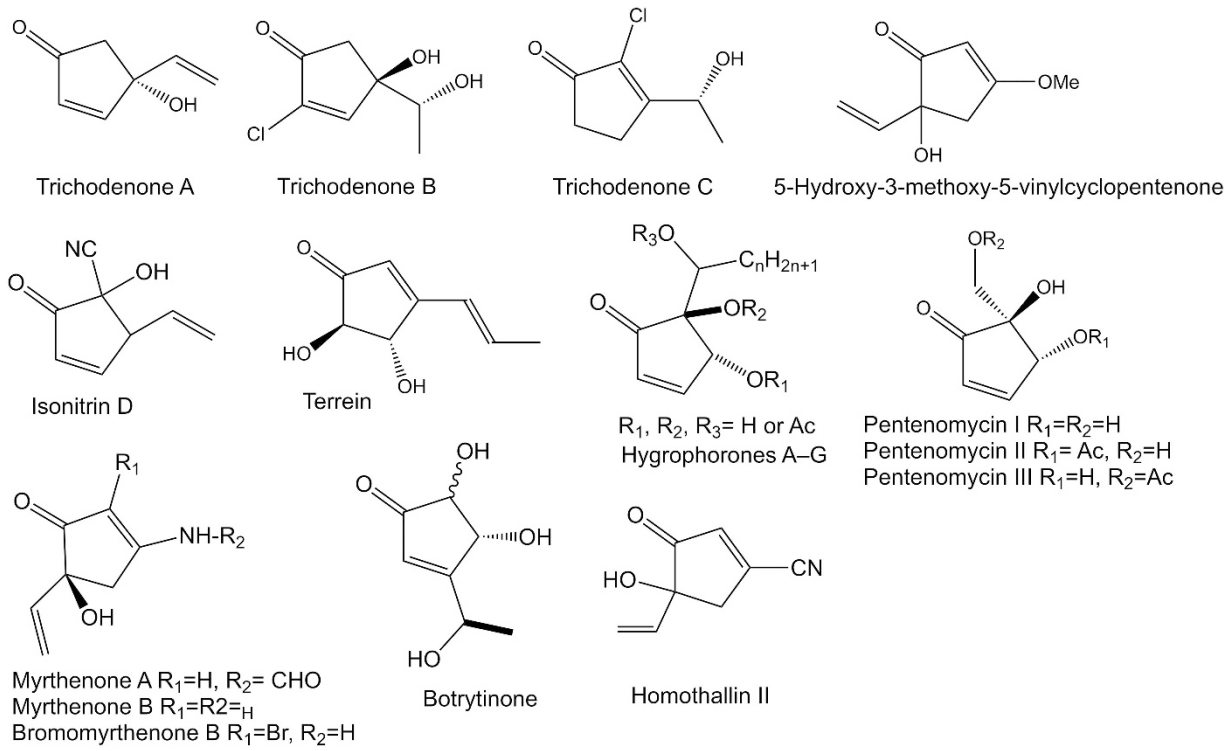

Figure 1 Fungal bioactive cyclopentenone derivatives from terrestrial and marine origin.

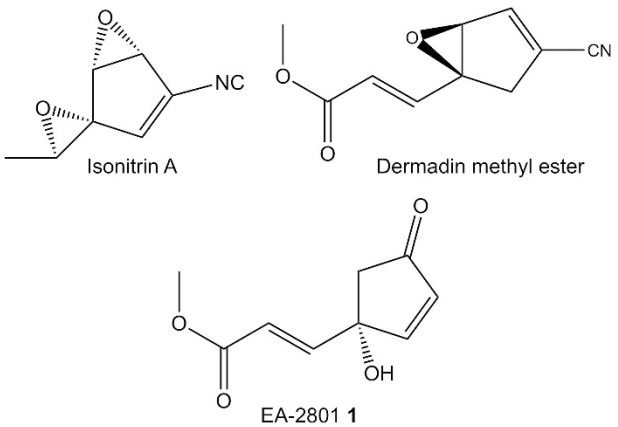

Figure 2 Compounds isolated from the AgSF/SPE (agar-supported cultivation coupled with solid-phase extraction) of *Trichoderma atroviridae* UB-LMA.

doublet methylene and four doublets double bond. The ^{13}C and DEPT-145 NMR spectra revealed the presence of 9 carbon atoms including one hydroxylated carbon C-4 at 77.7 p.p.m., and two carbonyl carbons C-8 and C-1 at 166.4 and 205.7 p.p.m., respectively. Furthermore, these spectra displayed four sp^2-hybridized C-atoms (C-2 at 133.9; C-3 at 163.4, C-6 at 120.2 and C-7 at 149.2 p.p.m.), one methylene group C-5 at 49.4 p.p.m. and one oxy-methyl group C-9 at 51.7 p.p.m. (Table 1).

^1H–^1H COSY and ^1H–^{13}C heteronuclear multiple bond correlation demonstrated the presence of a five-membered conjugated lactone, with a methyl acrylate side chain connected by a tertiary alcohol on C-4 (Figure 4).[23]

Compound **1** shared the same scaffold of trichodenone A,[10] except for the presence of the acetate on the side chain. The *(R)* absolute configuration of carbon 4 in trichodenone A was deduced from its total synthesis from (+)(4*R*)-4-*tert*-butyldimethylsilyloxy-2-cyclopenten-1-one with total retention of configuration.[20,24] We have synthesized compound **1** from the same TBDMS racemic precursor and purified the natural enantiomer by chiral supercritical fluid chromatography.[23] The engaged synthetic steps could not inverse the configuration in the 4 position, and according to the positive sign of the $[\alpha]_D$ of **1**, we can deduce that during the synthesis, compound **1** is derived from the (+)(4 R)TBDMS-cyclopentenone. We can then suggest that **1** corresponds to (+)methyl-(*R*,*E*)-3-(1-hydroxy-4-oxo-cyclopent-2-en-1-yl)-acrylate (Figure 5).

Compound **1** was tested at levels up to 1 mg ml^{-1} against KB cancer cell line along with one noncancer cell line MRC5, and did not exhibit cytotoxicity toward these two cell lines. Compound **1** exhibited a broad spectrum of antimicrobial activity against various Gram-positive and -negative pathogens with minimum inhibitory concentrations ranging from 32 to 128 μg ml^{-1} (Table 2).[21]

In conclusion, methyl (*R*,*E*)-3-(1-hydroxy-4-oxocyclopent-2-en-1-yl)-acrylate derivative (**1**) is a promising new antibiotic. The total synthesis of the racemic compound was achieved and enantiomers obtained by chiral supercritical fluid chromatography. Our ongoing efforts are dedicated to the chiral synthesis of the natural isomer as well as more powerful and easily bioavailable analogs.

METHODS

General experimental procedure

^1H and ^{13}C spectra were recorded using Brüker Avance-600 instruments operating at 600 MHz (Wissembourg, France). The Brüker Avance 600 MHz was equipped with a microprobe head (1.7 TXI). LC-ESI-MS analyses were performed on a simple-stage quadrapole Waters-Micromass (Guyancourt,

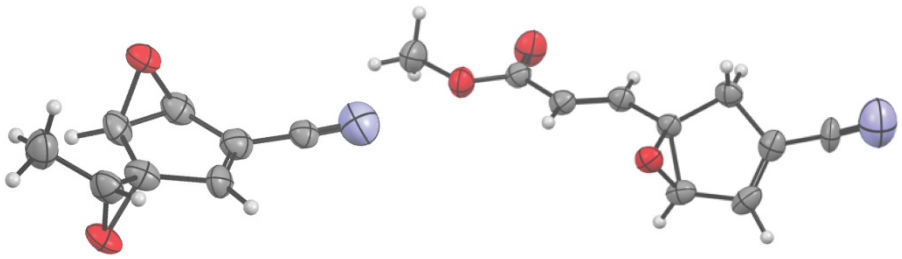

Figure 3 ORTEP (Oak Ridge Thermal-Ellipsoid Plot Program) diagram showing the crystal state conformation of isonitrin A (left) and dermadin methyl ester (right).

Table 1 ^{1}H and ^{13}C NMR data for compound 1 (recorded at 600/150 MHz in $CDCl_3$, δ in p.p.m., *J* in Hz)

No.	δ_C	*HSQC*	δ_H
1	205.7	C	—
2	133.6	CH	6.20 d (5.5)
3	163.4	CH	7.33 d (5.5)
4	77.7	C	—
5	49.4	CH2	2.63 d (12.0)
6	120.2	CH	6.17 d (16.0)
7	149.2	CH	6.91 d (16.0)
8	166.4	C	—
9	51.7	CH3	3.74 s

Abbreviation: HSQC, heteronuclear single quantum correlation.

France) ZQ 2000 mass spectrometer equipped with an ESI interface coupled to an Alliance Waters (Guyancourt, France) 2695 HPLC instrument with photodiode array and evaporative light scattering detection. The HR-ESI-MS spectra were recorded on a Waters-Micromass mass spectrometer equipped with ESI-time of flight. Optical rotations were measured at 25 °C on a JASCO (Lisses, France) P1010 polarimeter. IR spectra were obtained on a Perkin-Elmer (Villebon-sur-Yvette, France) Spectrum100 model instrument. HPLC consisted of a Waters device including an autosampler 717, a pump 600, a photodiode array 2998 and an evaporative light scattering detector, ELSD 2420. The HPLC analytical column used was a 3.5 µm, C-18 column (Sunfire 150 × 4.6 mm) operating at 0.7 ml min^{-1}. The preparative column was a 5 µm, C-18 (Sunfire 250 mm × 10 mm) operating at 4 ml min^{-1}. On both columns, the gradient consisted of a linear gradient for 50 min from H_2O to acetonitrile, both containing 0.1% formic acid. Silica gel 60 (6–35 and 35–70 ml) and analytical TLC plates (Si gel 60 F 254) were purchased from SDS (St Quentin Fallavier, France). Prepacked silica gel Redisep columns were used for flash chromatography using a Combiflash-Companion apparatus (Serlabo, Avignon, France). All other chemicals and solvents were purchased from SDS.

Microorganisms

The fungal strain was isolated from the bark of the yew *T. baccata*. Fungal strain *T. artroviride* CMU-LMA was identified through molecular techniques and DNA sequencing of small subunit and internal transcribed spacer ribosomal DNA. The strain was deposited with number BankIt1582103, 10260002.seq, KC171717 at Genebank.

The cultivation medium consisted of potato dextrose broth, Difco (potato starch 4 g l^{-1}, dextrose 20 g l^{-1}) supplemented with agar at a final concentration of 20 g l^{-1}. The spores-mycelium mixture was recovered from Petri plate pre-cultures in 20 ml of sterile potato dextrose broth, to which 35 g of XAD-16 (Rohm, Saint-Maximin, France) resin was added. This mixture was used to inoculate two 25 × 25 cm Petri plates that were then left to grow at 27 °C for 9 days in a microbial incubator.

Extraction and isolation procedures

Biomass and resin were scratched from the surface of the agar and extracted with 3 × 500 ml of ethyl acetate to afford 560 mg of evaporated extract. The extract was subjected to flash chromatography using a gradient of ethyl acetate/heptane (0:100–80:20) at 40 ml min^{-1} during 110 min to afford 5 fractions, according to their TLC profiles. Fraction 3 constituents (24 mg) were separated by preparative HPLC on a C-18 column (Sunfire, 250 mm × 10 mm) using a 10 min linear gradient from H_2O to acetonitrile containing both 0.1% formic acid. We obtained 6 mg of compound 1, 7 mg of isonitrin A and 6 mg of methyl-3-(3-isocyano-6-oxabicyclo[3.l.0]hex-2-en-5-y1)-2-propenoate.

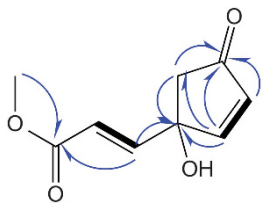

Figure 4 Key ^{1}H–^{1}H COSY (bold bond) and ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC; arrows) of compound **1**.

Compound 1. Colorless oil; $[\alpha]_D^{20}$+40.9 (c 0.2, CH_2Cl_2); IR ν_{max} 3392, 2950, 1724, 1658, cm^{-1}; HR-ESI-MS *m/z* 181.0495 $[M\text{-}H]^-$ (calculated from $C_9H_9O_4$ 181.0501).

Antibacterial assays and MIC

Antibacterial activity was measured using the disk diffusion zone method against *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031, *Enterobacter cloacae* ATCC 13047, *A. baumanii* ATCC 19606, *Staphylococcus aureus* ATCC 25923 and *Streptococcus pneumonia* ATCC 49619. Inhibition was compared with 10 µg gentamicin, 10 µg penicillin and 30 µg chloramphenicol.

The MICs were determined by serial microdilution essay according to literature.[25,26] Stock solution of EA-2801 was prepared in DMSO at 100 mg ml^{-1} final concentration. Nine concentrations were tested (1024, 512, 256, 128, 64, 32, 16, 8 and 4 µg ml^{-1}). Tests were performed in triplicate in a 96-well sterile microplates. The plates were inoculated with 5×10^5 CFUs per ml in Mueller-Hinton broth, sealed and incubated at 37 °C for 24 h. The growth was evaluated by absorbance at 660 nm on a microplate reader (Berthold Multimode Reader (Thoiry, France) (Tristar^2 SLB942). Each well contains 100 µl of Mueller-Hinton broth, 40 µl of target strain inoculum and 10 µl of EA-2801 in DMSO. Negative control (without inoculum) and positive control (without EA-2801) were incubated in the same condition as the essays. The MIC was defined as the minimum concentration of EA-2801 at which no growth was observed.

Cytotoxicity assays

A tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide)-based colorimetric assay was used to measure inhibition of the proliferation of the carcinoma cells KB, as previously reported.[27] All the tested compounds were formulated in DMSO and applied to cells in final DMSO concentration ranges from 1 to 3%.

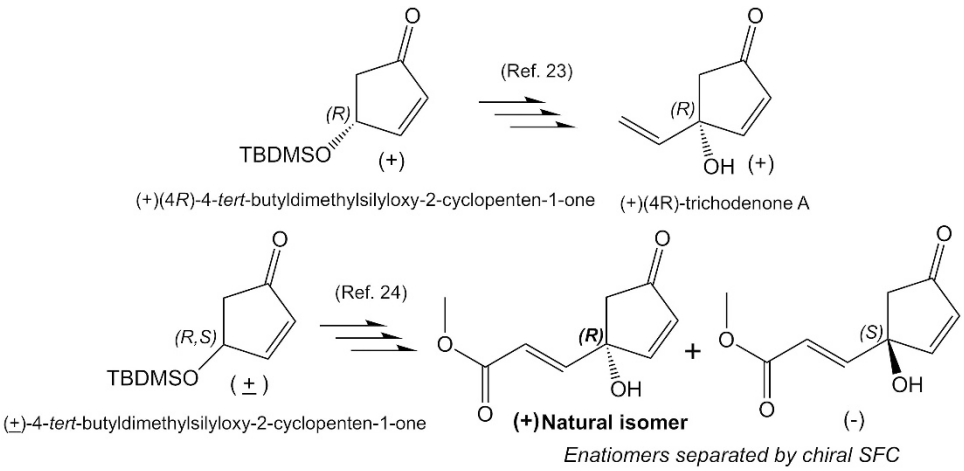

Figure 5 Arguments towards the absolute configuration of compound **1**.

Table 2 The MIC of EA-2801 1 against various pathogens

Target bacteria	*MIC* ($\mu g\ ml^{-1}$)
Escherichia coli ATCC 25922	64
Klebsiella pneumoniae ATCC 10031	32
Enterobacter cloacae ATCC 13047	128
Acinetobacter baumanii ATCC 19606	32
Staphylococcus aureus ATCC 25923	64
Streptococcus pneumonia ATCC 49619	128

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)