Bioorganic & Medicinal Chemistry Letters 24 (2014) 83–86

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Verrulactone C with an unprecedented dispiro skeleton, a new inhibitor of *Staphylococcus aureus* enoyl-ACP reductase, from *Penicillium verruculosum* F375

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ARTICLE INFO

Article history:
Received 8 September 2013
Revised 11 November 2013
Accepted 28 November 2013
Available online 4 December 2013

Keywords:
FabI
Antibacterial
S. aureus
Dispiro

ABSTRACT

An highly quaternary and unprecedented dispiro compound, verrulactone C, with the known compound, altenuisol, were isolated from a culture broth of the fungal strain *Penicillium verruculosum* F375 and their structures were established by various spectral analysis. Verrulactone C and altenuisol showed FabI-selective inhibition. Especially altenuisol had the high correlation between FabI-inhibition and whole cell antibacterial activity against *Staphylococcus aureus* and MRSA with MICs of 8–32 μg/mL.

The emergence of multidrug-resistant pathogens is a serious health problem worldwide.[1] One of strategies to overcome multidrug-resistant pathogens is to use new drugs that function through novel mechanisms of action. In this regard, bacterial enoyl-ACP reductase that catalyzes the final and rate-limiting step in the bacterial fatty acid synthesis (FAS) is an attractive antibacterial target since FAS is organized differently in bacteria and mammals, which potentially makes it possible to identify a selective antibacterial agent without mechanism-based toxicity.[2] Among four isoforms, FabI, FabK, FabL, and FabV in bacterial enoyl-ACP reductase, FabI is well conserved among the important pathogenic bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).[3] So far, several inhibitors of FabI that showed antibacterial activity against multidrug-resistant pathogens have been reported,[4–6] but new inhibitors with new skeleton are needed.

In the course of screening for FabI inhibitors, we previously discovered verrulactones A (**3**) and B (**4**) from the fermentation broth of *Penicillium verruculosum* F375.[7] Compounds **3** and **4** were dimeric compounds of alternariol class. Further investigation of fractions with weaker FabI-inhibitory activity from this fermentation led to discovery of an highly quaternary and unique dispiro metabolite named verrulactone C (**1**),[8] together with the known compound, altenuisol (**2**).[9] Originally the structure of natural altenuisol had been proposed to be 3-Me ether form,[10] but recently was revised to be 9-Me ether form by comparison of its ^{1}H NMR data with that of synthetic altenuisol.[11] The ^{13}C NMR assignment of altenuisol has not been reported yet. In this paper, we present the isolation, structure determination, and FabI-inhibitory and antibacterial activity of **1** and **2**.

Fermentation was carried out in 1-L Erlenmeyer flasks containing glucose 2%, polypeptone 0.5%, yeast extract 0.2%, KH_2PO_4 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.05% (adjusted to pH 5.7 before sterilization). A piece of agar from the mature plate culture of the producing strain was inoculated into a 500 mL Erlenmeyer flask containing 80 mL of sterile seed liquid medium with the above composition and cultured on a rotary shaker (150 rpm) at 28 °C for 3 days. For the production of verrulactones, 5 mL of the seed culture was transferred into one-liter Erlenmeyer flasks containing 100 ml of the above medium, and cultivated for 14 days using the same conditions. The culture broth (12 L) was added to the same volume of acetone and the cell debris was then removed by filtration. After the filtrate was evaporated to remove acetone, the resultant water phase was partitioned with an equal volume of EtOAc three times and the EtOAc layer was concentrated in vacuo. The resultant residue (2.8 g) was subjected to Sephadex LH-20 (Amersham Biosciences) column chromatography followed by elution with MeOH to give two weak-FabI inhibitory fractions followed by **3** and **4**-containing fractions. The first active fractions were pooled and concentrated in vacuo to give a yellow residue. The residue was applied to the second Sephadex LH-20 column chromatography and

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0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.11.071

eluted with MeOH. The active fractions were pooled and concentrated in vacuo. The residue dissolved in MeOH was purified by thin layer chromatography (TLC) on RP-18 F_{254} plates (Merck No 1.15389.0001: Darmstadt, Germany) developed with ACN–water (55:45) containing 0.1% TFA. The active band was further purified by HPLC column (10 × 250 mm, YMC C18) chromatography. The column was eluted with ACN–water (55:45) containing 0.1% TFA at a flow rate of 1.6 ml/min to afford **1** (17.2 mg) with a retention time of 32.6 min as a yellow powder. The second active fractions were pooled and concentrated in vacuo to give a yellow residue. The residue was applied to the second Sephadex LH-20 column chromatography and eluted with MeOH. The active fractions were pooled and concentrated in vacuo. The residue dissolved in MeOH was purified by RP-18 TLC developed with ACN–water (55:45) containing 0.1% TFA. The active band dissolved in MeOH was further purified RP-18 TLC with MeOH–water (80:20) containing 0.1% TFA to give **2** (5.1 mg) as a yellow powder.

The molecular formula of **1** was determined to be $C_{29}H_{18}O_{12}$ on the basis of high resolution ESI-MS [(M−H)−, 557.0734 *m/z* (+1.40 mmu error)] in combination with ^1H and ^{13}C NMR data. The IR absorption at 1624 and 3440 cm^{-1}suggested the presence of carbonyl and hydroxyl moieties, respectively. The ^1H NMR spectrum (Table 1) of **1** with COSY spectrum exhibited resonances for two singlet aromatic protons, two *meta*-coupled aromatic protons, $-(CH_3)C{=}CH-$, two methoxyls, and four exchangeable protons. Compared to ^1H NMR spectrum, the ^{13}C NMR spectrum (Table 1) was so complicated; one methyl, two methoxyls, five olefinic methines, two sp^3 quaternary carbons, fifteen sp^2 quaternary carbons, two ester carbonyls, and two ketone carbonyls.

The presence of 1,2,3,5-tetrasubstituted benzene (ring A) in **1** was determined by the HMBC correlations (Fig. 2) of one *meta*-coupled proton (H-4″) at δ 6.24 with three sp^2 quaternary carbons at δ 104.1 (C-2″), 157.4 (C-3″), and 165.4 (C-5″), and an aromatic methine at δ 99.9 (C-6″); of the other *meta*-coupled proton (H-6″) at δ 6.10 with three sp^2 quaternary carbons at δ 148.1(C-1″), 104.1 (C-2″), and 165.4 (C-5″), and an aromatic methine at δ 103.1 (C-4″); and of one exchangeable proton (3″-OH) at δ 10.86 with C-2″, C-3″, and C-4″. In addition, the HMBC correlation of the methoxyl protons (5″-OCH_3) at δ 3.77 with C-5" together with the ROEs from 5″-OCH_3 to H-4″ and H-6″, and the HMBC correlation of H-4″ with a carbonyl carbon (C-7″) at δ_C 164.5 together with the low-field chemical shift of 3″-OH at δ_H 10.86 indicated the attachment of the methoxyl (5″-OCH_3) and the carbonyl (C-7″) at C-5″ and C-2″, respectively. The presence of a 3-methylcyclopentenone (ring B) was determined by the HMBC correlations of the allylic methyl protons at δ 1.81 (H_3-7′) of $-(CH_3)C{=}CH-$ with one sp^3 quaternary carbon at δ 91.2 (C-2′), an sp^2 quaternary carbon at δ 167.9 (C-3′), the olefinic methine carbon at δ 134.9 (C-4′), and one ketone carbonyl carbon at δ 200.1 (C-5′), and of the olefinic proton at δ 6.82 (H-4′) of $-(CH_3)C{=}CH-$ with the other sp^3 quaternary carbon at δ 68.1 (C-1′), C-2′, C-3′, C-5′, and C-7′.

Table 1
^1H and ^{13}C NMR data[a] of verrulactone C (**1**) and altenusiol (**2**)

	1		**2**	
Positon	δ_H, multi. (*J* in Hz)	δ_C, multi.	δ_H, multi. (*J* in Hz)	δ_C, multi.
1		109.6, C	7.51, s	108.7, CH
2		147.4, C		143.6, C
3		148.9, C		143.8, C
4	6.99, s	110.2, CH	6.79, s	103.2, CH
4a		141.4, C		148.8, C
6		163.2, C		164.6, C
6a		97.1, C		98.5, C
7		163.5, C		163.3, C
8	6.79, s	99.3, CH	6.54, d (2.4)	99.8, CH
9		165.1, C		166.4, C
10		107.5, C	7.02, d (2.4)	9.8, CH
10a		130.4, C		13.2, C
10b		106.6, C		108.6, C
1′		68.1, C		
2′		91.2, C		
3′		167.9, C		
4′	6.82, d (1.5)	134.9, CH		
5′		200.1, C		
6′		198.9, C		
7′	1.81, d (1.5)	13.0, CH_3		
1″		148.1, C		
2″		104.1, C		
3″		157.4, C		
4″	6.24, d (1.8)	103.4, CH		
5″		165.4, C		
6″	6.10, d (1.8)	99.9, CH		
7″		164.5, C		
9-OMe	3.81, s	56.3, CH_3	3.92, s	55.9, CH_3
5″-OMe	3.77, s	55.6, CH_3		
2-OH	11.09, s			
3-OH	10.76, s			
7-OH	11.21, s		11.5, s	
3″-OH	10.86, s			

[a] Recorded in DMSO-d_6.

The ring C was also determined by the HMBC correlations (Fig. 2) from one singlet aromatic proton at δ 6.79 (H-8) to four sp^2 quaternary carbon at δ 97.1 (C-6a), 163.5 (C-7), 165.1 (C-9), and 107.5 (C-10); from the low-field shifted proton at δ 11.21 (7-OH) to C-6a, C-7, and C-8; and from the methoxyl protons at δ 3.81 (9-OCH_3) to C-9. The ring E was assigned by the HMBC correlations of the other singlet aromatic proton at δ 6.99 (H-4) with four sp^2 quaternary carbons at δ 147.4 (C-2), 148.9 (C-3), 141.4 (C-4a), 106.6 (C-10b); of one hydroxyl proton at δ 11.09 (2-OH) with three sp^2 quaternary carbons at δ 109.6 (C-1), C-2, and C-3; and of the other hydroxyl proton at δ 10.76 (3-OH) with C-2, C-3, and C-4. The linkage of C-10a with C-10b was confirmed by the HMBC correlations from H-4 to C-10a. These spectral data indicated the presence of the alternariol moiety composed of rings C–E. The connectivity of rings A–C was also determined by the HMBC and NOE spectrum. The long-range correlation of H-6″ with C-2′ enabled the establishment of the linkage of C-1″ with C-2′. The linkage of C-10 with C-1′ was confirmed by the HMBC correlation of H-8 with C-1′. The long-range corrleations from 2-OH to the remaining ketone carbonyl carbon at δ 198.9 (C-6′) in HMBC optimized for 3 Hz measured at 800 MHz in $CDCl_3$ indicated the attachment of C-6′ to C-1. Considering the molecular formula and the chemical shifts for C-1′ (δ_C 68.1) and C-2′ (δ_C 91.2), C-6′ and the C-7″ bonded oxygen atom should be attached to the sp^2 quaternary carbons C-1′ and C-2′ of the cyclopentenone ring, respectively, to form the dispiro skeleton to satisfy the unsaturation requirement of **1**. The planar structure of **1** was established as shown in Fig. 1.

The relative configuration of **1** was determined by the ROESY and 1D NOE spectrum (Fig. 2). In the ROESY spectrum, an ROE between 7-OH and H-4″ was observed, suggesting that the rings A and C were on the same side. In order to confirm the stereochemistry, the 1D NOE difference experiment irradiated at δ 6.10 (H-6″) was performed at 900 MHz. As a result, H-6″ showed NOEs with H-8 as well as H-4′ with the same intensity even though weak. These spectral data clearly indicated that H-6″ and H-8 were on the same side. Thus, the relative configuration of **1** were assigned as 1′*R** and 2′*S**.

The molecular formula of **2** was determined to be $C_{14}H_{10}O_6$ on the basis of high resolution ESI-MS [(M−H)−, 273.0398 *m/z* (−0.1 mmu error)] in combination with ^1H and ^{13}C NMR data (Table 1). The IR and UV spectra were similar to those of **1**. The ^1H NMR data of **2** with HMQC indicated the presence of two isolated aromatic methines (δ_H 6.79, s; δ_C 103.2 and δ_H 7.51, s; δ_C 108.7), two *meta*-coupled aromatic methines (δ_H 6.54, d, J = 2.4; δ_C 99.8 and δ_H 7.02, d, J = 2.4; δ_C 97.8), one methoxyl (δ_H 3.92, s; δ_C 55.9), and one intramolecularly hydrogen bonded phenolic proton (δ_H 11.5, s). The spectral data suggested that **2** was an