

Antibacterial Activity of Two Canthin-6-one Alkaloids from *Allium neapolitanum*

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The emergence of multidrug-resistant strains of many human pathogens has led to an urgent need for the discovery and development of new antimicrobial agents. As part of an ongoing investigation into the antibacterial properties of the Alliaceae, the isolation of 1 (canthin-6-one), 2 (8-hydroxy-canthin-6-one) and 3 (5(ζ)-hydroxy-octadeca-6(E)-8(Z)-dienic acid) from *A. neapolitanum*, a perennial bulbous herb found in open pastures of the Mediterranean is reported. Compounds 1 and 2 were isolated by Sephadex LH-20 from fractions exhibiting a positive reaction with Dragendorff's reagent on TLC, compound 3 was isolated after HPLC purification of Sephadex fractions. Structures were elucidated by extensive 1D and 2D NMR experiments and are in accordance with published data, however, the ¹³C NMR data for compound 2 and the ¹H and ¹³C NMR data for compound 3 are reported here for the first time. Canthin-6-one alkaloids are well-known constituents of the Simaroubaceae and Rutaceae, and display a wide range of biological activities. These metabolites are reported as constituents of the Alliaceae here for the first time, and displayed minimum inhibitory concentrations (MICs) in the range 8–32 µg/mL against a panel of fast-growing *Mycobacterium* species and 8–64 µg/mL against multidrug-resistant (MDR) and methicillin-resistant (MRSA) strains of *Staphylococcus aureus*. Compound 3 displayed antimycobacterial activity in the range of 16–32 µg/mL. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: *Allium neapolitanum*; antibacterial; canthin-6-one; *Mycobacterium*; *Staphylococcus aureus*.

INTRODUCTION

The genus *Allium* is represented by 550 species distributed in the temperate, tropical or often semi-arid regions of the world. Many species are cultivated for culinary, medicinal and ornamental use. *Allium sativum* (garlic) has been widely studied for its pharmaceutical properties with allicin being identified as the major antibacterial component in the 1940s (Cavallito *et al.*, 1944a; 1944b; 1945). Garlic is popularly known as 'Russian penicillin' and was of clinical use in the treatment of tuberculosis (TB) patients in the early 20th century (Bolton *et al.*, 1982). The World Health Organization (WHO) has estimated that 8 million people each year become infected with TB and approximately 2 million die (WHO, 2000). The disease was declared a global emergency in 1993 with HIV/AIDS induced immunosuppression being a major factor in the acceleration of mycobacterial infection. There has also been a rise in infection caused by non-tuberculous mycobacteria (Falkinham, 1996) and the recovery of fast-growing strains from patients with cystic fibrosis is becoming more common (Bange *et al.*, 1999). In recent decades,

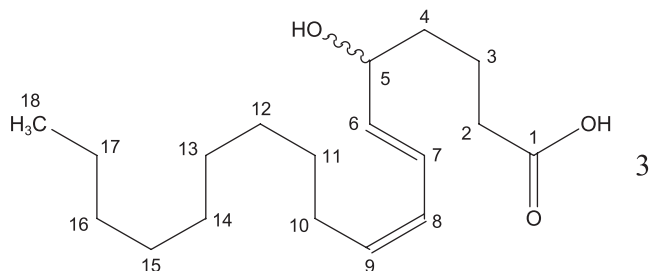
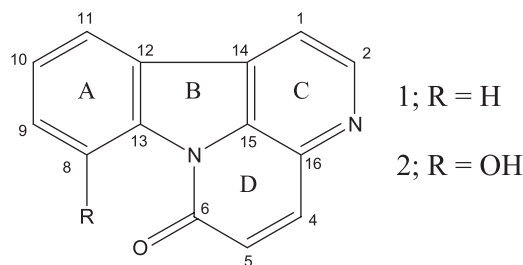
single-drug and multi-drug resistant strains of non-tuberculous mycobacteria have been documented resulting in an urgent need for new antimycobacterial agents with novel modes of action. As part of a project to identify antimycobacterial natural products from the subterranean parts of the Liliales, the bioassay-guided fractionation of the chloroform extract of *A. neapolitanum* against fast-growing strains of *Mycobacterium* is reported. The isolation of a number of flavonoids and flavonoid glycosides has been reported from this perennial bulbous herb (Carotenuto *et al.*, 1997).

MATERIALS AND METHODS

Instruments and reagents. NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer. Chemical shift values (δ) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants (J values) are given in Hertz. Mass spectra were recorded on a Finnigan MAT 95 high resolution, double focusing, magnetic sector mass spectrometer. Accurate mass measurement was achieved using voltage scanning of the accelerating voltage. This was nominally 5 kV and an internal reference of heptacosane was used. Resolution was set between 5000 and 10 000. IR spectra were recorded on a Nicolet 360 FT-IR spectrophotometer and UV spectra on a Thermo Electron Corporation Helios spectrophotometer.

Plant material. The fresh bulbs of *A. neapolitanum* were obtained from Gee Tee Garden Products,

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Lincolnshire, UK, and a herbarium specimen (GO'D/013B) was deposited at the Centre for Pharmacognosy and Phytotherapy.

Extraction and isolation. 6.3 kg of macerated bulb material underwent cold extraction with hexane, chloroform and methanol (3 L). The chloroform extract (3.4 g) was subjected to vacuum liquid chromatography (VLC) on silica gel eluting with hexane and 10% increments of ethyl acetate to yield 12 fractions. The fractions eluted with 30% and 20% hexane exhibited MIC values of 32 µg/mL against *Mycobacterium fortuitum* and, combined, underwent a further fractionation by Sephadex (LH-20) eluting fractions with 95% methanol to yield compounds **1** (13.6 mg) and **2** (4.4 mg). Sephadex fraction 2 underwent a final purification with reversed-phase preparative HPLC (5–95% acetonitrile, 50 mL/min) to yield compound **3** (16.3 mg).

Antibacterial assay. *Mycobacterium fortuitum*, *M. smegmatis*, *M. abscessus* and *M. phlei* were acquired from the NTCC. *M. smegmatis* mc²2700, which expresses the *M. tuberculosis* FAS1 gene, was provided by O. Zimhony (Zimhony *et al.*, 2004). Strains were grown on Columbia blood agar (Oxoid) supplemented with 7% defibrinated horse blood (Oxoid) and incubated for 72 h (120 h for *M. abscessus*) at 37 °C prior to minimum inhibitory concentration (MIC) determination. *S. aureus* strains were cultured on nutrient agar and incubated for 24 h at 37 °C. Strain XU212, which possesses the TetK tetracycline efflux protein, was a generous gift from E. Udo (Gibbons and Udo, 2000). SA-1199B, which overexpresses the *norA* gene encoding the NorA MDR efflux protein was provided by G. Kaatz (Kaatz, 1993). EMRSA-15 which possesses the *mecA* gene was provided by Dr Paul Stapleton, ULSOP. Bacterial inocula equivalent to the 0.5 McFarland turbidity standard were prepared in normal saline and diluted to give a final inoculum density of 5×10^5 cfu/mL. The inoculum (125 µL) was added to all wells and the microtitre plate was incubated at 37 °C for 24 h for *S. aureus* strains and 72 h for *M. fortuitum*, *M. smegmatis* and *M. phlei*.

For *M. abscessus* the plate was incubated for 120 h. The MIC was recorded as the lowest concentration at which no bacterial growth was observed as previously described (Gibbons and Udo, 2000).

Ethambutol and isoniazid were used as positive controls for the mycobacterial strains and norfloxacin was used for *S. aureus*.

Canthin-6-one (1). Amorphous yellow solid; λ_{max} (log ϵ): 398.0 (3.97), 383.0 (3.95), 319.5 (4.19), 240.0 (4.64) nm; IR ν_{max} (thin film) cm^{-1} : 2917, 2358, 1652, 1600, 1436; HR-MS (m/z): 221.0755 [$M + H$]⁺ (calc. For $C_{14}H_8ON_2$, 220.0636).

8-Hydroxy-canthin-6-one (2). Amorphous yellow solid; λ_{max} (log ϵ): 381.5 (3.76), 374.0 (3.66), 364.0 (3.93), 318.0 (3.90), 295.5 (3.77), 292.5 (3.77), 261.0 (3.90) nm; IR ν_{max} (thin film) cm^{-1} : 3286 (br), 3060, 1669, 1632, 1434, 1333, 1142, 844, 794, 749; ¹H NMR and ¹³C NMR (CDCl_3): see Table 1; HR-MS (m/z): 237.0664 [$M + H$]⁺ (calc. For $C_{14}H_8O_2N_2$, 236.0585).

5(ζ)-Hydroxy-octadeca-6(E)-8(Z)-dienioic acid (3). Colourless oil; $[\alpha]_{\text{D}}^{20}$ 0° (c 0.65, MeOH). UV (MeOH): λ_{max} (log ϵ): 276.0 (3.45), 232.0 (4.22) nm. IR (film) ν_{max} : 2928, 2856, 1707, 987 cm^{-1} ; ¹H-NMR and ¹³C-NMR (MeOD): Table 2; HR-MS (m/z): 295.2267 [M]⁻ (calc. for $C_{18}H_{31}O_3$, 295.2273).

RESULTS AND DISCUSSION

Bioactivity guided isolation of the active chloroform extract (16 µg/mL) yielded compound **1** as a yellow solid, which displayed a positive reaction to Dragendorff's reagent on analysis by TLC. High-resolution ESI-MS showed the [$M + H$]⁺ ion at m/z 221.0755 indicating a molecular formula of $C_{14}H_8N_2O$. The ¹H and ¹³C-NMR data were in accordance with the published data for canthin-6-one previously isolated from *Ailanthus altissima* (Koike and Ohmoto, 1985).

Compound **2** was isolated as a yellow solid and assigned a molecular formula of $C_{14}H_8N_2O_2$ [$M + H$]⁺ (237.0664) with HRESI-MS. A positive reaction was also observed with Dragendorff's reagent, and the ¹H-NMR data (Table 1) were in close agreement with the published literature for 8-hydroxy-canthin-6-one previously isolated from *Ailanthus excelsa* (Cordell *et al.*, 1978). However, the full NMR data for this metabolite were not reported so a full analysis of the resonances of **2** was undertaken. The ¹H and ¹³C-NMR data were similar to those for compound **1**; however, ring A was seen to be an ABC tri-substituted aromatic system and a deshielded hydroxyl singlet was present at δ 11.95 in the ¹H-NMR spectrum. In addition to the hydroxyl group, the ¹H-NMR data for compound **2** displayed seven signals in the aromatic region (δ 7.10–8.90). Three protons, two doublets (δ 7.58 and δ 7.23) and one triplet (δ 7.45) were equivalent to an ABC aromatic system as seen through COSY correlations. Two further *ortho* coupled aromatic systems were also evident; one indicative of a *cis* double-bond adjacent to a carbonyl group at δ 8.18 and δ 7.10 (J = 10.0 Hz) and the other consistent with positioning next to an aromatic nitrogen δ 8.90 and δ 8.00 (J = 5.0 Hz). In addition to seven

Table 1. ^1H (500 MHz) and ^{13}C NMR (125 MHz) spectral data and ^1H - ^{13}C long-range correlations of **2** recorded in CDCl_3

No	^1H	^{13}C	2J	3J
1	8.00 <i>d</i> (5.0)	117.0		
2	8.90 <i>d</i> (5.0)	147.2		
4	8.18 <i>d</i> (10.0)	141.3		C-6, C-15
5	7.10 <i>d</i> (10.0)	127.0		C-16
6	–	160.7		
8	–	146.8		
9	7.23 <i>d</i> (8.0)	119.6	C-10	C-11
10	7.45 <i>t</i> (8.0)	128.7		C-8, C-12
11	7.58 <i>d</i> (7.5)	113.4	C-10, C-12	C-9, C-14
12	–	126.9		
13	–	141.4		
14	–	127.5		
15	–	131.6		
16	–	136.9		
OH	11.95 <i>s</i>	–		C-9

Table 2. ^1H (500 MHz) and ^{13}C NMR (125 MHz) spectral data and ^1H - ^{13}C long-range correlations of **3** recorded in MeOD

No.	^1H	^{13}C	2J	3J
1	–	177.8		
2	2.27 <i>t</i> (7.5)	35.1	C-1	
3	1.60 <i>m</i>	26.3		C-1
4	1.53 <i>m</i>	38.5		
	1.47			
5	4.09 <i>dd</i> (13.0, 6.5)	73.4	C-4, C-6	C-3, C-7
6	5.62 <i>dd</i> (15.0, 7.0)	137.3	C-5	C-4, C-8
7	6.50 <i>dd</i> (15.4, 11.0)	126.5	C-8	C-5, C-9
8	5.98 <i>t</i> (11.0)	129.4	C-7	C-6, C-10
9	5.41 <i>dt</i> (15.0, 10.5, 7.5)	132.9	C-10	C-7, C-11
10	2.20 <i>dd</i> (13.0, 7.5)	28.4	C-9, C-11	C-8
11	1.39 <i>m</i>	30.7		C-9
12–15	1.31 <i>m</i>	30.7		
16	1.31 <i>m</i>	33.0		
17	1.31 <i>m</i>	23.7		
18	0.91 <i>t</i> (7.0)	14.0	C-17	C-16

methine carbons, the ^{13}C -NMR spectrum revealed the presence of six aromatic quaternary carbons (δ 113.4–147.2), and one deshielded signal consistent with a carbonyl carbon (δ 160.7).

Ring A was defined as an ABC aromatic system by COSY correlations. The doublet at δ 7.23 (H-9; J = 8.0 Hz) displayed a COSY correlation to the triplet at δ 7.45 (H-10; J = 8.0 Hz) which in turn coupled to the doublet at δ 7.58 (H-11; J = 7.5 Hz). H-10 displayed a 3J HMBC correlation to the hydroxylated quaternary carbon C-8 (δ 146.8) and H-10 displayed a 3J correlation to C-12 (δ 126.9).

Ring C was consistent with an *ortho* coupled pair of hydrogens (H-1, δ 8.00 and H-2, δ 8.90; J = 5.0 Hz) positioned on a pyridine ring. H-1 displayed an HMBC correlation to the quaternary carbon C-15 (δ 131.6), completing the assignment of ring B, while H-2 showed an HMBC correlation to the C-14 quaternary (δ 127.5). The only NOESY correlation present was between H-1 and H-11 concluding the β -carboline skeleton of the canthin-6-one structure. The D ring was finally assigned as a pyridone through further HMBC correlations. H-4 and H-5 (δ 8.18 and δ 7.10) gave a coupling constant

of 10.0 Hz suggesting they were positioned α and β to a carbonyl carbon and a 3J HMBC correlation between H-4 to C-6 (δ 160.7) confirmed this. Furthermore 3J correlations between H-4 and C-15 (δ 131.6), and H-5 to C-16 (δ 136.9) revealed the quaternary carbons of the C $\rightarrow$ D ring junction. The remaining quaternary carbon C-13 was assigned by comparison with the published literature for canthin-6-one (Koike and Ohmoto, 1985). The ^{13}C NMR data have not been published previously for this metabolite and are reported here for the first time.

Compound **3** was isolated as a colourless oil and assigned a molecular formula of $\text{C}_{18}\text{H}_{31}\text{O}_3$ [$\text{M} + \text{H}$] $^+$ (295.2267) with HRESI-MS. The ^1H -NMR data (Table 2) were reminiscent of a monohydroxylated unsaturated fatty acid recently described by Stavri *et al.* (2006).

A deshielded methylene triplet at δ 2.27 (H-2; J = 7.5 Hz) displayed a strong 2J correlation to a carbonyl carbon (δ 177.8) of the carboxylic acid. A weaker 3J correlation was evident from a second downfield methylene (δ 1.60 *m*) positioned β to the carbonyl carbon. Non-equivalent methylene hydrogens at δ 1.53 (C-4) displayed a 2J correlation to C-3 (δ 26.3). An oxymethine

Table 3. MICs of **1**, **2** and **3** and standard antibiotics in µg/mL

Strain	1	2	3	Ethambutol	Isoniazid	Norfloxacin
<i>M. fortuitum</i> ATCC 6841	16	16	32	4	0.25	ξ
<i>M. smegmatis</i> ATCC 14468	8	16	32	0.25	2	ξ
<i>M. smegmatis</i> mc ² 2700	8	2	16	0.25	2	ξ
<i>M. phlei</i> ATCC 11758	8	8	16	0.5	2	ξ
<i>M. abscessus</i> ATCC 19977	16	32	ξ	128	128	ξ
<i>S. aureus</i> 1199B	8	16	>128	ξ	ξ	32
<i>S. aureus</i> XU212	8	8	>128	ξ	ξ	16
<i>S. aureus</i> EMRSA-15	32	64	>128	ξ	ξ	0.5

ξ, not tested.

hydrogen (H-5; δ 4.09) displayed 2J correlations to the methylene of C-4 (δ 38.5) and to an olefinic carbon at C-6 (δ 137.3). 3J correlations from H-5 to the C-3 methylene (δ 26.3) and the C-7 olefinic carbon (δ 137.3) were also evident.

The olefinic hydrogens were ordered through COSY correlations. The double doublet of H-6 (δ 5.62; J = 15.0, 7.0 Hz) coupled to the double doublet of H-7 (δ 6.50; J = 15.5, 11.0 Hz) through a large coupling constant which signified a *trans* configuration. H-7 then coupled to a triplet of H-8 (δ 5.98; J = 11.0 Hz) which in turn displayed a coupling to a doublet of triplets of H-9 (δ 5.41; J = 10.5, 7.5 Hz). The coupling constant between H-8 and H-9 was smaller than that for the H-7/H-8 double bond suggesting a *cis* configuration. H-9 displayed a further coupling to a methylene at δ 2.20 (H-10) which coupled to a methylene at δ 1.39 (H-11) and finally into a methylene envelope at δ 1.31. 2J and 3J correlations from H-9 to C-10 (δ 28.4) and C-11 (δ 30.7), respectively, confirmed this. The terminal methyl exhibited a COSY coupling to the methylene envelope and 2J and 3J HMBC correlations into methylene carbons at δ 23.7 and δ 33.0, respectively.

The specific optical rotation data (α = 0.00; c = 0.65, MeOH) was inconclusive for the stereochemical assignment of C-5. This is in accordance with the findings of Stavri *et al.* (2006) where it was suggested that the racemate could be due to a non-enzymatic hydrolysis of a 2-3 double bond to afford 3(ζ)-hydroxy-octadeca-4(*E*)-6(*Z*)-dienoic acid (a 4-5 double bond in the case of compound **3**) resulting in the non-specific orientation of the hydroxyl group.

The ^1H and ^{13}C -NMR data were very similar to those for 3(ζ)-hydroxy-octadeca-4(*E*)-6(*Z*)-dienoic acid isolated from *Scrophularia deserti* (Stavri *et al.*, 2006) and this is the first time the NMR data for 5(ζ)-hydroxy-octadeca-6(*E*)-8(*Z*)-dienoic acid have been reported.

Compounds **1** and **2** exhibited the most pronounced antibacterial activity with MIC values of 8–64 µg/mL against the *S. aureus* strains and 2–32 µg/mL against a

range of fast-growing *Mycobacterium* species (Table 3). The antimycobacterial activity compared favourably with the control antibiotic isoniazid against *M. smegmatis* (mc²2700), *M. phlei* and *M. abscessus*. The MIC values for **1** and **2** were comparable against most bacterial strains tested, however, **2** displayed enhanced activity against the *M. smegmatis* (mc²2700) strain expressing the *M. tuberculosis* FASI gene, when compared with that for **1**. Furthermore, the activity of **2** was greater against *M. smegmatis* (mc²2700) than *M. smegmatis* (ATCC 14468) suggesting that the C-8 hydroxyl substituent may play a role in the inhibition of FASI. This is an intriguing target for new antimycobacterial agents as mycobacterial FASI is a unique but important enzyme in mycolic acid synthesis (Zimhony *et al.*, 2004). In addition, these compounds are planar and it may also be deduced that the mode of action could be through DNA intercalation. Canthin-6-one type alkaloids are well known constituents of the Simaroubaceae and Rutaceae and antimicrobial activity, particularly from the genus *Zanthoxylum*, is well reported (Islam and Ashan, 1997; Thouvenel *et al.*, 2003). This is the first reported isolation of these natural products from the Alliaceae and may be of chemotaxonomic value. Compound **3** displayed activity in the range 16–32 µg/mL against the fast-growing *Mycobacterium* species but no activity at the concentrations tested against the *S. aureus* strains. These data are consistent with those reported for the antibacterial activity of 3(ζ)-hydroxy-octadeca-4(*E*)-6(*Z*)-dienoic acid (Stavri *et al.*, 2006) suggesting that the position of the olefinic and hydroxyl groups may have little influence on the antibacterial activity.

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