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Agrochelin, a new cytotoxic alkaloid from the marine bacteria *Agrobacterium* sp.

Librada M. Cañedo,[a] Jesús A. de la Fuente,[a] Cristina Gesto,[a] María J. Ferreiro,[b] Carlos Jiménez[c] and Ricardo Riguera[b,*]

[a]*Instituto Biomar S.A., 24005 León, Spain*

[b]*Departamento de Química Orgánica, Universidad de Santiago, 15706 Santiago de Compostela, Spain*

[c]*Departamento de Química Fundamental e Industrial, Universidade de A Coruña, 15071 A Coruña, Spain*

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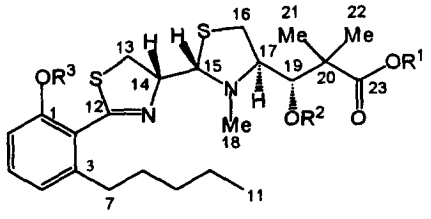
Abstract

Agrochelin (**1**), a new cytotoxic thiazole alkaloid, has been isolated from the fermentation broth of a marine unicellular bacterium belonging to genus *Agrobacterium*. Its structure was determined from spectral data and chemical transformations. Agrochelin showed chelating properties to the Zn^{2+} ion.

Keywords: marine alkaloids; thiazolines; antitumour; Zn^{2+} chelation.

Although there is great interest in the chemistry of cultured marine organisms, only relatively few compounds derived from marine bacteria have been identified to date.[1] In the course of a program devoted to the isolation of anti-cancer agents from marine microorganisms, we selected a marine *Agrobacterium* for studies on the basis of the cytotoxic activity of its extracts.

The $CHCl_3$–MeOH extract of the mycelium of marine *Agrobacterium* sp. was washed with hexane and partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fraction was chromatographed on silica gel and MPLC followed by further purification by C18 reversed phase chromatography (85% MeOH) to afford pure Agrochelin (**1**).[2]



1 $R^1 = R^2 = R^3 = H$
2 $R^1 = H$, $R^2 = R^3 = Ac$
3 $R^1 = Me$, $R^2 = R^3 = H$

* Corresponding author.

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Table 1
^{13}C and ^{1}H NMR (125 and 500.13 MHz) chemical shifts (ppm) for **1–3** in Cl_3CD

	δ_C mult			δ_H mult (*J* in Hz)		
C	**1**	**2**	**3**	**1**	**2**	**3**
1	159.6 s	147.8 s	159.7 s	——	——	——
2	116.1 s	126.5 s	116.2 s	——	——	——
3	143.7 s	143.0 s	143.8 s	——	——	——
4	121.4 d	126.9 d	121.4 d	6.70 d (7.8)	7.12 d (7.8)	6.70 d (7.8)
5	132.2 d	129.9 d	132.2 d	7.20 t (7.8)	7.32 t (7.8)	7.20 t (7.8)
6	115.4 d	120.1 d	115.4 d	6.84 d (7.8)	6.94 d (7.8)	6.85 d (7.8)
7	35.3 t	32.8 t	35.4 t	2.93 m	2.68 m	2.89 m 2.99 m
8	32.1 t	30.8 t	32.2 t	1.60 m	1.60 m	1.59 m
9	31.8 t	31.7 t	31.9 t	1.33 m	1.32 m	1.35 m
10	22.4 t	22.4 t	22.8 t	1.33 m	1.32 m	1.35 m
11	14.0 q	13.9 q	14.1 q	0.89 t (6.9)	0.88 t (6.7)	0.90 t (7.0)
12	172.1 s	165.9 s	171.8 s	——	——	——
13	35.0 t	35.9 t	35.1 t	3.14 dd (7.5, 11.4) 3.46 dd (8.7, 11.3)	3.44 d (9.2)	3.13 dd (7.7, 11.3) 3.46 dd (8.7, 11.2)
14	77.8 d	77.9 d	78.1 d	4.72 q (8.7)	4.96 dd (6.4, 9.1)	4.75 q (8.6)
15	79.3 d	80.8 d	79.6 d	4.18 d (9.3)	4.44 d (6.4)	4.19 d (9.4)
16	36.5 t	34.4 t	36.3 t	2.93 m 3.27 dd (7.2, 11.6)	3.09 m	2.82 dd (4.5, 11.6) 3.22 dd (7.1, 11.6)
17	73.0 d	70.6 d	72.9 d	3.37 m	3.37 m	3.41 m
18	46.2 q	42.9 q	46.7 q	2.62 s	2.61 s	2.62 s
19	77.0 d	78.2 d	77.2 d	3.49 d (7.0)	5.20 d (4.4)	3.54 d (6.8)
20	46.0 s	45.9 s	46.1 s	——	——	——
21	22.1 q	23.8 q	22.5 q	1.28 s	1.27 s	1.28 s
22	22.0 q	21.5 q	20.3 q	1.25 s	1.25 s	1.22 s
23	180.0 s	178.9 s	177.0 s	——	——	3.70 s
CO<u>2</u>Me	——	——	52.0 q	——	——	——
<u>C</u>OMe	——	169.4 s 170.3 s	——	——	——	——
CO<u>Me</u>	——	21.1 q	——	——	2.29 s, 2.12 s	——

The molecular formula of the cytotoxic **1** was established by HRFABMS of its $[M+H]^+$ pseudo molecular ion at *m/z* 467.2042 as $C_{23}H_{34}N_2O_4S_2$; the presence of eight unsaturations in the molecule was indicated.

The ^{13}C and DEPT NMR spectra displayed 23 distinct resonances including one carbonyl group at 180.0 ppm, one quaternary olefinic carbon at 172.1 ppm characteristic of a thiazoline ring, six aromatic carbons (three CH and three quaternary), six methylene carbons (four resonating between 35.3 and 22.4 ppm due to a saturated carbon chain and two sulphur-bearing methylenes at 36.5 and 35.0 ppm), and four methines between 79.3 and 73.0 ppm (Table 1). The remaining five signals corresponded to four methyls and one sp^3 quaternary carbon.

The presence in **1** of a 1,2,3-trisubstituted benzene ring with position 1 occupied by a phenolic OH was easily deduced from the proton and carbon chemical shifts, the coupling of the proton aromatic signals, and the bathochromic shift observed in the UV when the spectrum was taken in methanolic NaOH. The combined use of ^{1}H–^{1}H COSY, TOCSY, and HMQC of **1** allowed the assignment of four spin systems **a–d** (Fig. 1a, in bold): a three-proton system of the aromatic ring, eleven protons corresponding to a five-carbon saturated chain, and two CH_2CHCH proton systems. The three remaining singlets were identified as three methyl groups. Furthermore, a ^{15}N NMR spectrum showed the presence of two well separated nitrogens at –80 and –320 ppm (CH_3NO_2=0 ppm) identified as C=N and NMe, respectively.

Long range C–H correlations (HMBC) were used to assemble the skeletal fragments through quaternary carbons and heteroatoms obtained from the other experiments (Fig. 1a). The HMBC correlations between the methylene H-7 to the C-2, C-3, and C-4 aromatic carbons connected the five-carbon saturated

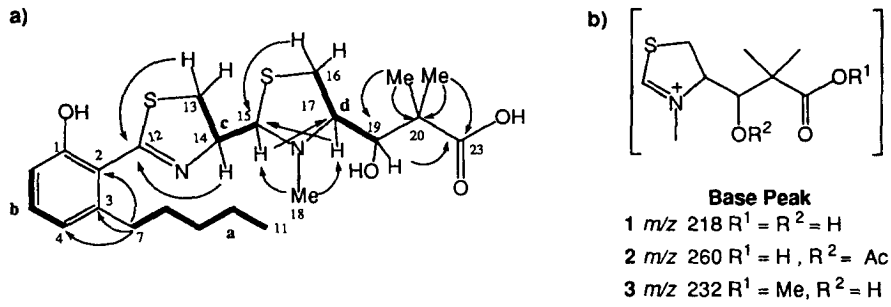


Figure 1. (a) 2D NMR data of Agrochelin (**1**). The partial structures shown in thick lines were obtained from COSY and TOCSY experiments, and the connectivities by HMBC. (b) Base peak fragmentation in FABMS (+) of **1–3**

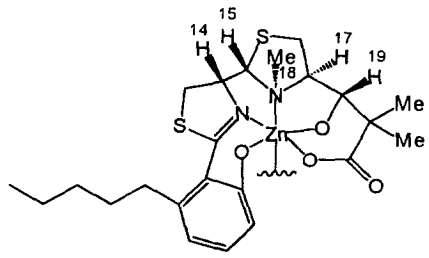
chain (**a** unit) to C-3 of the aromatic ring (**b** unit). HMBC cross peaks between quaternary olefinic carbon C-12 to the methylene protons H-13 and the methine proton H-14, suggested the presence of a thiazoline moiety in **1**. Long range correlations between the NMe carbon at δ_C 46.2 (δ_H 2.62) to the methine protons H-15 and H-17, and between C-15 and the methylene protons H-16 connected the **c** and **d** units through the nitrogen and suggested the presence of a second thiazoline system. Finally, the HMBC correlations from the methyl protons H-21 and H-22 to the quaternary carbon C-20, the oxymethine C-19, and the carbonyl C-23, and between the oxymethine proton H-19 and the carbonyl C-23 established the other end of the molecule and allowed us to assign its structure as **1**. Long range (2–3 bonds) N–H correlations were observed between the *N*-methylated nitrogen at –320 ppm and protons H-18, H-16, H-14 and H-19 and between the imine nitrogen at –80 ppm and protons H-15 and H-13 which corroborated structure **1**.

Several chemical transformations were carried out to confirm the proposed structure. Thus, acetylation of **1** gave the diacetylated derivative **2** whose molecular formula was established as $C_{27}H_{38}N_2O_6S_2$ by HRFABMS of its pseudo molecular ion $[M+H]^+$ at *m/z* 551.2229 (Δ 2.0 amu). Comparison of the proton and carbon chemical shifts of the aromatic ring of **2** to those of **1** indicated that acetylation of the phenolic OH had occurred. On the other hand, the carbon resonance at 77.0 ppm assigned to C-19 in the ^{13}C NMR of **1** was shifted downfield in **2** by 1.2 ppm, while C-17 (73.0) and C-20 (46.0) resonances were shifted upfield by 2.4 and 0.1 ppm, respectively. These changes are characteristic for α- and β-effects of an acetyl group and confirmed the position of the second OH at C-19.

Treatment of **1** with diazomethane gave the methyl ester **3** whose positive FABMS showed the pseudo molecular ion $[M+H]^+$ at *m/z* 481 corresponding to $C_{24}H_{36}N_2O_4S_2$. Its NMR spectra showed the expected additional Me group (δ_H 3.70, δ_C 52.0) and the upfield carbon shift of C-23 to 177.0 ppm in relation to that of **1**. Furthermore, the HMBC correlation between the protons of the additional methyl ester to the carbonyl carbon confirmed the methylation of the carboxylic acid of **1**.

In full accordance with these conclusions, the FABMS (+) of compounds **1–3** showed their base peaks at *m/z* 218, 260 and 232 amu, respectively, that corresponded to the thiazoline ion generated by cleavage of the C-14/C-15 bond.

Similarly to the metal-containing micacocidins A–C from *Pseudomonas* sp.,[3] the iron-transporting yersiniabactin[4] and yersiniophore[5] from *Yersinia* strains, and anguibactin[6] from the marine fish pathogen *Vibrio anguillarum*, agrochelin was shown to form a complex with Zn^{2+} ions. Thus, when a methanolic solution of $ZnCl_2$ was added to **1** in methanol, the mass spectra of the solution showed intense ions at 629 and 441 amu, corresponding to the molecular ion of the complex and the ion generated by cleavage of the C-19/C-20 bond as in micacocidin A. Moreover, the UV absorption maxima shifted from 210 to 234 nm and a new band due to a charge transfer appeared at 360 nm.[7] Similarly, the CD spectra in the presence of Zn^{2+} showed a broad Cotton effect at 355 nm due to the charge transfer of the metal ion. In



Proton	1	1-Zn complex
14	4.81	4.96
15	4.33	4.45
17	3.37	3.60
18	2.58	2.76
19	3.49	3.64

Figure 2. Proposed structure for Agrochelin-Zn^{2+} complex and selected ^1H NMR data for the **1** and **1**-Zn^{2+} complex in CD_3CN

addition, new CD bands indicative of a conformational change were observed. NMR experiments were also used to study the effect of Zn^{2+} on the structure of agrochelin. Thus, the ^1H NMR spectra showed an intense downfield shift for those protons close to the coordination points (Fig. 2). All this information strongly suggests the formation of a Zn^{2+} complex as depicted in Fig. 2, with the two nitrogens as donor atoms similar to that reported for micacocidin A.[3]

Our efforts to obtain crystals useful for X-ray analysis of **1** either as the free acid, an ester, a salt or a Zn^{2+} complex, all failed, and therefore, the only stereochemical information available comes from the NOESY spectra of **1** in the presence of Zn^{2+} which showed a strong NOE between H-14 and H-15 and between H-17 and N-Me but not between H-15 and H-17 which suggests the stereochemical arrangement 14*R**, 15*S**, 17*R**, 19*S** which is different to that reported for micacocidin A.

Agrochelin (**1**) showed strong in vitro cytotoxicity (from 0.05 to 0.2 μg/mL) against mouse (P-388) and human (A-549, HT-29, MEL-28) tumour cell lines but the acetylated derivative **2** was found to be much less active (from 3 to 7 μg/mL).

Acknowledgements

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2. Compound **1**: Amorphous yellow oil. [α] −20.5 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} 210 nm (lg ε 5.18), 264 (4.80); CD (*c* 1×10^{-4} M, MeOH) λ_{max}/nm ($\Delta\varepsilon$/cm^2 mol^{-1}): 222 (5.25), 244 (−2.32), 267 (−3.63), 336 (0.32); HRFABMS *m/z*: 467.2042 $[M+H]^+$ (calcd for $C_{23}H_{35}N_2O_4S_2$: 467.2038); 218.0856 (calcd for $C_9H_{16}NO_3S$: 218.0851). ^{13}C and ^1H NMR data: see Table 1.
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7. **1**–Zn complex: ^1H NMR (400.13 MHz, CD_3CN) δ (ppm): 0.91 (m, 3H), 1.20 (s, 3H), 1.23 (s, 3H), 1.35 (m, 4H), 1.61 (m, 2H), 2.76 (br s, 3H), 2.85 (m, 2H), 3.12 (dd, *J*=6.2, 12.2 Hz, 1H), 3.33 (m, 1H), 3.39 (m, 1H), 3.65 (m, 3H), 4.45 (d, *J*=7.0 Hz, 1H), 4.96 (q, *J*=7.1 Hz, 1H), 6.82 (d, *J*=7.7 Hz, 2H), 7.26 (t, *J*=7.9 Hz, 1H); UV (MeOH) λ_{max} 234 nm (lg ε 5.18), 266 (4.85), 360 (4.55); CD (*c* 0.95×10^{-4} M, MeOH) λ_{max}/nm ($\Delta\varepsilon$/cm^{2}mol^{-1}): 222 (7.78), 257 (−9.80), 276 (1.49), 294 (−2.63), 355 (3.59).