Tetrahedron Vol. 45, No. 12, pp. 3873 to 3878, 1989
Printed in Great Britain.

0040-4020/89 $3.00+.00

CLATHRIDINE AND ITS ZINC COMPLEX, NOVEL METABOLITES FROM THE MARINE SPONGE *CLATHRINA CLATHRUS*

Patrizia Ciminiello, Ernesto Fattorusso*, Silvana Magno and Alfonso Mangoni

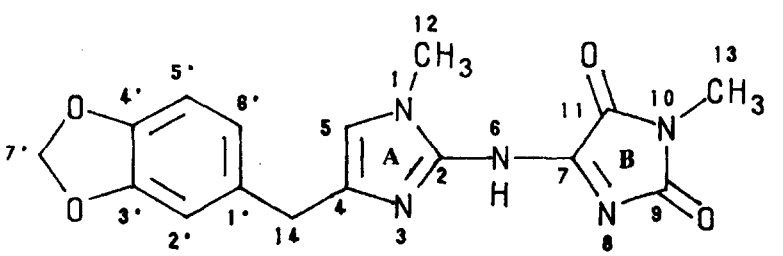
Dipartimento di Chimica delle Sostanze Naturali - Università di Napoli
Via D. Montesano 49, 80131 Napoli, Italy

(*Received in UK* 20 *March* 1989)

Summary: The structure of an imidazole-containing compound, clathridine (**1**), was established from chemical evidence and by extensive NMR analysis. Clathridine was isolated from the sponge *Clathrina clathrus* and exhibited antimycotic activity. The sponge also contains smaller quantities of a Zn-complex of clathridine (**4**), which has been identified on the basis of its spectroscopic properties and synthesized from **1** with $ZnSO_4$. A hypothesis for the stereostructure of **4** is also given.

We have recently reported[1] the occurrence of three new 7-keto-sterols from the lipophilic fraction of *Clathrina clathrus* (Schmidt), an encrusting bright yellow sponge of the class Calcispongia, consisting in a branching network of narrow-walled tubes, which prefers to grow in shade places away from the light. We have now isolated clathridine (**1**), a nitrogenous metabolite, present in large amount in the same organism, which showed *in vitro* antimycotic activity against *Candida albicans* and *Saccharomyces cerevisiae* (40 μg per disk). Structure **1** has been established on the basis of extensive NMR studies, including bidimensional techniques, and chemical evidence. The sponge, in addition to clathridine, contains smaller amounts of its zinc-complex, which was proved to be a genuine metabolite of the marine organism. This seems to be a quite interesting feature, since zinc is a metal which plays a role in biological systems second in importance only to iron and is essential for all life forms[2]. There are now over one hundred metalloenzymes known to require zinc for their functions, representing each of the six categories of enzymes designated by the International Union on Biochemistry Commission on enzymes. Consequently a large number of zinc-chelating substances possesses interesting biological properties[3].

Specimens of *C. clathrus* were collected in June 1987 in the Procida Channel, near Napoli, at a depth of 20/25m by SCUBA techniques. A chloroform extract of the lyophilized material was partitioned by column chromato-



1

Table 1. NMR data for **1**.

Pos.	$^1H(CF_3COOD)$	$^1H(CDCl_3)$	$^{13}C(CF_3COOD)$	$^{13}C(CDCl_3)$	COLOC(CF_3COOD)
2			140.51		H5, H12
4			133.39		H5, H14
5	6.90 (1H,s)	6.52	120.53	117.49	H12, H14
7			146.59		
9[a]			154.98		
11[a]			159.17		H13
12	3.70 (3H,s)	3.70	33.83	31.98	
13	3.03 (3H,s)	3.17	25.28	24.59	
14	3.87 (2H,s)	3.79	31.40	34.42	H2'
1'			129.39		H5' or H6', H14
2'	6.65 (1H,s)	6.71	109.94	109.17	H5' or H6'
3'[b]			147.88		H2', H5' or H6',H7'
4'[b]			148.92		H2', H7'
5'	6.75 (1H,d,J=7.8Hz)[c]	6.75	109.94	108.23	
6'	6.69 (1H,d,J=7.8Hz)[c]	6.70	123.34	121.58	H2', H14
7'	5.82 (2H,s)	5.92	101.87	100.85	

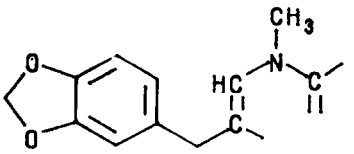
a,b) The positions can be reversed.
c) The values can be reversed.

graphy on silica gel under medium pressure. A fraction which was eluted with 3% MeOH in $CHCl_3$, after crystallization from $CHCl_3$, afforded compound **1** as orange-yellow prisms (m.p. 260-262°C dec.) which was sparingly soluble in most organic solvents. The mass spectrum of compound **1** showed a molecular ion peak at m/z 341.1116, corresponding to the molecular formula $C_{16}H_{15}N_5O_4$. The IR spectrum contained an N-H absorption at ν_{max} 3260 cm^{-1} and carbonyl absorptions at ν_{max} 1793, 1737 and 1716 cm^{-1}. The UV spectrum showed absorptions at λ_{max} 371 nm (ε=14700) and 285 nm (ε=5400). Decisive information on the structure **1** was obtained by an extensive analysis of ^1H- and ^{13}C-NMR spectra of clathridine. Owing to the paucity of protons in the molecule, extensive use was made in the stucture elucidation of long-range ^1H-^{13}C couplings. The spectra were performed in $CDCl_3$, in which **1** is only sparingly soluble, and/or in CF_3COOD.

Compound **1** possesses a 3,4-methylenedioxybenzyl (piperonyl) unit, as indicated by the mass spectrum (intense ion at m/z 135, $C_8H_7O_2$), by the proton NMR spectrum ($CDCl_3$), which includes two methylene singlets at δ 3.79 and 5.92, and three aromatic protons resonating, respectively as a singlet at δ 6.71, and as an AB system at δ 6.70 and 6.75, and by the ^{13}C-NMR spectrum, which shows all the signals expected for a piperonyl unit [see Table 1; the assignments were based on ^{13}C-^1H shift correlated 2D NMR spectroscopy *via* ^1J,[4] which established direct correlation, and *via* $^{2,3}J$ (COLOC)[5] through which confident assignment for non-protonated carbons were also accomplished].

As a result of inspection of molecular formula and ^1H- and ^{13}C-NMR spectra, the remaining part of the molecule had to be comprised of two carbonyl functions (δ_C159.17 and 154.98),one methinic [δ_H6.52 (s), δ_C120.53] and three fully substituted (δ_C146.59, 140.51 and 133.39) sp^2 carbon atoms, five nitrogen atoms and two N-methyl groups [δ_H3.17 (s), δ_C25.28 and δ_H3.70 (s), δ_C33.83]. Unfortunately, their relative positions could not be established either through spin decoupling work or by a COSY experiment, since most of the carbons in **1** were non-protonated. Therefore further information regarding the skeletal network was sought from two- and three-bond proton-carbon

couplings. Correlation of H_2-14 with carbons C-4 and C-5, of H-5 with C-4 and C-2, and of H_3-12 with C-5 and C-2 allowed us to extend the piperonyl partial structure to



Insertion of a nitrogen atom at position 3 and consequently the definition of the imidazolic nature of the ring **A** was dictated by proton-carbon long-range couplings (H_3-15 with C-2 and C-4) showed by a COLOC experiment on the methyl-derivative **2,** obtained from **1** by methylation with diazomethane.

The location of a third nitrogen atom on C-2 was deduced from alkaline hydrolysis of **1**, which yielded the amino-derivative **3**, whose structure was deduced from its ^{1}H-NMR spectrum [δ 6.74 and 6.71 (2H, AB system, J=7.5Hz), 6.71 (1H, s), 5.99 (1H, s), 5.94 (2H, s), 3.70 (2H, s), 3.44 (3H,s)] and mass spectrum, which indicated the molecular formula $C_{12}H_{13}N_3O_2$.

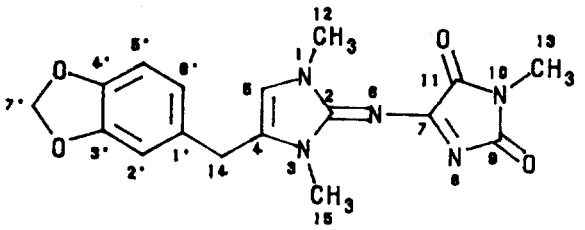
The above results account for all the skeletal atoms of clathridine except those of the ring **B**, which must contain the CO-NCH_3-CO moiety. This was indicated by long-range couplings of H_3-13 with C-9 and C-11 which, even if not clearly detectable in the 2D ^{13}C-^{1}H NMR spectrum of **1**, were undoubtedly present in that of **2**. At this stage, taking into account the molecular formula, only one nitrogen and one quaternary carbon atoms remained to be accomodated. This can only be accomplished through their incorporation in a second imidazole-type ring, linked to the rest of the molecule by an -NH as depicted in **1**. Clathridine is therefore structurally related to Naamines recently isolated by Karmely and Kashman from *Leucetta chagosensis*, a sponge from Red Sea[6].

The $CHCl_3$ extract from *C. clathrus* contained smaller amounts of a further nitrogenous metabolite, which was isolated in a pure form by HPLC on an SiO_2 column. Comparison of its most significant spectroscopic properties (UV, IR, ^{1}H-NMR) with those of clathridine revealed a strong structural analogy between these two compounds. In

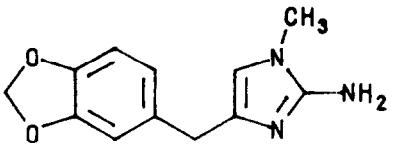
Table 2. NMR data for **2**.

Pos.	^{1}H-NMR($CDCl_3$)	^{13}C-NMR($CDCl_3$)	COLOC($CDCl_3$)
2		150.81	H5, H12, H15
4		129.66	H5, H14, H15
5	6.37(1H,s)	115.67	H12, H14
7		167.54	
9[a]		165.54	H13
11[a]		166.89	H13
12	3.53(3H,s)	34.19	
13	3.10(3H,s)	24.66	
14	3.77(2H,s)	30.41	
15	3.40(3H,s)	31.19	
1'		128.19	H5', H14
2'	6.64(1H,s)	108.84	H14
3'[b]		147.36	H2' o H6'
4'[b]		148.54	H2' o H6'
5'	6.78(1H,d,J=9.2Hz)	108.78	
6'	6.63(1H,d,J=9.2Hz)	121.69	H14
7'	5.98(2H,s)	101.32	

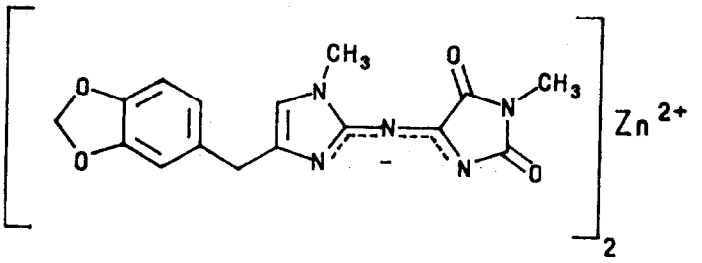
a,b) The positions can be reversed.



2



3



4

particular the ^{1}H-NMR spectrum (see experimental) is very similar to that of **1**, the only significative difference being confined to the splitting of the two methylene signals into two AB systems, whose very different coupling constants (16.5 Hz for H_2-14 and 1.5 Hz for the methylenedioxy group) fully agree with the values reported in literature for geminal coupling of similar methylene groups[7].

Useful information for the assignment of the structure **4** to the compound under investigation was obtained from its mass spectrum, which showed a molecular ion cluster at m/z 744, 746 and 748 (relative intensities 100, 56 and 37) clearly indicative of the presence of one zinc atom in the molecule. The high-resolution mass spectrum measurement on the peak at m/z 744 (744.1430) established the molecular formula $C_{32}H_{28}N_{10}O_8Zn$, thus suggesting that compound **4** could derive by two deprotonated clathridine molecules and one Zn^{2+} ion.

This hypothesis was definitively confirmed by the synthesis of **4**, performed by treatment of a dichloromethane solution of clathridine with an excess of an aqueous 0.1M $ZnSO_4$ solution under vigorous stirring for 1 hr, which gave **4** in quantitative yield. This compound must be a coordination zinc derivative rather than simply a salt, as proved by its UV spectrum (λ_{max} 399(s), 372, 361 and 285 nm), which was very different from that of the clathridine anion (λ_{max} 359 and 284 nm), and by its proton NMR spectrum which shows that in both methylene groups the hydrogens are not magnetically equivalent. The well-known capacity of imidazole derivatives to chelate transition metals and particularly zinc ion[3], to give ionic or neutral complexes, could account for the formation of compound **4** in the sponge *C. clathrus*. The possibility that **4** could be formed during chromatographic separation upon SiO_2, as reported for other metal-chelating naturally-occurring compounds[8] was ruled out since the ^{1}H-NMR spectrum of the $CHCl_3$ extract from the sponge, before any work-up, showed to contain the characteristic signals of **4**.

About stereostructure of **4** unequivocal evidence is not yet available; however the structure depicted in Fig. 1 could be reasonably hypothesized, taking into account that most zinc complexes have a tetrahedral geometry. In addition this structure completely agrees with the ^{1}H-NMR spectrum of **4**, since in the proposed structure the zinc represents a chiral center.

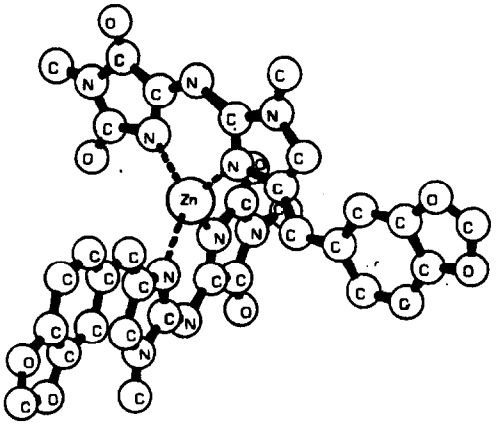


Figure 1. Hypothesized stereostructure for the zinc-complex of clathridine (**4**)

EXPERIMENTAL

General methods

EIMS were obtained at 70 eV on a Kratos MS80 mass spectrometer. IR spectra were recorded on a Perkin-Elmer Model 399 spectrophotometer in chloroform solution. UV spectra were performed on a Perkin-Elmer Model 550S spectrometer in methanol solution. ^{1}H- and ^{13}C-NMR experiments were performed on a Bruker WM-250 spectrometer in $CDCl_3$ and/or CF_3COOD solution. Proton chemical shifts are referenced to the residual chloroform signal (δ 7.24) in $CDCl_3$ solution and to acetone (δ 2.22) as internal standard in CF_3COOD solution. Carbon-13 chemical shifts are referenced to the solvent ($^{13}CDCl_3$= 77ppm; $^{13}CF_3COOD$= 115.7ppm). The multiplicity of ^{13}C resonances was determined by DEPT experiments which were performed using polarization transfer pulses of 90° and 135°, obtaining in the first case only signals for -CH groups and in the other case positive signals for -CH and -CH_3 and negative ones for -CH_2 groups. Polarization transfer delays were adjusted to an average C-H coupling of 135 Hz. The shift correlations with polarization transfer *via* ^{1}J coupling[4] were carried out adjusting the fixed delays to give maximum polarization for $J_{C,H}$= 135 Hz. The long range heteronuclear correlations[5] were performed with maximum polarization for $J_{C,H}$= 8 Hz, leading to ^{2}J and ^{3}J spots in the same spectrum. Medium pressure liquid chromatography was performed on a Büchi 861 apparatus using a SiO_2 (230-400 mesh) column, and chloroform-methanol mixtures as eluent. High performance liquid chromatography was performed on a Varian 5020 apparatus equipped with an UV detector, using a Hibar LiChrosorb Si60 (7x250 mm) column and chloroform-methanol 97:3 as eluent.

Extraction

Specimens of *C. clathrus* were collected by hand in the Bay of Naples at -20/25m during November 1987. Freshly collected animals (40 g, dry weight after extraction) were homogenized, then lyophilized and extracted with Et_2O (600 ml for 2 times), to remove fats and steroids, and with $CHCl_3$ (600 ml , 3 times) at room temperature for 5 days.

Isolation of **1**

The $CHCl_3$ extract was evaporated to yield a dark brown viscous oil (792 mg), which was chromatographed on a silica gel column under medium pressure (MPLC) using a solvent gradient system from chloroform to methanol. The early fractions which were eluted with $CHCl_3/CH_3OH$ 97:3, were combined to give 230 mg of an oil enriched in clathridine (**1**). Final purification was achieved by crystallization from $CHCl_3$ which produced 118 mg of **1**:
m.p. 260-262°C (dec.)
HRMS: found m/z 341.1116, calc. for $C_{15}H_{15}N_5O_4$ 341.1121;
MS: m/z (relative intensities) 341 (100) [M^+], 326 (2.3) [M-CH_3], 256 (12.5), 228 (16), 135 (6.5) [piperonyl], 113 (8.5);
IR ($CHCl_3$): ν_{max} 3260, 1793, 1737, 1716, 1670, 1620, 1443, 1392, 1309, 1117 cm^{-1};
UV (CH_3OH): λ_{max} 371 (ε=14700), 285 nm (ε=5400);
^{1}H- and ^{13}C-NMR: see Table 1.

Isolation of **4**

The mother liquors from the crystallization to obtain **1** (see above) were taken to dryness and chromatographed by HPLC, thus affording 8.7 mg of the pure compound **4**. Physical properties are as follows:

m.p. 158-160°C (yellow microcrystals from $Et_2O/CHCl_3$);
HRMS: found m/z 744.1430, calc. for $C_{32}H_{28}N_{10}O_8{}^{64}Zn$ 744.1377;
MS: m/z (relative intensities) 748 (46), 746 (65), 744 (100) [M^+]; 407 (13), 405(20), 403 (29) [M-clathridine]; 342 (9) [clathridine+H];
IR ($CHCl_3$): ν_{max} 1771, 1720, 1620, 1482, 1445, 1163 cm^{-1};
UV (CH_3OH): λ_{max} 399 (ε=13300, s), 372 (ε=22500), 361 (ε=22700), 285 nm (ε=9500);
^{1}H-NMR ($CDCl_3$): δ 6.63 (1H, s), 6.50 and 6.25 (2H, AB system, J=8.2 Hz), 6.24 (1H, s), 5.90 and 5.88 (2H, AB system, J=1.5 Hz), 3.80 (3H, s), 3.49 and 3.35 (2H, AB system, J=16.1 Hz), and 3.03 (3H, s).

Preparation of **4** from **1**

A CH_2Cl_2 solution of **1** (5.3 mg in 10 ml) and 0.1M aqueous $ZnSO_4$ (10 ml) were kept under stirring at room temperature for 1 hr. The organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated, thus affording 5.6 mg of **4**, which was identical to natural **4** by comparison of spectral and chromatographic data.

Methylation of **1** to obtain **2**

A suspension of 115 mg of **1** in an 1:1 ether/methanol mixture (20 ml) was treated with an excess of ethereal diazomethane under stirring for 1 hr, then evaporated. The residue was purified by TLC (0.5 mm SiO_2, eluent EtOAc/CH_3OH 9:1). The band at Rf 0.4 was scraped out and eluted with methanol, thus obtaining, after evaporation, 24 mg of **2**:
m.p. 181-183°C (pale yellow microcrystals from EtOAc);
MS: m/z 355 [M^+], 340 [M-CH_3], 270, 244, 220, 188, 149, 135 [piperonyl];
IR ($CHCl_3$): ν_{max} 2950, 1714, 1585, 1445, 1398, 1360, 1142 cm^{-1};
^{1}H- and ^{13}C-NMR: see Table 2.

Hydrolysis of **1**

A suspension of 8 mg of **1** in 1M aqueous NaOH was stirred at reflux. After 30 min workup was commenced by extraction with $CHCl_3$ (3 x 5 ml). The organic layers were combined, dried over Na_2SO_4 and evaporated under vacuum. The residue, a viscous oil, was purified by HPLC ($CHCl_3/CH_3OH$ 7:3), thus affording 2 mg of **3**:
HRMS: found m/z 231.0997, calc. for $C_{12}H_{13}N_3O_4$ 231.1009;
MS: m/z 231 [M^+], 216 [M-CH_3], 207, 179, 149, 135 [piperonyl];
^{1}H-NMR ($CDCl_3$): δ 6.74 and 6.71 (2H, AB system, J=7.5 Hz), 6.71 (1H, s), 5.99 (1H, s), 5.94 (2H, s), 3.70 (2H, s), and 3.03 (3H, s).

ACKNOWLEDGEMENT

This work is a result of research supported by M.P.I. (Italy). We wish to thank Dr. M. Pansini (Università di Genova, Italy) for identifying the sponge. Mass spectra data were provided by "Servizio di Spettrometria di Massa del CNR e dell' Università di Napoli". The assistance of the staff is gratefully appreciated.

REFERENCES

1. Aiello, A.; Ciminiello, P.; Fattorusso, E.; Magno, S. *Steroids*, in press.
2. Prasad, A. S. *Ann. Rev. Pharmacol. Toxicol.* **1979**, *20*, 393-426.
3. Wilkinson, G. (ed.), "Comprehensive Coordination Chemistry", Pergamon Press, Oxford, **1983**, Vol. V, pp. 926-1022.
4. Bax, A.; Morris, G. *J. Mag. Res.* **1981**, *42*, 501.
5. Kessler, H.; Griesinger, C.; Zambsck, J.; Loosli, R. *J. Mag. Res.* **1984**, *57*, 331.
6. Carmely, S.; Kashman, Y. *Tetrahedron Letters* **1987**, *28*, 3003.
7. Pretsch, E.; Clerc, T.; Seibl, J.; Simon, W. "Tables of Spectral Data for Structure Determination of Organic Compounds", Springer-Verlag, Berlin, **1983**, pp. H20 and H70.
8. Cuppels, D. A.; Stipanovic, R. D.; Stoessl, A.; Stothers, J. B. *Can. J. Chem.* **1987**, *65*, 2126.