Bull. Pharm. Sci., Assiut University, Vol. 30, Part 2, 2007, pp. 149-157.

IMIDAZOLE ALKALOIDS FROM THE INDOPACIFIC SPONGE *PERICHARAX HETERORAPHIS*

A. A. Ali[1], H. A. Hassanean[1], Ehab S. Elkhayat[2], Ru AnGelie Edrada[3], Rainer Ebel[3], and Peter Proksch[3]

[1]*Department of Pharamcocnosy, Faculty of Pharmacy, Assiut University, 71526 Assiut, Egypt*
[2]*Department of Pharamcocnosy, Faculty of Pharmacy, Al-Azhar University (Assiut branch , 71524 Assiut, Egypt)*
[3]*Institut für Pharmazeutische Biologie, Heinrich-Heine-Universität, Universitätsstr 1, 40225 Düsseldorf, Germany*

المسح الحيوي للأجزاء المختلفه من الخلاصه الكحوليه لاسفنج البيريكاراكس هيتيرورافيس الذى تم جمعه من المحيط الهندي اثبت أن جزء خلات الاثيل له تأثير مميت على ربيان الماء المالح بنسبة ٧٨% عند تركيز ٥ جزء في المليون. كما ان له تأثير مضاد للبكتريا من نوع: باسيلاس سبتيليس واستافيلاس ايوريوس عند تركيز ١٠٠ ميكروجرام ، هذا بالاضافة الى تأثيره المضاد لفطر كلادوسبوريوم هربارم عند نفس التركيز.

الدراسه الكيميائيه وعزل المواد الفعالة الموجودة في جزء خلات الاثيل تم من خلالها التعرف على ثلاثة مركبات هي: بريكلاثريدين أ والذي يفصل لاول مره من الاسفنجيات البحريه بالاضافة الى لويسيتامين أ ولويسيتامين ب اللذين يفصلان لاول مره من اسفنج البيريكاراكس هيتيرورافيس.

*Chemical investigation of the sponge Pericharax heteroraphis (Polejeff) collected in Indonesia, has led to the isolation of three imidazole alkaloids, preclathridine-A (**1**), leucettamine-B (**2**) and leucettamine-A (**3**). The structures of the isolated compounds were unambiguously established by ^{1}D and ^{2}D NMR and mass data. This is the first report of this class of compounds from the P. heteroraphis sponge. Investigation of the antimicrobial activities of the isolated compounds showed that leucettamin-A (**3**) was active against the gram-positive bacteria Staphylococcus aureus and the fungus Cladosporium herbarum, while other compounds were inactive.*

Received in 4/9/2007, Received in revised form in 12/12/2007 & Accepted in 13/12/2007

INTRODUCTION

Three 2-iminoimidazole alkaloids, preclathridine-A (**1**), leucettamine-B (**2**) and leucettamine-A (**3**) have been isolated for the first from the indopacific sponge Pericharax heteroraphis (Polejeff). The preliminary biological screening of the fractions obtained from the crude alcoholic extract of the calcareous yellow sponge, led to selection of the ethyl acetate fraction for further isolation work. Where the ethyl acetate fraction showed 78% mortality in the brine shrimp assay at a concentration of 5 ppm, and antimicrobial activity, against *B. subtilis* and *S. aureus* in concentration of 100 µg (inhibition zone of 8 and 9 mm respectively) and fungistatic activity of 7 mm against *C. herbarum*, as shown in Table 1.

The imidazole alkaloids have been recently reported to have interesting biological activities as: antifungal[1], cytotoxic[2&3], antiviral activity against HSV-1 and HIV-14 and an anti-inflammatory activity through cyclo-oxygenase inhibition[5]. The first isolation of 2-aminoimidazole alkaloids from marine sponges was done by Carmely *et al*. as he reported the isolation of the naamine A and its congeners from the Leucetta chagosensis[6&7] sponge, followed by the work done by Ciminiello et al that reported the isolation of clatheridines from the Clathrina clathrus sponge[8].

A literature survey of the sponge P. heteroraphis showed reports on the associated microbial population, without any chemical investigation of its metabolites[9]. In this paper, we report the first isolation of compound (**1**) from marine sponges and the first isolation of compounds (**2**) and (**3**) from the P. heteroraphis sponge.

EXPERIMENTAL

General experimental procedures

^{1}H and ^{13}C-NMR spectra were recorded on Bruker DRX 500 spectrometer. Mass spectra were measured on Finningan MAT 8430. Sephadex LH_{20} (25-100 mm mesh size, Merck). TLC was performed on TLC plates pre-coated with silica-gel F_{254} (Merck, Darmstadt, Germany). Semi-preparative HPLC was performed on HPLC system (Merck, Darmstadt, Germany) coupled with UV detector L7400 (UV detection was at 240 nm), the separation column (8 x 250 mm) pre-packed with Eurosphere C_{18} (Knauer, Berlin, Germany). The compounds were eluted with solvent system of H_2O/ MeOH (30/70), at flow rate of 5 ml/min.

Sponge material

The sponge *P. heteroraphis* belongs to the class Demospongiae, order Clathrinida, Family Desmacellidae. It was collected in Kapoposang island (Pulau Kapoposang), Indonesia in 1997 at a depth of 44 feet. It was kindly identified by Dr. W. M. Van Soest. A voucher specimen was kept in ethanol under the registration number ZMA POR. 17168.

Isolation

The sponge was stored in ethanol immediately after collection then freezed and kept frozen at -04°C till the time of processing. Prior to extraction, the sponge was freeze dried, ground (31.1 g), and then was exhaustively extracted with methanol (3 x 700 ml). The methanol extract was evaporated under vacuum to afford 2.31 g of reddish brown residue. The dried extract was partitioned between distilled water (200 ml) and the following solvents: n-hexane (0.27 g), ethylacetate (0.68 g) and n-butanol (0.19 g), finally the remaining aqueous layer was evaporated (1.03 g). The obtained fractions were screened for their biological activities using brine shrimp mortality and antimicrobial assays[10]. The ethyl acetate fraction was chromatographed on sephadex LH_{20} and eluted with methanol to afford 5 fractions. The second fraction afforded leucettamina-A (**3**) as a single component that showed a single spot on TLC with an R_f of 0.54 (dichloromethane/methanol/water, 65/30/5). The third and fourth fractions were combined together (two spots, R_f values 0.61 and 0.65, dichloromethane/methanol/water, 65/30/5), and subjected for further purification by semi-preparative HPLC to afford Preclathridine-A (**1**) and leucettamine-B (**2**).

Bioassays

Antimicrobial assay[10]

Sterile filter paper discs were impregnated with 100 and 50µg of the obtained fractions and with 10 and 5 µg of the pure isolated compounds. Then the impregnated discs were placed on agar plates previously inoculated with: *Bacillus subtilis*, *Staphylococcus aurous*, *Escherichia coli*, *Candida albicans* and *Cladosporium herbarum*. Benzyl penicillin and gentamycin were run against the bacterial strains as a positive control. The plates were checked for inhibition zones after 24 h of incubation at 37°C; the antimicrobial activity was recorded as a clear zone (mm) of inhibition surrounding the disc. The test sample was considered active when the inhibition zone was greater than 7mm.

Table 1: Antimicrobial activity of the fractions of *P. heteroraphis* extract.

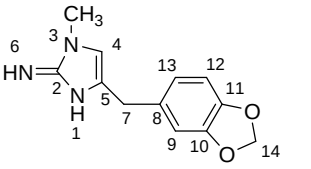
Fracction	*S. eureus*		*E. coli*		*B. subtilis*		*C. herbarum*	
	50 µg	100 µg	50 µg	100 µg	50 µg	100 µg	50 µg	100 µg
n-Hexane	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*
EtoAc	n.a*	9	n.a*	n.a*	n.a*	8	n.a*	7
n-BuOH	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*
Aq.	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*	n.a*

* n.a. : not active.

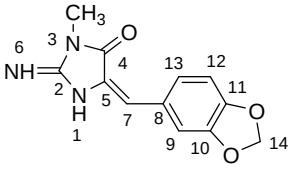
Brine shrimp assay[11]

Eggs of *Artemia salina* (Dohse, Aquaristik GmbH, Bonn, Germany) were hatched in small tanks filled with artificial seawater which was prepared with a commercial salt mixture (Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany) and distilled water. After 48 hrs, 20 napulii were transferred into 10 ml tubes containing 250 and 500 µg of the tested fractions and 20 µL of DMSO. Artificial sea-water was added to final volume of 5 mL. Control vials containing DMSO alone were prepared in the same way. The lethality percent at each dose level, including the control was determined after 24 hrs.

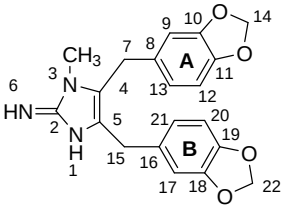
RESULTS AND DISCUSSION



1



2



3

Fig. 1: Structures of the isolated compounds **1-3**.

Compound 1, was isolated as a yellow amorphous solid; UV λ_{max} (MeOH) 202 and 285 nm. The EIMS showed a molecular ion peak at m/z 231 $[M+H]^+$, with significant fragment ions at m/z 216 $[M-15]^+$ and 135 $[M-96]^+$. The ^{1}H-NMR (Table 2), showed the presence of two exchangeable protons at δ_H12.13 (1H, br.s) and 7.47 (1H, s), as well as three aromatic protons of the ABX system; at δ_H 6.85 (1H, d, *J*= 7.88 Hz), δ_H 6.82 (1H, d, *J*= 1.58 Hz) and δ_H 6.71 (1H, dd, *J*= 1.58 and 7.88 Hz), in addition to a methylene group at δ_H 5.97 (2H, S) and a methyl attached to hetero-atom at δ_H 3.34 (3H, S). The ^{13}C-NMR (Table 3), showed the presence of 12 carbons. The methylene resonance at 100.8 ppm was assigned to a methylenedioxy residue[5,12], and the methyl resonance at 31.7 ppm was assigned to an N-methyl substituent[2].

The HMBC (Fig. 2), cross peaks of the N-methyl with C-2 (δ_C 146.3) and *C*H-4 (δ_C 113.8), and the cross peak of C*H*-4with C-2 and C-5 (δ_C 146.3 and 125.5, respectively) afforded an N-methyl-2-imino-imidazoline moiety. The cross peaks of CH_2-14 with two oxygenated quaternary carbons C-10 and 11 (δ_C 147.3 and 145.9, respectively) afforded its connection to the benzenoid structure through the two oxygens which identified a piperonyl moiety[8]. The cross peaks of CH_2-7 with the aromatic carbons C-8, C-9 and C-13 (δ_C 131.2, 109.0 and 121.5, respectively) afforded its connection to the piperonyl moiety. Finally, the cross peaks of CH_2-7 with C-5 and

Table 2: ^{1}H-NMR data of compounds **1-3** in DMSO-d_6 at 500 MHz.

Pos.	**1**	**2**	**3**
1	12.13 (1H, br.s)	12.23 (1H, br.s)	12.09 (1H, br.s)
2	-	-	-
3	-	-	-
4	6.61 (1H, s)	-	-
5	-	-	-
6	7.47 (1H, s)	7.5 (1H, s)	7.43 (1H, s)
7	3.66 (2H, s)	6.64 (1H, s)	3.89 (2H, s)
8	-	-	-
9	6.82 (1H, d, *J*= 1.58 Hz)	6.83 (1H, d, *J*= 1.58)	6.63 (1H, d, *J*=1.58)
10	-	-	-
11	-	-	-
12	6.85 (1H, d, *J*= 7.88 Hz)	6.86 (1H, d, *J*= 7.88 Hz)	6.81 (1H, d, *J*= 7.88 Hz)
13	6.71 (1H, dd, *J*= 1.58, 7.88)	6.71 (1H, dd, *J*= 1.58,7.88)	6.69 (1H, dd, *J*= 1.58, 7.88)
14	5.97 (2H, s)	5.97 (2H, s)	5.97 (2H, s)
15	-	-	3.78 (2H, s)
16	-	-	-
17	-	-	6.79 (1H, d, *J*= 1.58 Hz)
18	-	-	-
19	-	-	-
20	-	-	6.83 (1H, d, *J*= 7.88 Hz)
21	-	-	6.59 (1H, dd, *J*= 1.58, 7.88)
22	-	-	5.95 (2H, s)
N-CH_3	3.34 (3H,s)	3.36 (3H,s)	3.15 (3H,s)

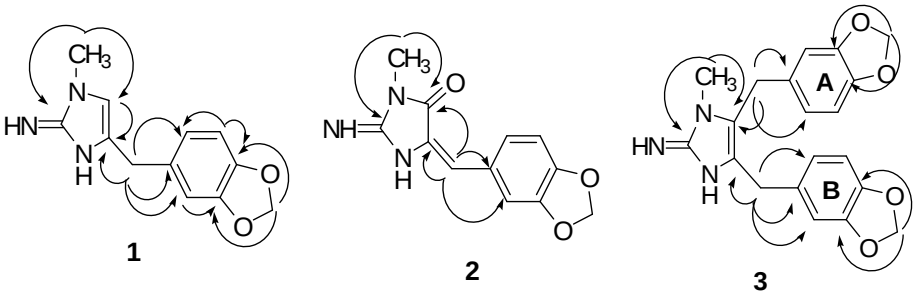


Fig. 2: Important HMBC correlations of compounds **1**-**3**.

Table 3: ^{13}C-NMR data of compounds **1**-**3** in DMSO-d_6 at 125 MHz.

Pos.	**1**	**2**	**3**
1	-	-	-
2	146.3	161.9	146.1
3	-	-	-
4	113.8	172.0	121.8
5	125.5	141.8	122.1
6	-	-	-
7	29.6	115.8	27.1
8	131.2	132.7	131.0
9	109.0	109.1	108.4
10	147.3	148.4	147.6
11	145.9	148.0	145.7
12	108.2	108.2	108.3
13	121.5	126.0	121.0
14	100.8	100.6	100.8
15	-	-	28.5
16	-	-	132.0
17	-	-	108.8
18	-	-	147.4
19	-	-	146.0
20	-	-	108.2
21	-	-	121.3
22	-	-	101.0
N-CH_3	31.7	26.8	29.4

CH-4 assigned compound **1** as: 2-Imino-3-methyl-5-(3,4-methylene-dioxybenzyl)-imidazole. The mass data afforded further evidence on the structure, as the fragment ion at m/z 216 indicated the loss of methyl functionality, and the intense fragment at m/z 135 referred to the piperonyl moiety, which in the same time indicated the loss of the methylimidazoline moiety [M-96]$^{+}$ [13].

In fact the structure of compound (**1**) is a resonating form of the 2-amino-3-methyl-5-(3,4-methylene-dioxybenzyl)-imidazole previously known as Preclathridine-A that was first isolated from the nudibranch *Notodoris garineri*[4]. However, this is the first isolation of Preclathridine-A from sponges, in addition to the first isolation of the 2-imino form of this compound.

Compound 2, was isolated as a yellowish white amorphous solid; UV λ_{max} (MeOH) 201.7, 238.3 and 366.2 nm. The EIMS, showed a molecular ion peak at m/z 245 $[M]^+$. The ^{1}H-NMR spectrum (Table 2), showed the signals associated with N-methyl-2-iminoimidazole structure as it showed two exchangeable protons at δ_H 12.23 and 7.5 ppm (1H, s, each) and the methyl signal at δ_H 3.36 (3H, s), as in compound (**1**). Moreover, the spectrum showed the presence of a piperonyl moiety in the structure as it showed the signals of the methylene-dioxy residue at δ_H 5.97 (2H, s), and the ABX system of an ortho-coupled proton at δ_H 6.86 (1H, d, *J*= 7.88 Hz), meta-coupled proton at δ_H 6.83 (1H, d, *J*= 1.58 Hz) and ortho-meta coupled proton at δ_H 6.71 (1H, dd, *J* = 7.88 and 1.58 Hz). The ^{13}C-NMR spectrum (Table 3), showed resonances associated with 12 carbons. In comparison with compound (**1**), the spectrum was quite similar, except the presence of a carbonyl at δ_C 172.0 ppm in addition to a down field shift of the carbons associated with iminoimidazoline moiety. Aided with HMQC, six of the twelve carbons were associated with nine protons, and the olefinic carbon at δ_C 115.8 was associated with a proton at δ_H 6.64 (1H, br.s). The HMBC experiment (Fig. 2), showed the cross peaks of the N-methyl with C-4 and C-2 (δ_C 172.0 and 161.9, respectively), which confirmed the N-methyl-iminoimidazolone structure. The cross peaks of the olefinic proton C*H*-7 (δ_H 6.64) with C-4, 5, 8, 9 and C-13 (δ_C 172.0, 141.8, 132.7, 109.1 and 126.0, respectively). Accordingly, compound (**2**) was assigned as: 2-imino-3-methyl-5-(3,4-methylenedioxybenzyl)-imidazole-4-one, which is the imino form of the previously known compound Leucettamine-B [2-amino-3-methyl-5-(3,4-methylenedioxybenzyl)-imidazole-4-one] that was first isolated from the Pauluan sponge; *Leucetta microraphis*[5]. However, this is the first isolation of the imino form of leucettamine-B from *P. heteroraphis* sponge, in addition to the first isolation of the imino form of leucettamine-B form marine sponges.

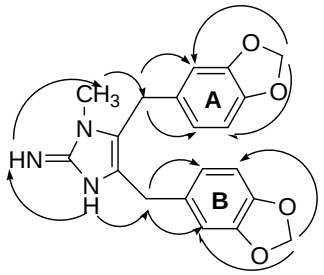
Compound 3, was isolated as a brownish amorphous solid; UV λ_{max} (MeOH) 202.7, 237 and 287.3 nm. The EIMS showed a molecular ion peak at m/z 365 $[M]^+$, with significant fragment ions at m/z 231 and 135. The ^{1}H-NMR (Table 2), showed two exchangeable protons at δ_H 12.09 (1H, br.s) and 7.43 (1H, s) and an N-methyl at δ_H 3.15 (3H, s), which in comparison with compound (**1**) were assigned for N-methyl-2-imino-imidazoline structure. In addition, the proton spectrum revealed the presence of two pipronyl moieties as it showed signals assigned for two benzylic methylenes at δ_H 3.89 and 3.78 (2H, br.s each), and two methylenedioxy moities at δ_H 5.97 and 5.95 (2H, s each), and two ortho coupled aromatic protons at δ_H 6. 83 and 6.81 (1H, d, *J*= 7.88 Hz, each), two meta coupled aromatic protons at δ_H 6.79 and 6.63 (1H, d, *J*= 1.57 Hz,

each), in addition to the two ortho-meta coupled protons at δ_H 6.69 and 6.59 (1H, dd, *J*= 7.88, 1.57 Hz, each). This was strengthened by the mass data as the difference from compound (**1**) was 134 mass units, which could be referred to another pipronyl moiety.

The ^{13}C-NMR (Table 3), showed the presence of twenty carbons, aided with DEPT experiments it revealed the presence of nine quaternary carbons, six methines and four methylenes, in addition to an N-methyl. On comparison to compound (**1**), the methine at δ_C 113.9 ppm was absent and there was an additional quaternary carbon at δ_C 121.8ppm, suggesting a disubstituted imidazole ring[2].

The HMBC (Fig. 2) supported by HMQC data, showed a cross peaks of the N-methyl with the two quaternary carbons C-2 and 4 (at δ_C 146.1 and 121.8, respectively), which confirmed the N-methyl-iminoimidazole structure disubstituted at C-4 and C-5. The cross peaks of the two aromatic protons *H*-12 and *H*-9 (δ_H 6.81 and 6.63, respectively) with C-10 and 11 (δ_C 147.6 and 145.8, respectively), and the cross peaks of the methylenedioxy C*H*$_2$-14 with C-10 and C-11, and the cross peaks of methylene C*H*$_2$-7 with C-8, 13 and C-9 (δ_C 131.0, 121.0 and 108.4, respectively) identified system A. In the same way, cross peaks of the two aromatic protons C*H*-17 and C*H*-20 (δ_H 6.79 and 6.83, respectively) with C-18 and C-19 (δ_C 147.4 and 145.9, respectively), and the cross peaks of the methylenedioxy C*H*$_2$-22 (δ_H 5.95) with C-18 and C-19, in addition to the cross peaks of C*H*$_2$-15 (δ_H 3.78) with the aromatic carbons C-16,17 and C-21 (δ_C 132.0, 108.8 and 121.3, respectively) identified system B. The cross peaks of C*H*$_2$-7 with the quaternary carbon at δ_C 121.8 (*C*-4), and the correlation of C*H*$_2$-15 with C-5 (δ_C 122.1), established the structure of compound (**3**).

The ROESY experiment (Fig. 3), has confirmed the structure as it showed the diagnostic through space correlations of the benzylic methylene C*H*$_2$-7 with the aromatic protons at H-9 and H-13 which identified system A. And the through space correlations of the second benzylic methylene C*H*$_2$-15 with the aromatic protons H-17 and H-21 as system B. The correlations of N*H*-1 with N*H*-6, which in turn correlated with the N-methyl, and the correlations of N*H*-1 with C*H*$_2$-15, in addition to the correlations of the N-methyl, with C*H*$_2$-7, confirmed structure **3** as 4,5-Bis(1,3-benzodioxol-5-ylmethyl)-1-methyl-imidazol-2-imine, which is a resonating form of 4,5-Bis(1,3-benzodioxol-5-ylmethyl)-1-methyl-imidazol-2-amine previously known as leucettamine A, which was first isolated from the Pauluan sponge; *Leucetta microraphis* sponge[5]. However, this is the first isolation of leucettamine A from *P. heterraphis* sponge, in addition to the first isolation of the 2-imino form of this compound from marine sponges.



3

Fig. 3: Important NOE correlations of compound **3**.

The antimicrobial activity of the isolated compounds was tested on an agar plate diffusion assay against *S. aureus*, *B. subtilis* and *E. coli* as antibacterial, and against *C. albicans* and *C. herbarum* as antifungal compound. Leucettamine-A (**3**) displayed moderate antibacterial activity at a concentration of 10µg against the gram-positive bacteria S. *aureus* (9mm), as well as a fungistatic activity at the same concentration towards the pathogenic plant fungus *C. herbarum*, as it exhibited distinct but an overcastted inhibition zone.

REFERENCES

1- D. C. Dunbar, J. M. Rimoldi, A. M. Clark, M. Kelly and M. T. Hamann, Tetrahedron, 56, 8795 (2000).
2- H. Gross, S. Kehraus, G. M. Koenig, G. Woerheide and A. D. Wright, J. Nat. Prod., 65, 1190 (2002).
3- R. K. Akee, T. R. Carroll, W. Y. Yoshida, P. J. Scheuer, T. J. Stout and J. Clardy, J. Org. Chem., 55, 1944 (1990).
4- P. Molina, P. M. Fresneda and M. A. Sanz, ibid., 64, 2540 (1999).
5- G. W. Chan, S. Mong, M. E. Hemling, A. J. Freyer, P. H. Offen J. W. Westly, C. W. DeBrosse, H. M. Sarau and J. W. Westley, J. Nat. Prod., 56, 116 (1993).
6- S. Carmely and Y. Kashman, Tetrahedron Letters, 28, 3003 (1987).
7- S. Carmely, M. Ilan and Y. Kashman, Tetrahedron, 45, 2193 (1989).
8- P. Ciminiello, E. Fattorusso, S. Magno and A. Mangoni, ibid., 45, 3873 (1989).
9- C. R. Wilkenson, Marine Biology, 49, 161, 169, 177 (1987).
10- E. Elkhayat, R. Edrada, R. Ebel, V. Wray, R. van Soest, S. Wiryowidagdo, H. M. Mohammed, W. E. G. Muller and P. Proksch, J. Nat. Prod., 67, 1809 (2004).
11- B. N. Meyer, N. R. Ferrigni, J. E. Putnam, L. B. Jacobsen, D. E. Nichols and J. L. Mclauglin, Planta Medica, 45, 31 (1982).
12- H. He and D. J. Faulkner, J. Org. Chem., 57, 2176 (1992).
13- K. A. Alvi, B. M. Peters, L. M. Hunter and P. Crews, Tetrahedron, 49, 329 (1993).