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ETZIONIN, A NEW ANTIFUNGAL METABOLITE FROM A RED SEA TUNICATE

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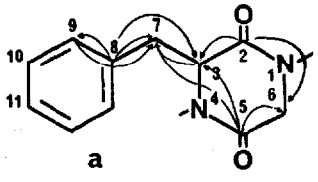
Abstract - The structure of etzionin (1), a new diketopiperazine hydroxamate derivative isolated from a Red Sea tunicate has been determined by combined one and two dimensional NMR and mass spectral techniques.

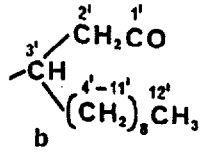
In the course of screening for novel antifungal agents the 5%MeOH, $CHCl_3$ extract of an unidentified red tunicate collected in the Northern part of the Gulf of Eilat (the Red Sea) was found to possess both cytotoxic and antifungal activities (IC_{50} (P 388) 5μg/ml, MIC (*C. albicans*) 31μg/ml)[1]. Repeated chromatography on a Sephadex LH-20 column eluted with $CHCl_3$; petrolether; MeOH 1:2:1 afforded compound **1** (0.01% dry wt of the organism) which was responsible for the antifungal activity of the tunicate (MIC 3μg/ml)[1].

Compound **1**, designated etzionin[2] (Rf=0.1, on a silica gel plate eluted with $CHCl_3$; MeOH 8:2 and visualized by UV) gave a blue color with $Co(SCN)_2$ and a red one with $FeCl_3$ pointing to a nitrogenous metabolite possessing a chelating enolic or hydroxamic OH group[3].

Etzionin, **1**, which was difficult to purify, exhibited broad lines in the ^{1}H-NMR spectrum[4] typical for many polar polyamino and/or hydroxy compounds. Acetylation of compound **1** with a mixture of Ac_2O/Pyridine at 60° for 4h furnished diacetate **2**[5] which was easier to purify chromatographically and, in contrast to **1**, gave a well defined NMR spectrum. The NMR data suggested a mono substituted phenyl group, five carbonyls and a long aliphatic chain. Furthermore, the ten degrees of unsaturation of **2** required an additional ring. Intensive NMR studies including COSY, CH-correlations, COLOC and d-NOE experiments proposed for **2** the following three segments (**a**-**c**):



a



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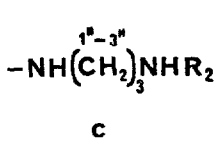


c

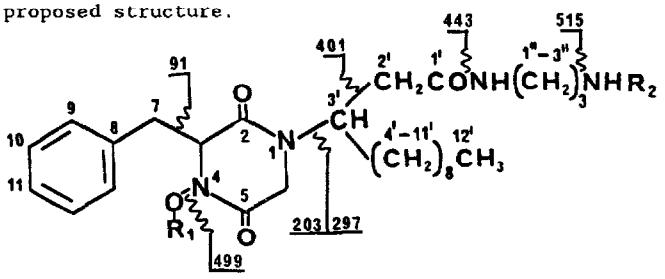
(the above three fragments do not include a second acetyl group).

Additionally, a long range HH-correlation between H-6 and H-3'suggested that portion **b** is connected to N(1) of the diketopiperazine ring of **a** and therefore the amide carbonyl of **b** (δ_c 170.1s) has to be linked to the amino group of **c**.

Diacetate **2** turned out to be relatively unstable; it decomposes on standing for several days or during a few minutes in dilute ammoniacal methanol to give a mono acetate **3**[6]. Whereas compound **2** gave a negative $FeCl_3$ test, the mono acetate **3** exhibited a positive one.

The best rationale for the above data is the presence of a hydroxamate group in 1. The relatively acidic hydroxamate OH was one of the two groups which reacted with Senger's reagent[3], and could also be methylated with CH_2N_2 furnishing from 3 the C-3"-acetamido-N(4)-O-methyl, derivative (4).

The location of the hydroxamate at N(4) of 2 was mainly suggested on the basis of the 203/297 fragments in the mass spectrum[5]. These and the other fragmentations are in full agreement with the proposed structure.

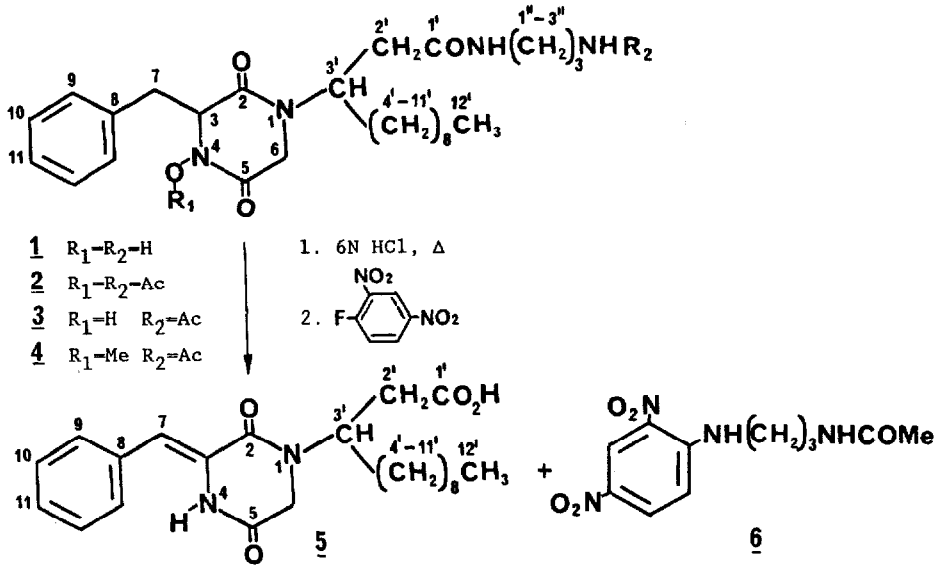


Unequivocal confirmation of the structure of 1 including the location of the hydroxamate functionality was obtained from the acidic hydrolysis degradation products of compound 2 (refluxing in 6N HCl for 24h). Following the usual work-up the crude reaction mixture was treated with fluoro-2,4-dinitro-benzene and then submitted to chromatography on a silica gel column. Elution with 5% MeOH in CH_2Cl_2 afforded two major compounds: acid-5[7] and a 2,4-dinitrophenylamine derivative 6[8]. Compound 6 was readily identified as the 1-acetamido-3-(2,4-dinitrophenyl)propane-1,3-diamine thus confirming the 1,3-diaminopropane moiety of 1. The 3-(2-benzylidene-1,4-diketopiperazinyl)dodecanoic acid structure for 5 was proposed on basis of its NMR and mass spectra[7].

Negative $FeCl_3$ test and no acetylation suggested that compound 5 lost its hydroxamic OH group. Furthermore, the NMR data pointed clearly to the presence of an additional double bond (δ_H 7.01s, δ_C 125.6s&116.7d). The location of the latter bond between the phenyl and C-2 of the diketopiperazine was deduced from: a. disappearance, in the ^1H NMR, of the benzyl ABX system of 2; and b. a bathochromic shift in the UV spectrum (from 260 in case of 2, to 310 (11400)nm). Loss of the hydroxamic OH and obtaining of a styrene moiety confirmed the suggested N(4) location of the OH group. The formation of the 3(7)Z-double bond in 5[7] is best explained by acidic elimination of the N(4)OH to the corresponding imine followed by tautomerization of the double bond to furnish the conjugated α-amino cinammamide moiety. Hydrogenation of 5 over Pd/C yielded the expected 3,7-dihydro derivative 8[9].

Marine 1,4-diketopiperazines, most likely derived from two amino acids, have already been reported[10]. Compound 1 is unique in its hydroxamate functionality on one hand, and in the β-amino dodecanamide chain on the other hand. Diketopiperazine hydroxomates are known as fungal metabolites[11], this again raises the well known question of the real source (the microorganism vs. the host animal) of this and many other marine metabolites. Biogenetically, a condensation between phenylalanine and glycine is expected to form the diketopiperazine. Also common is the 1,3-diaminopropane segment. The coupling route between the heterocycle and the dodecanamide at its β-position is less clear, a possible

precursor is the $\alpha\beta$-unsaturated acid. An alternative route will start with the β-glycine dodecanamide.



The antifungal activity of etzionin against the pathogenic yeast Candida albicans is of interest: an MIC of 3 μg/ml in RPMI-1640 and 12.5 μg/ml in Sabouraud dextrose broth shows good inhibition of fungal growth. Activity was also seen against Aspergillus nidulans and Bacillus subtilis. There was no activity against gram negative bacteria. Further work will determine the utility of this compound in animal models.

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References and Notes

1. The biological activity was determined in the laboratories of Harbor Branch Oceanographic Institution Ft. Pierce, Florida by Drs. Neal Burres and Peter McCarthy. The P388 IC_{50} of compound 1, 10μg/ml suggests that it is not the only active cytotoxic compound in the extract.
2. Etzionin was named after Etzion Gaver - the biblical name of the port of Eilat.
3. Compound 1 yields with fluoro-2,4-dinitrobenzene a yellow amorphous N,O-di-dinitrobenzene derivative, mp 62°C; δ_H ($CDCl_3$, coupling constants in Hz): 8.93d(1H,2.6), 8.75brs(1H), 8.40dd(1H,9.2,2.5), 8.25dd(1H,9.4,2.5), 7.30m(5H), 6.95d(1H,9.6), 6.39brs(1H), 4.73t(1H,3.3), 4.2brs(1H), 3.71d(1H,17.3), 3.65dd(1H,14.3,4.9), 3.50m(4H), 3.33dd(1H,14.3,2.9), 2.68m(1H), 2.63d(1H,17.3), 2.33dd(1H,15.5,5.8), 2.00 quintet(2H,4.5), 1.70m(4H), 1.60brt(long chain), 0.88t(3H,6.4); δ_C 148.1 and 155.5 - the two carbons of the dinitrobenzene moiety attached to the N and O atoms respectively.
4. Etzionin, 1, a foaming oil, δ_H ($CDCl_3$+CD_3OD) 7.5-7.1br, 6.3-5.5br, 4.5-3.2br, 2.3-1.2br, 0.90brt(J=7.0).

5. Compound 2, a foaming oil, $[\alpha]_D$+14(c,0.1,MeOH) ν_{max} 1698,1667,1654cm^{-1}; λ_{max}(MeOH) 260(1300)nm; HRMS (FAB,MH$^+$): 559.3562($C_{30}H_{47}N_4O_6$,-6.6mmu), 517($C_{28}H_{45}N_4O_5$-5.6mmu), 501($C_{28}H_{45}N_4O_4$-4.7mmu), 443($C_{25}H_{35}N_2O_5$,-1.6mmu), 401($C_{23}H_{33}N_2O_4$, +0.1mmu), 383($C_{23}H_{31}N_2O_3$ +0.6mmu); CIMS(NH_3): 559 (MH$^+$, 19%), 499(M-$C_2H_3O_2$, 100%), 443(M-$C_5H_{11}N_2O$, 9%), 297($C_{17}H_{33}N_2O_2$, 60%), 203($C_{11}H_{11}N_2O_2$, 57%), 91(C_7H_7, 5%), δ_H($CDCl_3$, 360.13MHz) (J in Hz): 7.25m(H-10-12), 7.15m(H-9,13), 6.30brs(NH), 4.50t(H-3, 3.8), 4.10brs(H-3'), 3.55d and 2.50d(AB,H_2-6, 17.1), 3.20m(H-7,1",3"), 2.45dd and 2.15dd (H_2-2', 14.3, 6.5), 2.28s(OAc), 1.98s(NAc), 1.29 (H-4'-11'), 1.60q(H-2",6.2), 0.90t(H-12',6.9); δ_c ($CDCl_3$, 90.5MHz) (C-No): 160.0s(2), 64.5d(3), 166.1s(OAc), 164.0s(5), 45.8t(6), 36.0t(7), 134.0s(8), 129.9d(9), 128.8d(10), 127.4d(11), 170.1s(1'), 38.7t(2'), 54.4d(3'), 29.2t, 31.5t(4'-11'), 13.7q(12'), 36.0t(1"), 30.4t(2"), 36.0t(3").

6. Compound 3: a solid oil, DCI(i-Bu): 517(MH$^+$, 100%), 499(M-H_2O, 79%); δ_H($CDCl_3$): 3.5-3.0m(7H), 1.98s(3H); all other resonance lines as for 2.

7. Compound 5: EIMS: 400(M$^+$, 10%), 382(M-H_2O, 31%), 341(M-$C_2H_3O_2$, 8%), 202($C_{11}H_{10}N_2O_2$, 100%), 106(C_7H_8N, 2%); ν_{max}($CHCl_3$): 3500-2500br, 2900, 1695, 1628, 1200cm^{-1}; λ_{max}(MeOH): 225(8000), 310(11400); δ_H($CDCl_3$, coupling constants in Hz): 8.00s(NH, exch. by D_2O), 7.44m(2H), 7.35m(3H), 7.01s(1H), 6.09brs(OH, exch. by D_2O), 4.57m(1H), 4.15s(2H), 2.82dd(1H,15.6,10.5), 2.57dd(1H,15.6,5.0), 1.70m(2H), 1.25brt(14H, long chain), 0.87t(3H,6.5); δ_c($CDCl_3$): 172.1s, 162.5s, 158.7s, 132.9s, 129.4d(x2), 128.7d, 128.3d(x2), 125.6s, 116.7d, 54.2d, 47.5t, 38.6t, 31.8t, 31.4t, 29.4t(x4), 26.3t, 22.7t, 14.1q. The Z-configuration of the double bond was deduced from the chemical shift of H-7; an E configaration would give a ca. 6.6ppm value.

8. Compound 6: EIMS: 282(M$^+$, 2%), 252(M-NO, 9%), 167($C_6H_3N_2O_4$, 27%), 116($C_5H_{12}N_2O$, 100%); ν_{max}($CHCl_3$): 1680, 1600; δ_H($CDCl_3$, coupling constants in Hz): 9.13shd(1H, 2.7), 8.80brs(1H,exch. by D_2O), 8.26dd(1H,9.5,2.6), 6.90d(1H,9.5), 5.90brs(1H, exch. by D_2O), 3.48q(2H,6.6), 3.43q(2H,6.4), 2.03s(3H), 1.94q(2H,6.6); δ_C($CDCl_3$): 171.1s, 148.2s, 135.9d, 124.3d, 113.7d, 42.9t, 38.7t, 29.2t, 23.2q.

9. Disappearance of the singlet of 5 at δ 7.01, and the appearance instead of an ABX system at δ 4.50, 3.20 ppm.

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