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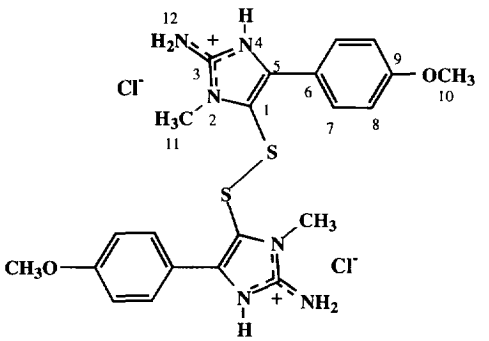
POLYCARPINE DIHYDROCHLORIDE: A CYTOTOXIC DIMERIC DISULFIDE ALKALOID FROM THE INDIAN OCEAN ASCIDIAN *POLYCARPA CLAVATA*

Heonjoong Kang and William Fenical*

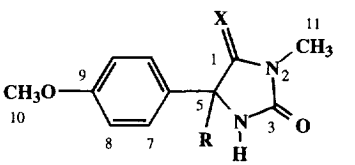
Scripps Institution of Oceanography
University of California at San Diego
La Jolla, California 92093-0236

Summary - Polycarpine dihydrochloride (**1**), a new cytotoxic dimeric disulfide alkaloid, and four related compounds (**2-5**) have been isolated from extracts of the ascidian *Polycarpa clavata* (Hartman, 1919) collected in Western Australia. The structure of the new alkaloid was determined on the basis of comprehensive spectral studies and by interconversion to its free base and several degradation products. On silica chromatography, **1** was converted to the corresponding unstable free base (**2**) which decomposed, via cleavage of the disulfide bridge, into monomeric derivatives. Polycarpine dihydrochloride is cytotoxic against the human colon tumor cell line HCT-116 at 0.9 μg/ml. Copyright © 1996 Elsevier Science Ltd

Ascidians, or tunicates, are clearly one of the most interesting groups of chemically-prolific marine invertebrates.[1] Recent chemical and biological investigations have revealed the importance of their secondary metabolites in providing a chemical basis for survival of the adults and their larvae in predator-rich habitats.[2] As part of continuing studies of the chemical adaptations of taxonomically-diverse ascidians, we encountered the solitary ascidian *Polycarpa clavata* in Western Australia living conspicuously on shallow sandy reef flats highly exposed to carnivorous predators. Our prior investigation of the related ascidian *P. aurata* resulted in the isolation of polycarpamines A-E, unusual, bioactive metabolites containing a high percentage of sulfur and novel functional groups.[3] On this basis, the metabolites of *P. clavata* have now been fully explored leading to the isolation of a novel dimeric disulfide alkaloid, polycarpine dihydrochloride (**1**). Alkaloid **1** was only obtained under mild chromato-



1 - polycarpine dihydrochloride
2 - polycarpine (free base)



3 - R = OMe, X = S
4 - R = OH, X=S
5 - R = OMe, X = O

graphic conditions. If silica gel was used, for example, **1** was readily converted to the free base **2,** and three degradation products (**3-5**) which appear to be artifacts of the isolation process.

Freeze-dried *P. clavata* (101g dry weight) was extracted twice with 70 % MeOH/CH_2Cl_2. The combined extract was concentrated and partitioned into hexane, ethyl acetate, n-butanol, and water. Gel-filtration of the n-butanol fraction through Sephadex LH-20 (50 % MeOH/CH_2Cl_2), followed by high speed countercurrent chromatography ($CHCl_3$/MeOH/H_2O = 4/4/3), gave polycarpine dihydrochloride (**1**) as the sole metabolite. Size-exclusion chromatography of the ethyl acetate fraction (LH-20; 50 % MeOH/CH_2Cl_2) and reversed-phased HPLC (ODS-silica) with 45 % H_2O/MeOH and 30 % H_2O/MeOH yielded alkaloids **3-5**. A separation scheme, with the same n-butanol fraction, but using silica flash chromatography (CH_2Cl_2/MeOH gradient) and reversed-phased HPLC (20, 25, 40 % H_2O/MeOH), gave only the free base **2**.

Polycarpine dihydrochloride (**1**), a water-soluble compound, was obtained as orange rods, mp 201-203°C. The molecular formula, $C_{22}H_{24}N_6O_2S_2$, established by HRFABMS [$(M+H)^+$ *m/z* 469.1503, dev. 4.8 ppm] showed 14 degrees of unsaturation. The isotopic cluster observed confirmed that **1** contained two sulfur atoms.[4] Since only 11 carbon signals were observed in the ^{13}C NMR spectrum of **1** (Table), a symmetry element appeared to be present.

Table. NMR assignments for polycarpine dihydrochloride (1) and free base 2.

No.	1[a]			2[a]		
	^1H	^{13}C[b]	HMBC (8 Hz)	^1H	^{13}C[b]	HMBC (8 Hz)
1 (1')		108.7			110.1	
3 (3')		147.2			152.3	
4 (4')	13.5 (bs, 2H)					
5 (5')		137.1			ND	
6 (6')		117.3			124.6	
7 (7', 7", 7"')	7.42 (bd, 4H, *J* = 8.5 Hz)	127.4	C5 (C5'), C7' (C7, C7"', C7"), C9 (C9')	7.37 (bs, 4H)	129.2	C7' (C7, C7"', C7"), C9 (C9')
8 (8', 8", 8"')	6.98 (d, 4H, *J* = 8.5 Hz)	113.9	C6 (C6'), C8' (C8, C8"', C8"), C9 (C9')	6.86 (d, 4H, *J* = 8.5 Hz)	114.7	C6 (C6'), C8' (C8, C8"', C8"), C9 (C9')
9 (9')		160.3			161.6	
10 (10')	3.87 (s, 6H)	55.5	C9 (C9')	3.83 (s, 6H)	56.3	C9 (C9')
11 (11')	3.20 (bs, 6H)	28.8	C1 (C1'), C3 (C3')	3.14 (bs, 6H)	29.3	C1 (C1'), C3 (C3')
12 (12')	7.68 (bs, 4H)					

Data for **1** were acquired in DMSO-d_6 while data for **2** were acquired in MeOH-d_4. [a]All ^1H NMR experiments were performed at 500 MHz, while all ^{13}C experiments were performed at 50 MHz. [b]Assignment of signals was based on HMQC and HMBC experiments at 500 MHz.

Exposure of **1** to acetate salts in water led to a new compound (**1a**) possessing two acetate counter ions, thus demonstrating the dicationic character of this metabolite.[4] The high content of nitrogen and its water solubility suggested 2-aminoimidazolium functionalities, which were further indicated by IR absorptions at 3351(NH_2), 2833, 2762, 2704 (NH_2^+, ammonium bands),[5] 2390-2000 (immonium bands), 1673 (C=N), 1503 (Ar-NH_2), and 1256 (C-N) cm^{-1}. The ^1H NMR spectrum showed only four signals in MeOH-d_4, an O-methyl singlet, an N-methyl singlet, and bands for a *para*-disubstituted benzene ring. The long wavelength UV absorption at 394 nm indicated that the benzene ring was conjugated to at least one additional heterocyclic ring system. The final structure assignment for polycarpine dihydrochloride was achieved by combined spectral methods, particularly by 2D-NMR heterocorrelation methods (Table) and NOE experiments. Key to the structure were HMBC correlations linking the aromatic proton at C-7 to C-5 (and hence to the entire aromatic ring), and correlation of the C-11 N-methyl protons to C-1 and C-3. Based on the extended conjugation of **1** indicated in the UV spectrum, two connectivities of C-1-C-5 and N-4-C-5 were indicated. To meet the molecular formula, a disulfide bridge linking two identical halves, was positioned

between C1 and C1'. Consideration of the overall data led to structure **1** as the only reasonable candidate. Finally, electron dispersive spectroscopy (EDS) of polycarpine dihydrochloride crystals established the presence of sulfur and chlorine, confirming **1** as a hydrochloride salt. The results of computer-molecular modeling of **1** suggested that the N-methyl groups were very close to the C-8 aromatic protons in the energy-minimized conformation. Indeed, irradiation of the C-11 N-methyl group resulted in significant NOE enhancement of the C-8 aromatic proton.[4] The ^{13}C chemical shifts of the imidazolium ring were significantly different from those values reported in other natural products.[6] We concluded that the imidazolium and phenyl rings provide extended conjugation within one-half of the molecule. This proposal was also supported by long wavelength absorptions (394 and 259 nm) in the UV spectrum of **1**.

Polycarpine (**2**), the corresponding free base, was obtained as an orange noncrystalline solid. The ^1H NMR spectrum of **2** was similar to that from **1**. Likewise, the FAB mass spectrum (*m/z* 469) indicated the same molecular formula. However, there were distinct NMR differences between **1** and **2**. The ^1H NMR spectrum of **2** showed significant broadenings of two signals at δ 3.14 (bs, 6H) and 7.37 (bs, 4H). Only seven resonances were observed in the ^{13}C NMR spectrum. This observation suggested that **2** existed as a tautomeric mixture in solution. The bathochromic shift of **1** (λ_{max} 394 nm) in the UV spectrum, in comparison with **2** (λ_{max} 370 nm), was consistent with the fact that **1** is the salt form of **2**. In the IR spectrum of **2**, the ammonium bands (2833, 2762, and 2704 cm^{-1}) and immonium bands (2390-2000 cm^{-1}) were not observed. Instead, there were three additional bands at 1663, 1629, and 1549 cm^{-1} due to the absorption by the NH_2 and C=N groups of 2-aminoimidazole functionalities.[7] Furthermore, the carbon signal at C3 (C3') of **2**, in comparison with that of **1**, was shifted downfield since carbon C3 (C3') in **2** was more deshielded than the same carbon in **1**. The proposed relationship of **1** and **2** was confirmed by conversion of **1** into **2** by silica chromatography ($CHCl_3$/MeOH). Acetylation of compound **2** gave a diacetate **2a**, as would be expected.[4]

Compounds **2-5** were confidently assigned by a combination of spectral methods.[4] An intriguing aspect of compounds **3**, **4**, and **5** was that they were racemic, thus suggesting that these compounds could be artifacts produced during extraction or purification steps. To test the hypothesis, compound **2** was freshly generated by silica flash chromatography of **1** and monitored in methanol by ^1H NMR for several days. After 1 day, two additional monomeric derivatives had been produced. Indeed, the decomposed product had a thiohydantoin functional group which gave ^{13}C signals at δ 209 and 159. Treatment of **1** with a mild base (NH_4OH) in H_2O/MeOH yielded very similar results. Decomposition could be rationalized by nucleophilic addition of H_2O or MeOH to C5 of the imidazole ring of **1** followed by cleavage of the disulfide to give several monomeric derivatives.

To demonstrate that polycarpine dihydrochloride was the sole natural product produced by this animal, freshly defrosted *P. clavata* was dissected into tunic, branchial sac and other anatomical parts, and each was extracted with methanol and immediately evaluated by NMR. The branchial sac was found to be the only tissue containing polycarpine dihydrochloride (**1**). Polycarpine dihydrochloride was cytotoxic toward the human colon tumor cell line HCT-116 at 0.9 μg/ml.

Acknowledgments

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4. For **1**: orange rod, 270 mg, 0.3% dry wt.; mp 201-203 °C; LRFABMS obsd. 491 $(M + Na)^+$, 469 $(M + H)^+$, 437, 236; LRFAMS: Isotope Cluster Abundance: obsd: 469 (100%), 470 (30), 471 (15), 472 (4); calc: 469 (100), 470 (29.1), 471 (13.4), 472 (3); HRFABMS obsd. $(M + H)^+$ *m/z* 469.1503, $C_{22}H_{25}N_6O_2S_2$, dev. 4.8 ppm; UV (MeOH) 202 nm (log ε 4.45), 259 (4.28), 394 (3.58); UV (MeOH + NaOH) 203 (log ε 4.63), 224 (sh), 278 (4.09); IR (NaCl) 3351, 3267, 3097, 2833, 2762, 2704, 2390-2000 (w), 1673, 1606 (phenyl), 1503, 1435 (phenyl), 1256, 1182 (C-O), 834 (*para*-sub. benzene) cm^{-1}; NOEDS δ 3.87 (irrad.): δ 6.98 (1 %); δ 3.20 (irrad.): δ 7.42 (1 %) and 7.68 (4.2); ^{13}C NMR (MeOH-d_4) δ 29.9, 56.7, 111.7, 115.9, 129.6, 139.2, 149.1, 163.4. For **1a**: orange solid; HRFABMS obsd. $(M + H)^+$ *m/z* 469.1475, $C_{22}H_{25}N_6O_2S_2$, dev. -1.2 ppm; IR (NaCl) 3312, 3124, 2840, 2787, 2702, 1673, 1607, *1558*, 1504, 1415, *1335*, 1251, 1179, 833 cm^{-1}; ^1H NMR (MeOH-d_4): δ 2.00 (s, 6H), 3.14 (bs, 6H), 3.85 (s, 6H), 6.87 (d, 4H, *J* = 8.5 Hz), 7.37 (bd, 4H, *J* = 8.5 Hz). For **2**: orange amorphous solid; 13 mg, 0.013% dry wt.; LRFABMS (glycerol): obsd. *m/z* 469 $(M + H)^+$, 437, 236 (100), 204 (98); HRFABMS (thioglycerol): obsd. *m/z* 236.0856, $C_{11}H_{14}ON_3S$, dev. -0.7 ppm; UV (MeOH) 202 nm (log ε 4.50), 222(sh), 260 (4.12), 370 (3.57); UV (MeOH + NaOH) 205 nm (log ε 5.26), 280 (4.04); IR (NaCl) 3330, 1663, 1629, 1607, 1549, 1498, 1459, 1244, 1174, 834 cm^{-1}; NOEDS δ 7.37 (irrad.): δ 6.86 (8.9 %) and 3.12 (1). For **2a**: HRFABMS obsd. $(M + H)^+$ *m/z* 553.1700, $C_{26}H_{29}N_6O_4S_2$, dev. -1.5 ppm; ^1H NMR ($CDCl_3$ with three drops of MeOH-d_4): δ 2.20 (s, 6H), 3.20 (bs, 6H), 3.85 (s, 6H), 6.90 (d, 4H, *J* = 8.5 Hz), 7.65 (bs, 4H). For **3**: crystal (needle); 78 mg, 0.078% dry wt.; mp 127-129 °C; HRFABMS obsd. $(M + H)^+$ 267.0821 , $C_{12}H_{15}N_2O_3S$, dev. 6.6 ppm; UV (MeOH) 203 nm (log ε 4.14), 222 (4.17), 279 (4.10); IR (NaCl) 3287, 1755, 1609, 1511, 1465, 1313, 1252 cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.75 (bs, 1H, H-4), 7.38 (d, 2H, *J* = 8.5 Hz, H-7/H-7'), 6.91 (d, 2H, *J* = 8.5, H-8/H-8'), 3.74 (s, 3H, H-10), 3.19 (s, 3H, H-11), 3.15 (s, 3H, H-12); ^{13}C NMR (DMSO-d_6): δ 202.5 (C-1), 155.6 (C-3), 93.6 (C-5), 130.0 (C-6), 127.6 (C-7/7'), 113.4 (C-8/8'), 159.7 (C-9), 55.2 (C-10), 28.8 (C-11), 49.8 (C-12). For **4**: amorphous solid; 10 mg, 0.01% dry wt.; HRFABMS obsd $(M + H)^+$ *m/z* 253.0649, $C_{11}H_{13}N_2O_3S$, dev. 0.8 ppm; UV (MeOH) 202 nm (log ε 3.99), 220 (3.91), 278 (3.88); IR (NaCl) 3285, 1736, 1610, 1511, 1461, 1306, 1249, 1170 cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.60 (bs, 1H, H-4), 7.36 (d, 2H, *J* = 8.5 Hz, H-7/H-7'), 6.89 (d, 2H, *J* = 8.5, H-8/H-8'), 3.73 (s, 3H, H-10), 3.16 (s, 3H, H-11), 7.28 (bs, 1H, H-12); ^{13}C NMR (DMSO-d_6): δ 206.7 (C-1), 155.3 (C-3), 89.5 (C-5), 131.7 (C-6), 127.5 (C-7/7'), 113.2 (C-8/8'), 159.4 (C-9), 55.1 (C-10), 28.8 (C-11). For **5**: amorphous solid; 13 mg, 0.013% dry wt.; HRFABMS obsd. $(M + H)^+$ *m/z* 251.1038, $C_{12}H_{15}N_2O_4$, dev. 2.5 ppm; UV (MeOH) 203 nm (log ε 4.01), 225 (3.79), 277 (3.28), 292 (3.19); IR (NaCl) 3239, 1783, 1713, 1608, 1510, 1452, 1390, 1303, 1249 cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.32 (bs, 1H, H-4), 7.40 (d, 2H, *J* = 8.5 Hz, H-7/H-7'), 6.95 (d, 2H, *J* = 8.5, H-8/H-8'), 3.75 (s, 3H, H-10), 2.87 (s, 3H, H-11), 3.16 (s, 3H, H-12); ^{13}C NMR (DMSO-d_6): δ 169.9 (C-1), 154.9 (C-3), 87.8 (C-5), 126.7 (C-6), 127.6 (C-7/7'), 113.7 (C-8/8'), 158.8 (C-9), 55.2 (C-10), 24.1 (C-11), 50.7 (C-12).

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