SPECIALIA

Hoplonemertine Worms - a New Source of Pyridine Neurotoxins

W. R. KEM[1], KATHERINE N. SCOTT[2] and J. H. DUNCAN[2]

Department of Pharmacology and Therapeutics, University of Florida College of Medicine, The J. Hillis Miller Health Center, Gainesville (Florida 32610, USA); and Veterans Administration Hospital and Departments of Pharmacology and Radiology, University of Florida College of Medicine, Gainesville (Florida 32610, USA), 23 December 1975.

Summary. Two pyridine bases were isolated from the marine hoplonemertine *Amphiporus angulatus* (Fabricius) and identified by mass and PMR-spectroscopy as 2,3′-bipyridyl and 3,2′; 3′,2″; 4″,3‴-tetrapyridyl (Nemertelline). Nemertelline, the first tetrapyridyl natural product to be reported, shows a structural resemblance to the tobacco constituent nicotelline. The crustacean toxicity of 2,3′-bipyridyl is very similar to that of nicotine, but its mammalian toxicity is negligible.

BACQ[3,4] first discovered the presence of neurotoxic substances in a phylum of carnivorous marine worms, the nemertines[5]. Extracts of armed (hoplo-) nemertines acted like nicotine upon vertebrate skeletal muscle and autonomic ganglia. A nicotinoid toxin, anabaseine (I, Figure 1), has been isolated from the Pacific coast hoplonemertine *Paranemertes*[6]. We report here the isolation, structure elucidation, and initial pharmacological evaluation of the 2 most abundant pyridyls occurring in *Amphiporus angulatus* (Fabricius). Nemertelline, the most abundant compound, is the first tetrapyridyl to be isolated from a living organism. The main paralytic constituent 2,3′-bipyridyl, is highly active upon crustaceans in comparison with vertebrates.

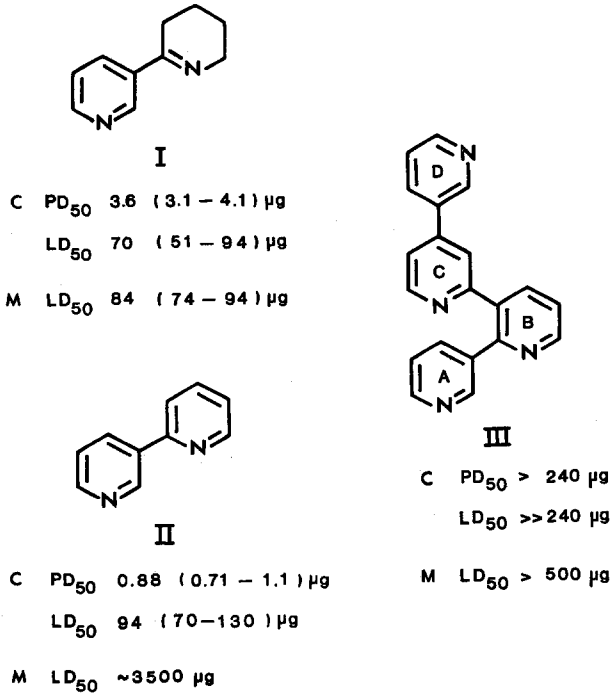

Fig. 1. Chemical structures and neurotoxic activities of the *A. angulatus* pyridyl toxins. I. anabaseine[6]; II, 2,3′-bipyridyl; III, nemertelline. Neurotoxicity was assessed by quantal bioassays on crayfish (C) and mice (M); median effective doses (µg, based upon a 20 g animal) and their fiducial limits (±2 SEM) were calculated by the SPEARMAN-KARBER method[7]. Animals were weighed and then injected with 0.06 ml per 10 g animal weight toxin solution. The crayfish (*Procambarus clarkii*, Dahl Co., Berkeley, Calif.) median paralytic dose (PD_{50}) was assessed by righting ability 15 min after injection, while the median lethal dose (LD_{50}) was determined 24 h after toxin injection. Mouse (Swiss-Webster, males, Flow Laboratories, Dublin, Va.) lethality was determined 30 min after i.p. toxin injection, (0.9% NaCl), since no further casualties were observed at 24 h. Mouse paralysis was not measured since it only occurred immediately preceding death. Bioassay results for S-(−)-nicotine are as follows: crayfish PD_{50}, 0.85 (0.71–1.0), LD_{50} 74 (50–110) µg; mouse LD_{50} 140 (120–170) µg.

Figure 1), has been isolated from the Pacific coast hoplonemertine *Paranemertes*[6]. We report here the isolation, structure elucidation, and initial pharmacological evaluation of the 2 most abundant pyridyls occurring in *Amphiporus angulatus* (Fabricius). Nemertelline, the most abundant compound, is the first tetrapyridyl to be isolated from a living organism. The main paralytic constituent 2,3′-bipyridyl, is highly active upon crustaceans in comparison with vertebrates.

A. angulatus was collected during low tides along rocky areas of the New Hampshire and Maine coasts. A methanol-acetic acid (99:1, v/v) extract of 68 worms (5.7 g dry wt.) provided a few milligrams of each of the 2 most abundant pyridyl constituents. The initial purification procedure was similar to the one previously described for anabaseine[6]; the basic constituents were resolved into 2 main UV-absorbing fractions by alumina preparative layer chromatography with ethyl acetate development. With a crayfish bioassay[7] we found that the high (0.8) Rf fraction contained > 90% of the paralytic activity, whereas large doses of the low (0.6) Rf fraction only caused minor paralysis. The major compound in each alumina fraction was separated from other pyridine constituents on silica gel layers with butanol-acetic acid-water (8:1:1); the eluted samples were then converted to free bases prior to spectroscopic analysis.

The low resolution mass spectrum (DuPont 21-491; direct probe introduction) of the main alumina high Rf compound contained a conspicuous molecular ion ($M^{+\cdot}$) at *m/e* 156 and fragments of M-1, M-26 and M-78, suggesting loss of hydrogen, cyano and pyridyl radicals, respectively, from a bipyridyl structure[8]. High resolution mass measurements (AEI-MS30) provided the empirical

[1] We thank Doctors N. MEINKOTH and N. RISER for collecting specimens, Dr. F. KUFFNER for providing the nicotelline sample, Dr. C. WESTERMAN for recording initial PMR-spectra, and Dr. R. KING for furnishing the computer simulation program and recording the high resolution mass spectra, and Mr. J. BABASHAK for use of the Kontes densitometer. This research was supported by an NIH-GRS grant and by the Veterans Administration (No. MRIS-9416).

[2] Veterans Administration Hospital, Gainesville, Florida and Departments of Pharmacology and Radiology, University of Florida, College of Medicine, Gainesville, Florida 32610, USA.

[3] Z. M. BACQ, Bull. clin. Sci., Acad. R. Belg. (S) *22*, 1072 (1936).
[4] Z. M. BACQ, Archs Int. Physiol. *44*, 109 (1937).
[5] R. GIBSON, *Nemerteans* (Hutchinson University Library, London (1972), p. 224.
[6] W. R. KEM, B. C. ABBOTT and R. M. COATES, Toxicon *9*, 15 (1971.)
[7] W. R. KEM, Toxicon *9*, 23 (1971).

formula $C_{10}H_8N_2$ (*m/e* 156.0670) and corroborated this proposed fragmentation pattern. Since the 2 rings of anabaseine are connected by a 2,3′ bond, 2,3′-bipyridyl (II, Figure 1) was synthesized[9]. The thin layer Rf's and spot reactions, gas chromatographic retention times, UV-spectra, mass spectra, and PMR-spectra of the main *A. angulatus* high Rf constituent were identical with those of synthetic 2,3′-bipyridyl. The proton resonance assignments for the *Amphiporus* bipyridyl were confirmed by homonuclear decoupling experiments and by computer simulation of the spectrum, and were only consistent with the 2,3′-bipyridyl isomer.

The empirical formula $C_{20}H_{14}N_4$ (*m/e* 310.1201) of the main alumina low Rf compound suggested a tetrapyridyl structure. The 2 major mass spectral fragments (*m/e* 154 and 205) presumably arise by loss of a neutral bipyridyl molecule from $M^{+\cdot}$, and by the loss of cyanopyridine from the M-1 ion, respectively, as confirmed by high resolution measurements. Our structure assignment (III, 3,2′; 3′,2″; 4″,3‴-tetrapyridyl, Figure 1) is primarily based upon a detailed PMR-analysis, including computer simulation of the observed PMR-spectrum (Figure 2) and homonuclear decoupling experiments. Nicotelline, a tripyridyl tobacco alkaloid, also contains the B-C-D ring structure present in III, but lacks ring A[12]. The PMR-spectrum of nicotelline was strikingly similar to the proton resonances assigned to rings A, C and D of III, further confirming the proposed structure. A remarkable feature shared by both natural products is the C ring containing 3-pyridyl substituents at both the 2 and 4 positions. In order to stress this structural and perhaps biosynthetic similarity we have named the *A. angulatus* tetrapyridyl nemertelline.

Several other minor pyridine compounds have recently been isolated from *A. angulatus*, including anabaseine, another tetrahydrobipyridyl, a methylbipyridyl, a tetrahydrotetrapyridyl, and a methyltetrapyridyl; we are currently investigating their structures. The tissue concentrations of the 4 major *A. angulatus* toxins, as determined by quantitative TLC, were 540 µg nemertelline, 120 µg 2,3′-bipyridyl, 60 µg methylbipyridyl, and 14 µg anabaseine per g fresh weight (whole animals). Only the median proboscis segment contained similarly high concentrations of these compounds; an important difference was that the anabaseine concentration was very high (700 µg per g), accounting for nearly 30% of the

[8] R. A. Khmelnitskii, N. A. Klyuev and P. B. Terentev, Zh. org. Khim. *7*, 395 (1971).
[9] E. Leete, J. Am. chem. Soc. *91*, 1697 (1969).
[10] T. M. Spotswood and C. I. Tänzer, Aust. J. Chem. *20*, 1227 (1967).
[11] S. Castellano, C. Sun and R. Kostelnik, J. chem. Phys. *46*, 327 (1967), and references contained therein.
[12] F. Kuffner and N. Faderl, Mh. Chem. *87*, 71 (1956).

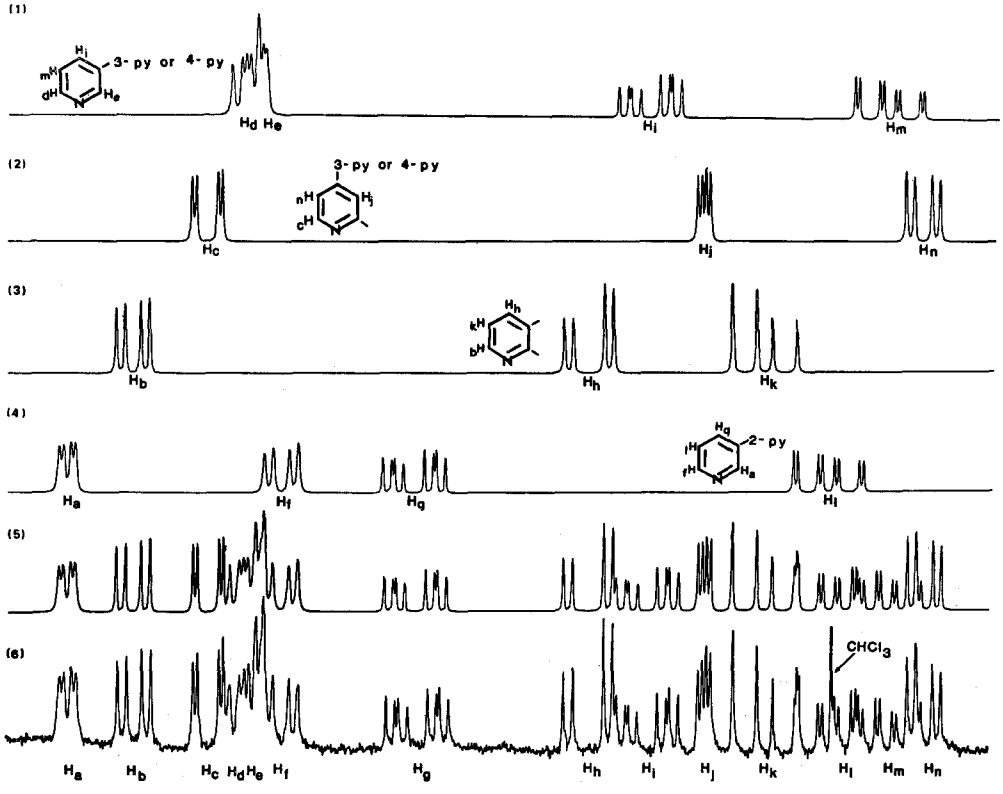

Fig. 2. PMR-spectrum of nemertelline. (1), (2), (3) and (4): Computer simulation of rings D, C, B and A, respectively. (5): Computer composite of (1), (2), (3), (4). (6): Experimental spectrum, 190 Hz wide sweep displayed. Inspection reveals a set of peaks (labelled H_a, H_f, H_g, H_l) identical to those observed for the 3-pyridyl ring of 2,3′-bipyridyl. Ring A of III is therefore a 3-substituted pyridyl, the substituent being a 2-pyridyl ring. Further inspection indicates the presence of another 3-substituted pyridyl spectrum (labelled H_d, H_e, H_i, and H_m). The chemical shifts of the protons in this ring require that the substituent on ring D be either a 3-pyridyl or a 4-pyridyl ring. The remaining 6 proton resonances were identified as those of a 2,4-disubstituted pyridine and a 2,3-disubstituted pyridine. In the 2,4-disubstituted ring, the chemical shifts of H_3 and H_5 are to high field, signifying that the substituent in the 4 position is either a 3-pyridyl or 4-pyridyl ring (Ref.[10,11]). The 4 computer simulated spectra when added together[5], are identical with the experimental spectrum of III. Although the 4 rings can be linked together 4 different ways, only one is consistent with the PMR-data, permitting unequivocal identification of the tetrapyridyl compound. PMR (90 MHz) spectra of both II and III were obtained in the Fourier transform mode on a Bruker HX 90 spectrometer equipped with a Nicolet 1083 computer. Solvent CS_2 (small $CHCl_3$ impurity); TMS, chemical shift reference; hexafluorobenzene, field-frequency lock material. The LAMP2 computer program (LAOCOON with magnetic equivalence and plot options) was used for computer simulations.

anabaseine in the entire animal, although the median proboscis contributes less than 1% of the body weight.

Our initial pharmacological assays with compounds I–III have provided several important results. First, 2,3'-bipyridyl rivals nicotine itself as a crustacean convulsant agent, although it is at least 20 times less lethal to mice than nicotine. Secondly, anabaseine is a potent paralyzing agent upon both crayfish and mice. The relative order of effectiveness in the mouse lethality bioassay, anabaseine > nicotine ≫ 2,3; -bipyridyl, has also been observed on the isolated frog rectus muscle preparation (KEM, unpublished results). According to current concepts about the molecular structure requirements for binding to arthropod and vertebrate nicotinic synaptic receptors, both 2,3'-bipyridyl and anabaseine would be predicted to have very little activity upon cholinergic synapses relative to nicotine, since both are weak bases (the pKa's of their most basic nitrogens are 4.4 and 6.7, respectively) and consequently would be largely unionized at physiological pH[13–15]. We suggest that the selective toxicity of 2,3'-bipyridyl may reflect substantial differences between arthropod and vertebrate receptor binding requirements, the planar, uncharged 2-pyridyl substituent binding more readily to crustacean than vertebrate receptors. If such differences do exist between the ligand-binding requirements of arthropod and vertebrate cholinergic receptors, they could provide a molecular basis for designing more selective insecticides[16].

Initial pharmacological experiments with pure nemertelline indicate that it has only a very small crustacean paralytic activity (Figure 1) and a very weak (less than 2,3'-bipyridyl) contractural action upon the frog rectus. Since it is the most abundant pyridine in *A. angulatus*, we suspect that nemertelline may act in some other manner, perhaps as a repellent to predators, which we cannot detect by our paralytic and lethality bioassays.

The *A. angulatus* pyridyls have tobacco alkaloid counterparts–anabaseine resembles myosmine, nemertelline resembles nicotelline, 2,3'-bipyridyl also occurs in tobacco, and a methylbipyridyl has recently been discovered in cured tobacco leaves[17]. This is a remarkable example of convergent biochemical evolution between an animal and a plant group. We have recently isolated the 3 most abundant pyridyls in a related hoplonemertine species (*Amphiporus ochraceus*) and found that they are all distinct from the compounds described here. Apparently the order Hoplonemertinea contains many pharmacologically active pyridyls. We expect that some of these will become useful chemical tools for investigating the mechanisms by which nicotinoids affect the nervous system.

[13] I. YAMOMOTO, in *Advances in Pest Control Research* (Ed. R. L. METCALFE; Interscience, New York 1965), vol. 6, p. 231.
[14] R. B. BARLOW and J. T. HAMILTON, Br. J. Pharmac. *18*, 543 (1962).
[15] K. SCHOFIELD, *Heteroaromatic Compounds: Pyrroles and Pyridines* (Plenum Press, New York 1967), p. 148.
[16] M. E. ELDEFRAWI, A. T. ELDEFRAWI and R. D. O'BRIEN, Agric. Food Chem. *18*, 1113 (1970).
[17] A. H. WARFIELD, W. D. GALLOWAY and A. G. KALLIANOS, Phytochemistry *11*, 3371 (1972).

The Structure of 4-Ketocedrol

Y. H. KUO, I. C. YANG, C. S. CHEN and Y. T. LIN[1]

Department of Chemistry, National Taiwan University, Roosevelt Road Section 4, Taipei (Taiwan, China),
24 November 1975.

Summary. 4-Ketocedrol was isolated from *n*-hexane extraction of *Juniperus squamata* Linn. and its formula established by physical and chemical evidence.

Material and methods. The neutral fraction of a *n*-hexane extract of wood of *Juniperus squamata* Lamb. was investigated. Besides α-cedrol and its derivatives, 8S, 14-cedrandiol, a new keto-derivative I was isolated by chromatography on neutral alumina[2]. This new derivative of cedrol was found to be 4-ketocedrol.

Results and discussion. I. m.p. 129–130°, $C_{15}H_{24}O_2$, $[\alpha]_D$ −21.5° (C. 1.4 in CH_3OH), exhibits IR-absorption bands at 3460 (—OH) and 1730 cm^{-1} (C=O). It shows NMR-spectrum signals at τ 9.08 (3H, d, J = 6.6 Hz, $=CHCH_3$), 9.03 and 8.62 (each of 3H, s, $=C(CH_3)_2$), and 8.75 (3H, s, $=C(OH)CH_3$). The structure of compound I was suggested by the similarity of its NMR-spectrum with that of α-cedrol II. When I was subjected to Huang-Minlon modification reduction, α-cedrol was obtained. From this result I is a ketocedrol. I gave III by heating in 99% formic acid at 75–80°. The lqiuid III exhibited an isolated carbonyl group (1730 cm^{-1}) and a trisubstituted double bond (1645, 850 cm^{-1}) in IR-absorption bands, no maximum absorption above 210 nm in UV-spectrum, and NMR-spectrum signals at τ 8.27 (3H, br s, CH_3—C=C—H), and 4.62 (1H, m, CH_3—C=C—H). By these data, the location of the carbonyl at C_9 and C_{10} in I can be excluded. Sodium borohydride reduction of I in MeOH gave IVa as the sole product, m.p. 143–144°, ν_{max} 3200 cm^{-1} (—OH); its NMR-spectrum exhibits signal at τ 5.74 (1H, m, $W_{1/2}$ = 13 Hz, =C(OH)H). Treatment of diol IVa with acetic anhydride in pyridine yielded a monoacetate IVb, m.p. 68–70°, ν_{max} 1725, 1715 and 3270 cm^{-1}. The NMR spectrum of this acetate shows signals at τ 7.95 (3H, s, CH_3COO—) and 4.77 (1H, m, $W_{1/2}$ = 14 Hz, =C(OAc)H). When IVb was treated with formic acid at 75–80° it gave Va, ν_{max} 1730, 3040 and 805 cm^{-1}; with NMR-signals at τ 7.93 (3H, s, CH_3COO—) and 4.73 (1H, m, $W_{1/2}$ = 12 Hz, =C(OAc)H). Vb, m.p. 82–85°, saponification product from Va, shows a multiplet centered at τ 5.61 ($W_{1/2}$ = 13 Hz) in the NMR-spectrum. Examination of the coupling pattern of the proton attached to the carbon atom carrying the hydroxyl or acetyl group (with great $W_{1/2}$ value) in IVa, IVb, Va or Vb, excludes the carbonyl at C_{11} in I. Therefore the

[1] The authors are indebted to National Council on Science Development of China for financial support.
[2] The separation procedure will be published elsewhere.