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J. Nat. Prod., **1993**, 56 (1), 9-14• DOI: 10.1021/np50091a002
• Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 1, pp. 9-14, January 1993

^{13}C-NMR ASSIGNMENTS AND CYTOTOXICITY ASSESSMENT OF ZOANTHOXANTHIN ALKALOIDS FROM ZOANTHID CORALS

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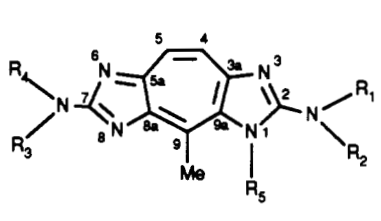
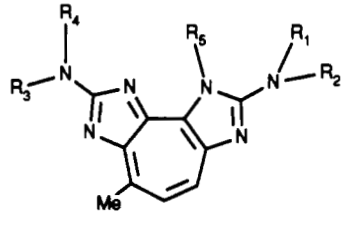
ABSTRACT.—Zoanthoxanthin alkaloids can vary among skeletal types **A**, **B**, and **C**. Three type C zoanthoxanthins were examined, including a new compound **1**, previously reported paragracine [**2**], and zoanthoxanthin **3**. Their nmr and cytotoxic properties are reported. We have used 2D nmr data to complete the assignments for **1** and suggest that these benchmark nmr assignments will allow future investigators to establish new metabolites of this class as a member of families A, B, or C with only a ^{13}C APT and a ^{1}H-^{1}H nmr spectra.

The zoanthoxanthins are unusual non-benzenoid aromatic zoochromic alkaloids (1). A recent survey of more than 80 different invertebrate species showed that these yellow fluorescent substances emanate solely from colonial anthozoans in both major families (Epizoanthidae and Zoanthidae) of the order Zoanthidea (1). They undoubtedly arise by a dimerization-rearrangement of arginine-derived C_5N_3 subunits, because three skeletons are known: 3*H*-zoanthoxanthin [**A**] (2), 4*H*-pseudozoanthoxanthin [**B**] (2), and 3*H*-pseudozoanthoxanthin [**C**] (3). Distinction among these isomeric framework possibilities can be tricky. Structural types **A–C** exist as equilibrating tautomeric pairs when the annular *N* is substituted with $R_5 = H$. Disymmetrical tautomers are possible for **A** when any one of the four substituents R_1–R_4 is different from the remaining three, whereas structures **B** and **C** are themselves tautomers when R_1–R_4 are identical. Seminal examples of **A–C** (each having an $R_5 = Me$) have been characterized by X-ray. Other derivatives are usually identified by comparing ^{1}H vinyl and Me shifts to those which co-occur with analogues that have been analyzed by X-ray crystallography. Alternatively, a decision among families A–C is sometimes made by assuming that these compounds are genus-specific. Surprisingly, no definitive ^{13}C-nmr assignments can be found for any members of this group, as none have been examined



A



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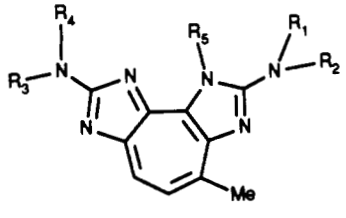
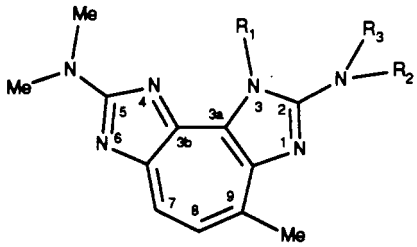


C

by the powerful 2D nmr techniques (4). Previous information about the bioactivity of zoanthoxanthins includes histamine-like action on the guinea-pig ileum (5) and papaverine-like activity (6). Our report below extends knowledge in both of these domains.

A colony tentatively identified as *Epizoanthus* sp. suspected as being a source of zoanthoxanthins attracted our attention during a field expedition in the Fiji Islands. We collected it in order to expand our ongoing study of bioactive zoochromic alkaloids (7–10). Our intent was to approach the structural characterizations of new zoanthoxanthins without making a biogenetically based assumption about its **A**, **B**, or **C** skeletal type. We felt that a succinct approach would involve generating substructures from ^{1}H multipicities and ^{1}H-^{13}C COSY nmr data, paying particular attention to $^{2-3}J_{CH}$ correlations. After making complete assignments for one or more zoanthoxanthins we could then demonstrate the feasibility of rapidly distinguishing between **A**, **B**, and **C** via $^{2-3}J_{CH}$ ^{1}H-^{13}C COSY nmr correlations of the Me and vinyl protons.

The non-encrusting coral yielded two 3*H*-pseudozoanthoxanthins, **1** and **2**. The former was a major component and it was characterized first. It was an amorphous yellow powder of $C_{14}H_{18}N_6$ molecular formula (hreims *m/z* 270.1587 $[M]^+$, Δ 0.6 mmu of calcd) and of $C_{14}H_{17}$ APT formula (11). Solutions containing **1** exhibited a distinctive yellow-blue fluorescence. Additional spectral data were in agreement with all three general structures **A**–**C**, and included uv absorption maximum (nm) at 308 (ϵ 30300) and 415 (ϵ 9375) and ^{1}H nmr (300 MHz, $CDCl_3$ with a drop of CD_3OD) resonances at δ 7.33 and 7.48 ppm (1H each, d, J = 10 Hz), δ 3.98 (N-Me), δ 3.21 (N-Me_2) and 3.11 (N-Me, s), and δ 2.64 (C-Me, s).



1 R_1=R_3=Me, R_2=H
2 R_1=R_2=H, R_3=Me
3 R_1=R_2=R_3=Me
4 R_1=Me, R_2=R_3=H

The 2D nmr spectra with $^{1}J_{CH}$ correlations and $^{2-3}J_{CH}$ correlations (Figure 1) as well as a coupled ^{13}C-nmr spectrum were rapidly collected for **1** (Table 1). Careful analysis of these data allowed framework **C** to be proposed for **1** and the positions of all the *N*-Me's to be specified. The $^{3}J_{CH}$ correlation from the protons of the C-Me to a protonated vinyl carbon (C-8) allowed **A** to be ruled out. The regiochemistry of the four *N*-Me's was deduced with the aid of long-range ^{13}C-^{1}H COSY nmr data. For example, diagnostic $^{3}J_{CH}$ correlations were observed from the Me protons attached to the annular N or the exocyclic ring N to C-2 as shown in Figure 1. The annular *N*-Me also exhibited a long-range COSY correlation to C-3a, and $^{3}J_{CH}$ correlations were visible from the *N*-Me_2 protons to C-5. A coupled ^{13}C-nmr spectrum revealed C-3b (δ 149.2) as a broad doublet ($^{3}J_{CH}$ = 10 Hz) ascribed to long-range coupling with H-7. The relative orientation established between H-7 and C-3b along with that deduced between the annular *N*-Me and C-3a was incompatible with framework **B**, yet entirely consistent with **C**.

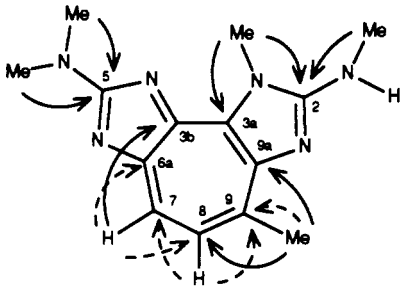


FIGURE 1. Long-range C-H correlations for **1**; ⟶ $^3J_{CH}$ - - - → $^2J_{CH}$

Several additional $^3J_{C\text{-}H}$ values in Hz and COSY correlations were obtained (Table 1 and Figure 1) in support of structure **1**, and these allowed all of its ^{13}C resonances to be unambiguously assigned.

The minor compound isolated in this study was concluded to be paragracine [**2**] based on the parallel trends in the ^{13}C-nmr shifts between **1** and **2**, as shown in Table 2, along with the correspondence of the ^1H-nmr data we collected versus those in the literature (12).

Conversion of **1** into the known **3** provided an opportunity to gather additional ^{13}C-nmr data for the pseudozoanthoxanthin framework; however a paucity of sample, coupled with the fact that inverse detection ^{13}C-nmr data was not available at the time this study was conducted, prevented the non-protonated carbons from being detected as shown by the data in Table 2. The insights gained above allowed the previously unassigned ^{13}C-nmr literature of the pseudozanthoxanthin derivative of structure **4** (13) to be fully deciphered as shown in Table 2.

The results outlined above illustrate that 2D nmr provides a concise way to place a newly isolated zoanthoxanthin within one of the general structural families A, B, or C. Our suggestion here is that the data of Table 2 now facilitates a rapid preliminary choice about which structural category A–C fits a newly isolated zoanthoxanthin. This can be done from just two nmr spectra: a ^{13}C-APT (used in combination with standard substituent additivity effects), and a ^1H-nmr spectrum. Obviously, a final and definitive

TABLE 1. ^{13}C- and ^1H-nmr Data for Compound **1** ($CDCl_3$/drop of CD_3OD).*

Position	^{13}C (mult)	$^1J_{C\text{-}H}$ (Hz)	$^3J_{C\text{-}H}$ (Hz)	^1H (mult, J in Hz)
2	157.1 (s)			
3a	131.2 (s)			
3b	149.2 (s)		10	
5	163.2 (s)			
6a	144.0 (s)		7	
7	116.2 (d)	157		7.48 (d, 10)
8	129.4 (d)	158		7.33 (d, 10)
9	150.2 (s)		8	
9a	136.2 (s)		mult.	
Me-2	28.4 (q)	138		3.11 (s)
Me-3	30.9 (q)	141		3.98 (s)
Me-5	37.0 (q)	138		3.21 (s)
Me-9	22.4 (q)	128		2.64 (s)

*300 MHz for ^1H; 75 MHz for ^{13}C.

TABLE 2. ^{13}C-nmr Data for Compounds **1–4**.

Carbon	Compound			
	1[a]	**2**	**3**[a]	**4**[b]
C-2	157.1 (s)	163.8 (s)	159.6 (s)	153.7 (s)
C-3a	131.2 (s)	132.8 (s)		130.1 (s)
C-3b	149.2 (s)	153.6 (s)[c]		149.1 (s)
C-5	163.2 (s)	165.0 (s)		
C-6a	144.0 (s)	140.3 (s)		139.1 (s)
C-7	116.2 (d)	120.5 (d)[d]	120.7 (d)	121.8 (s)
C-8	129.4 (d)	131.5 (d)[d]	129.3 (d)	137.5 (s)
C-9	150.2 (s)	154.3 (s)[c]		150.7 (s)
C-9a	136.2 (s)	138.4 (s)		136.9 (s)
Me-2	28.4 (q)	29.5 (q)[e]	38.1 (q)[c]	
Me-3	30.9 (q)		36.2 (q)	35.1 (q)
Me-5	37.0 (q)	38.4 (q)[e]	41.4 (q)[c]	39.4 (q)
Me-9	22.4 (q)	23.3 (q)[e]	23.1 (q)	23.5 (q)

[a]In $CDCl_3$/drop of CD_3OD.
[b]In CF_3CO_2D. Values are from Schwartz *et al.* (13).
[c,d]Values may be interchanged.
[e]Assigned by Komoda *et al.* (5).

conclusion could be reached after a long range ^{13}C-^{1}H COSY nmr spectrum was obtained.

Efforts to evaluate the bioactive potential of zoanthoxanthins were stimulated by prior observations that polyaromatic zoochromic alkaloids such as dercitin are in vivo active against both P-388 (leukemia) and B-16 melanoma tumors in mice (14). Compound **1** was active in cytotoxicity assays (in vitro) and exhibited these IC_{50}'s (μg/ml): HCT8 (human colon adenocarcinoma) = 1.61, A549 (human lung carcinoma) = 2.38, HT29 (human colon adenocarcinoma) = 0.824, and P-388 (mouse lymphocytic leukemia) = 1.77.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The nmr spectra were recorded at 99.5 MHz for ^{1}H, 25.0 MHz for ^{13}C, or at 300 MHz for ^{1}H, 75.0 MHz for ^{13}C. Multiplicities of ^{13}C nmr resonances were determined from APT or DEPT data, and COSY experiments were done at ^{1}H frequencies of 300 MHz. Low resolution eims data were obtained at U.C.S.C., while hrms data were obtained from the U.C. Berkeley MS laboratory. Hplc employed 10 μ ODS or 10 μ silica columns. All solvents were distilled for (hplc) use and were spectral grade for spectroscopy. Standard pulse sequences (4) were used for the ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, and long-range ^{1}H-^{13}C COSY experiments.

TAXONOMY, EXTRACTION AND ISOLATION.—The organism (coll. #87135) (0.5 kg) was collected by SCUBA from Namena Island (17°6′ S, 179°6′ E). Our voucher (deposited in the UC Santa Cruz Institute of Marine Sciences collection) was preliminarily identified by Dr. H. Chaney, Santa Barbara Museum. It was extensively reexamined, by Ms. M.C. Diaz (UCSC-IMS). Unfortunately only dried material was available which complicated the taxonomic analysis, as described below, so the identification at the genus level, *Epizoanthus* (Fam. Epizoanthidae, Order Zoanthidea), is tentative. In life the colonial anemone-like anthozoan was brown in color and was intertwined with a hydroid as shown in Figure 2A. This is a common habitat type for the genus *Epizoanthus*, and extensive descriptions along with photographs (Plates 17, 22, and 25) can be found in Mather and Bennett (16). Our voucher specimen displayed small polyps (1–2 mm wide and 2–3 mm high when contracted) arising from a common tissue mass overgrowing a branching erect hydroid (Figure 2A). Adjacent polyps are separated by 2–5 mm and branch out in opposite directions. A cross section of a polyp (Figure 2B) showed that sediment was heavily incorporated in the mesolgea and skin. Inspection of the actinopharynx (Figure 2B) clearly revealed 17–20 macronemic mesenteries and the absence of a siphonoglyph. This key observation allows distinction between

FIGURE 2. Morphology of *Epizoanthus* sp. sample #87135. A: External view of polyps growing over a branching erect hydroid. (P) Polyp that was dissected. B: Cross section of polyp shown in A at the actinopharynx (Ac) level showing the skin (SK) and mesogloea heavily charged with sand and the radial mesenteries (M).

the families Epizoanthidae/Parazoanthidae and Zoanthidae/Neozoanthidae. Further important histological features such as the presence of an endodermal sphincter (marker for family Epizoanthidae) or a mesogloeal sphincter (marker for family Parazoanthidae) could not be seen in our dried voucher. Alternatively, the gross morphology shown in Figure 2A along with habitat characteristics described above in comparison to those detailed by Verrill (15) and Mather and Bennett (16) for the order Zoanthidea favor identification as *Epizoanthus* (family Epizoanthidae) rather than *Parazoanthus* (family Parazoanthidae). In this regard *Epizoanthus* is noted as typically encrusting sponges and hydroids while *Parazoanthus* occurs mainly on sponges and gorgonians (16).

The hexacoral was extracted immediately with MeOH (2×) followed by concentration of the crude extract to yield 2.69 g of a crude oil. The entire oil was successively partitioned between aqueous MeOH and the series hexanes (1.5 g), CCl_4 (0.320 g), CH_2Cl_2 (0.200 g), and *n*-BuOH (0.120 g). The *n*-BuOH fraction was chromatographed on Sephadex LH-20 (MeOH) to give two products, **1** (30 mg) and **2** (4 mg), which were further purified by reversed-phase hplc [MeOH-H_2O (85:15)].

Compound **1**.—An amorphous yellow powder was obtained as above: uv (MeOH) λ max 415 (ε 9375), 373 (ε 5400), 308 (ε 30,300), 253 nm (ε 4400) nm; ir ($CHCl_3$) 3178, 1613, 1542, 1413, 1313, 1296 cm^{-1}; hreims *m/z* observed $[M]^+$ 270.1587 ($C_{14}H_{18}N_6$ Δ 0.3 mmu of calcd), 255.1354 ($C_{13}H_{15}N_6$, Δ 0.2 mmu of calcd); lreims *m/z* (%) $[M]^+$ 270 (32), $[M-Me]^+$ 255 (22), 241 (28); lrcims (isobutane) *m/z* (%) $[M+H]^+$ 271 (100), 257 (45), 241 (2), $[M-NMe_2]^+$ 227 (1); ^{13}C (75 mHz) and ^1H (300 MHz) see Table 1; assignments based on ^1H-^{13}C COSY ($J = 140$ Hz and $J = 9$ Hz).

Compound **2**.—Compound **2** was obtained as above and its physical properties match those of the literature (5): uv (MeOH) λ max 409 (ε 2125), 369 (ε 1625), 309 (ε 8250), 251 nm (ε 1875) nm; ir ($CHCl_3$) 3310, 1637, 1416, 1308, 1099 cm^{-1}; hrfabms *m/z* observed $[M]^+$ 257.1520 ($C_{13}H_{17}N_6$ Δ 0.8 mmu of calcd); ^1H nmr (300 MHz, $CDCl_3$) 7.79 (d, $J = 10.8$, H-8), 7.71 (d, $J = 10.5$, H-9), 3.93 (s, 6H), 3.18 (s, 3H), 2.89 (s, 3H).

METHYLATION OF COMPOUND **1**.—A solution of **1** (9 mg) in DMF (0.5 ml) was stirred with NaH (10 mg) for 10 min at room temperature, and a solution of CH_3I (50 mg) in DMF (0.5 ml) was added. The mixture was stirred for an additional 2 h at room temperature and was quenched with H_2O and extracted with $CHCl_3$. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated in vacuo to give compound **3**, which had identical spectroscopic data (uv, ir, ms, nmr) with that in the literature (uv, ms, ir).

ACKNOWLEDGMENTS

Research support was from NIH grant CA47135, and C.J. received a partial Postdoctoral Fellowship from Xunta de Galicia, Spain. We thank Mr. Jim Loo (U.C.S.C.) for assistance with nmr measurements

and Dr. J.J. Clement of Abbott Labs who kindly provided the cytotoxicity data. We also are grateful to the Fiji government for their cooperation.

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Received 13 January 1992