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Novel pteridine alkaloids from the sponge *Clathria* sp.

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Abstract—Three novel pteridine alkaloids, pseudoanchynazines A–C (**1**–**3**) have been isolated from the sponge *Clathria* sp. collected by trawling (−100 m) in a commercial clam fishery near the coasts of Rio Negro, Argentina, and their structures determined by a combination of chemical and spectroscopic techniques, with emphasis in 2D NMR and tandem mass spectrometry. Compounds **1**–**3** have two pteridine units and a tryptophan core with a methyl carbamate substituent. © 2002 Published by Elsevier Science Ltd.

1. Introduction

Marine sponges produce a wide array of bioactive secondary metabolites. It was long believed that the production of secondary metabolites had to be more important in tropical, shallow water species, due to their greater need of chemical defense against predators. For this reason, and the ease of collection, these samples received considerably more interest from chemists in the past. However, in recent years, there has been more evidence that deeper-water and temperate species are also capable of producing novel compounds. As part of our research program on bioactive secondary metabolites from South Atlantic marine invertebrates,[1–3] we began screening samples from commercial clam fisheries. The accompanying fauna obtained from these fishing operations provide access to samples of marine invertebrates which would be almost impossible to collect by other means due to a combination of factors such as great depths, rough weather and low visibility.

A sponge, *Clathria* sp. (formerly: *Pseudanchynoe* sp.) collected at 100 m depth, was the most abundant sample from the SW SAO bank, off the coasts of Rio Negro, Argentina. This sponge produces a family of novel pteridine alkaloids which we have named pseudoanchynazines A–C, **1**–**3**.[4] These compounds share some common structural features: every compound has two pteridine moieties, each of which bears a three carbon side chain. The three compounds have an unusual tryptophan unit with the α amino group blocked by a methyl carbamate and different pteridine substitution patterns. Pseudoanchynazine A has pteridine substitution at C-2, pseudoanchynazine B at N-1, and pseudoanchynazine C at C-4 (Fig. 1). In all cases, the tryptophan carboxyl forms an ester with the second pteridine unit.

Simple pteridine alkaloids have been previously isolated from sponges,[5–7] and the polychaete *Odontosyllis undecimdonta*.[8–13] On the other hand, in the marine environment, a methyl carbamate group has been reported in a variety of macrolide and enediyne antibiotics of bacteria and tunicates,[14–19] and also as an amino blocker in polyketides,[20] bromotyrosine derivatives,[21–23] and saxitoxins.[24] However, to the best of our knowledge, there are no reports of a structure that combines the methyl carbamate bound to the α amino group of a tryptophan unit, together with two lumazine derived fragments.

2. Results and discussion

The crude ethanolic extract of *Clathria* sp. (see Section 3) was concentrated to an aqueous suspension and extracted with EtOAc. Flash chromatography of the organic layer afforded compounds **1**–**4**, which were finally purified by reversed-phase HPLC.

Compound **4** had a low-abundance molecular ion *m/z* 250 by EIMS and a base peak at *m/z* 221 (loss of $C_2H_5^{\cdot}$). HRMS was performed on the latter ion giving a formula $C_9H_9N_4O_3$; indicating a molecular formula $C_{11}H_{14}N_4O_3$ for compound

Keywords: alkaloids; *Clathria* sp; pteridine; marine natural products; sponges.

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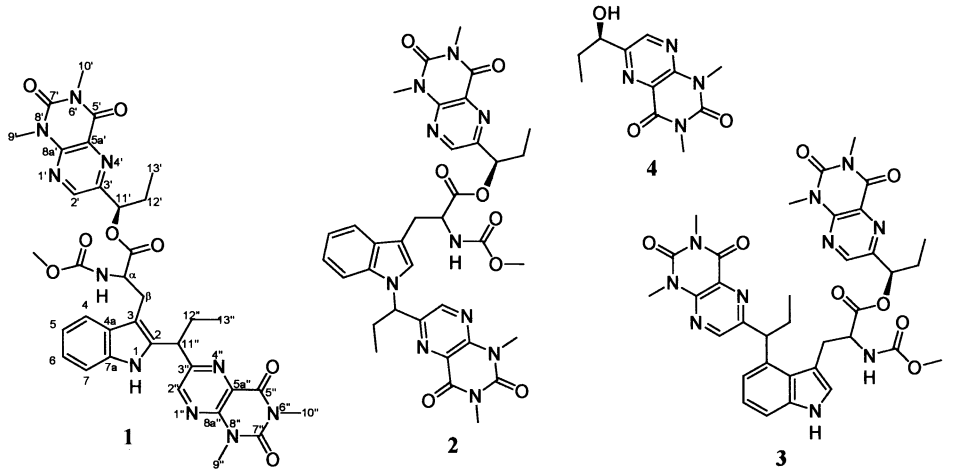

Figure 1. Structures of compounds **1**–**4**.

4. The ^{1}H NMR spectrum showed an oxygen bearing C-3 side chain (δ: 1.02 (t, 3H, J=7.5 Hz); δ: 2.00 (m, 2H), δ: 4.94 (dd, 1H, J=7.3, 5.3 Hz)) bound to a heteroaromatic ring (δ: 8.77, s, 1H), as well as two low-field methyls, which were assigned as heteroaromatic *N*-methyls by their ^{13}C chemical shifts (δ: 29.0 and 29.5). The ^{13}C NMR spectrum also confirmed the presence of a secondary alcohol and a heteroaromatic moiety (five signals in the range δ: 160 to δ: 145). Compound **4** crystallized as long needles, and was identified as 6-(1-hydroxypropyl)-1,3-dimethyl-1*H*-pteridine-2,4-dione by X-ray diffraction. The 11*S* enantiomer of **4** had been previously isolated from the marine polychaete *Odontosyllis undecimdonta*,[11] and was reported to have an optical rotation of −59.6° (*c* 1.079, MeOH). The optical rotation of **4** was observed to be: $[\alpha]_D^{25}$=59.1° (*c* 0.39, MeOH), thus indicating that **4** is the 11*R* enantiomer.

Compounds **1**–**3** had an identical molecular formula $C_{35}H_{38}N_{10}O_8$ established by HRFABMS. Some common structural blocks could be readily identified from the NMR spectra of these compounds: two pteridine rings with C-3 chains as in compound **4** plus a tryptophan moiety. The α amino group was substituted (δ: 4.84, dt, J=8.4, 5.5 Hz), although the nature of the substituent was not evident. Analysis of COSY spectra showed that in all cases, one of the pteridine units was esterified at C-11, while the other unit was connected by the same carbon either to nitrogen or an aromatic ring. A broad low-field methyl, which was assigned as a methoxy group by its ^{13}C chemical shift (δ: 52.3), could be detected in the three spectra together with the four pteridine *N*-methyls. The presence of an ester was also evident by IR and ^{13}C NMR (δ: 171.3) spectroscopy.

Acid methanolysis of **1** (Fig. 2) produced quantitatively two compounds, the previously found pteridine **4** and the methyl ester **5a**, which was the key to the structure elucidation of **1**–**3**. Compound **5a** had by EIMS a molecular ion at *m*/*z* 508, which could correspond to a molecular formula $C_{25}H_{28}N_6O_6$, indicative of 15 degrees of unsaturation. Inspection of the NMR spectra revealed that **5a** still had a pteridine nucleus and the tryptophan portion with a substituent at the α amino group. Two methoxy groups could be observed by NMR, together with the two *N*-methyls from the pteridine nucleus. Taking into account six double bond equivalents for the indole nucleus, seven for the pteridine ring and one for the tryptophan carbonyl, an additional unsaturation had to be present. An atom count indicated that this additional unsaturation had to be due to a carbonyl group. Taking into account the ^{13}C signals of the pteridine and tryptophan substructures, an additional low-field quaternary carbon was evident (δ: 156), which was assigned to the extra carbonyl group. The COLOC spectrum of **1** showed a correlation peak between the carbonyl at δ: 156 and the broad methoxyl singlet. Using ethanol under the same alcoholysis conditions, an ethyl ester **5b** was obtained

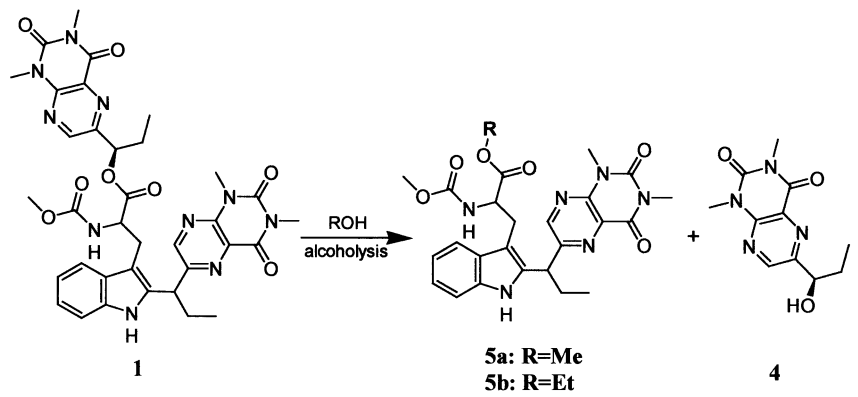

Figure 2. Acid catalyzed alcoholysis of compound **1**.

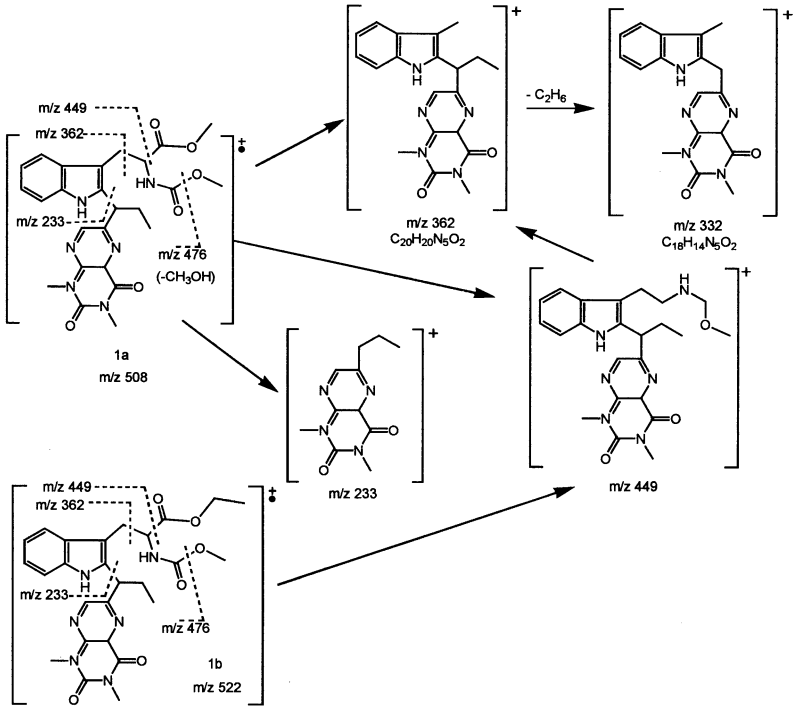

Figure 3. Analysis of compound **5a** by tandem mass spectroscopy.

which still had a broad methoxy group. The unusually low chemical shift of this carbonyl, together with the enhanced stability towards transesterification compared to the ester functionality already present in the molecule, suggested the presence of a methyl carbamate group at C-α of the tryptophan core. At the same time, inspection of the tryptophan ^{1}H NMR signals, revealed that H-2 was absent, indicating substitution at C-2. The COLOC spectrum of **1** showed a weak correlation between H-11″ of the pteridine and C-2 of the indole, thus connecting both substructures.

A detailed analysis of **5a** by tandem mass spectrometry (Fig. 3) was undertaken in order to confirm this tentative structure. Compound **5a** showed by EIMS a molecular ion at *m/z* 508, a base peak at *m/z* 362 and significant fragments at *m/z* 476, 449, 332, 318 and 233. A parent ion scan of *m/z* 362 gave signals at *m/z* 449, 476 and 508. HRMS of ion *m/z* 362 gave a formula $C_{20}H_{20}N_5O_2$ which could match a substructure consisting of the complete pteridine and indole rings plus C-β of the tryptophan chain. The ion *m/z* 476 arises from *m/z* 508 by loss of methanol, a typical fragmentation in methyl carbamates, while *m/z* 449 is formed by α-cleavage of the tryptophan carbonyl. The latter was confirmed by analysis of the ethyl ester **5b**, which also showed the fragment at *m/z* 449. A daughter-ion scan of *m/z* 362 gave ion *m/z* 332, with a formula $C_{18}H_{14}N_5O_2$ established by HRMS. This rare loss of ethane from *m/z* 362 can only be explained by the formation of a very stable carbocation, as would be expected in the case of α cleavage to both the indole and pteridine rings. A daughter ion scan of *m/z* 332 gave ions *m/z* 247, 219 and 192 which correspond to typical pteridine ring fragmentations. A remaining major fragment ion in the mass spectrum of **5a** is *m/z* 233, which matches the entire pteridine substructure plus the C-3 side chain, and arises from the cleavage of the bond connecting the indole and the pteridine moieties. All these results are outlined in Fig. 3.

Once the structure of **5a** was ascertained, compound **1** was easily identified. Inspection of the FABMS spectrum of **1** showed only two important fragment ions: *m/z* 362 and 233, which could be easily explained after the abovementioned fragmentation analysis of **5a**. A complete set of 2D NMR spectra (COSY, HETCOR and COLOC) confirmed this structure and the assignment of all proton and carbon resonances.

The structures of compounds **2** and **3** were very similar to **1**. In the ^{1}H NMR spectrum of compound **2**, indole H-2 was evident while NH-1 was absent. This, together with the downfield chemical shift for C-11″ and H-11″ (δ: 60.5 and 5.62 (dd, *J*=9.1, 5.8 Hz, respectively), indicated nitrogen substitution at C-11″; therefore, the identity of **2** as the N-1 substituted isomer of **1** was established. In the case of **3**, ^{1}H NMR signals indicated three connected protons in the indole nucleus, which placed the pteridine substituent either at C-4 or C-7. Analysis of the 2D NMR spectra, especially COLOC (H-11″, C-5 correlation) and Phase-Sensitive NOESY (NOE between H-11″ and H-5), indicated substitution at C-4. A complete assignment of all carbon and proton resonances was achieved by 2D NMR. Compound **1** was found to be mildly active against *E. coli* at 50 μg/disk.

3. Experimental

3.1. General methods

The IR and UV spectra were taken on a Nicolet Magna-IR 550 spectrometer and on a Hewlett–Packard 8451A diode

Table 1. ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR chemical shift assignments (δ) and coupling constant data (Hz) for compounds **1–3** in $CDCl_3$

Atom	Compound					
	1		**2**		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1		9.72 (s)				8.60 (s)
2	135.7		132.2	7.32 (s)	122.4	7.10 (s)
3	106.3		110.4		109.8	
4	118.1	7.53 (d, 7.3)	118.9	7.52 (d, 7.7)	134.3	
4a	127.5		128.2		128.1	
5	121.9	7.15 (m)	122.3	7.04 (m)	118.6	7.06 (d, 7.3)
6	119.8	7.08 (m)	120.0	7.06 (m)	124.7	7.12 (t, 7.1)
7	111.5	7.41 (d, 7.7)	109.6	7.23 (7.3)	110.3	7.21 (dd, 7.1, 0.4)
7a	135.9		136.6		137.3	
2′	144.5	7.39 (s)	145.8	8.54 (s)	146.2	8.58 (s)
3′	149.6		149.8		150.7	
5′	159.7		159.6		160.0	
5a′	126.0		126.0		130.8	
7′	150.2		150.4		150.2	
8a′	146.7		147.2		147.3	
9′	29.4	3.66 (s)	23.3	3.67 (s)	29.9	3.67 (s)
10′	29.2	3.51 (s)	29.0	3.52 (s)	29.1	3.53 (s)
11′	75.8	5.72 (dd, 7.3, 4.7)	76.5	5.85 (t, 6.6)	76.6	5.72 (t, 6.8)
12′	30.8	2.05 (m)	27.4	1.98 (m)	29.9	2.37 (m)
13′	12.0	0.8 (t, 7.3)	9.3	0.84 (t, 7.3)	12.6	1.01 (t, 7.3)
2″	147.9	8.72 (s)	145.5	8.05 (s)	147.7	8.48 (s)
3″	153.0		151.7		156.7	
5″	159.3		159.8		160.3	
5a″	125.7		126.0		129.1	
7″	150.2		149.8		150.5	
8a″	146.6		147.2		146.2	
9″	29.3	3.65 (s)	29.2	3.61 (s)	29.3	3.63 (s)
10″	29.0	3.48 (s)	29.0	3.54 (s)	29.0	3.51 (s)
11″	42.0	4.38 (t, 7.7)	60.5	5.62 (dd, 9.1, 5.8)	52.4	4.94 (t, 7.6)
12″	27.2	1.67 (m)	27.2	2.50 (m)	28.5	1.81 (m)
13″	8.7	0.62 (t, 7.1)	11.0	1.05 (t, 7.3)	9.2	0.68 (t, 7.5)
α-Trp	56.1	4.84 (dt, 8.4, 5.5)	54.7	4.79 (m)	55.2	4.84 (d, 7.5)
β-Trp	27.3	3.48 (m)	28.2	3.34 (m)	27.5	3.60 (m)
$NHCOCH_3$		5.60 (d, 8.4)		5.46 (d, 7.3)		5.72 (m)
$NHCOCH_3$	156.5		156.4		155.9	
$NHCOCH_3$	52.3	3.72 (s)	52.3	3.64 (s)	48.1	3.64 (s)
*C*O-Trp	171.3		171.6		172.1	

array spectrophotometer, respectively. Optical rotation values were obtained with a Perkin–Elmer 343 polarimeter. ^{1}H, ^{13}C and 2D NMR spectra were recorded on Bruker AC-200 and AM-500 spectrometers. Positive mode FAB (using glycerol as matrix and a Cesium gun) and HREI (at 70 eV) mass spectra were obtained on a VG Analytical ZAB-SEQ BEqQ type mass spectrometer. HPLC separations were achieved using a Thermo Separations pump and UV detector, a Shodex RI-71RI detector and a YMC RP-18 (20×250 mm) column.

3.2. Collection and isolation

The spherical yellow sponge *Clathria* sp., initially classified as the synonym *Pseudanchynoe* sp. (Burton, 1929),[25] (8 kg) was collected during commercial clam fishing activities at the SW SAO bank (−100 m).by the 'Erin Bruce' and 'Mr. Big' vessels, and was stored at −20°C until worked-up. The sample was classified by Lic. Elena Cuartas (Universidad Nacional de Mar del Plata), and a voucher specimen is deposited at the INIDEP benthos collection (Mar del Plata). The frozen sponge was blended and extracted with EtOH (2×10 L). A portion of the extract was vacuum dried and partitioned (EtOAc/H_2O) to give 11 g of dry residue from the organic phase. The residue was subjected to silica flash chromatography using mixtures of CH_2Cl_2/EtOAc and 10 fractions were collected. Reversed phase flash chromatography of fraction 5 (MeOH/H_2O), yielded 6-(1-hydroxypropyl)-1,3-dimethyl-1*H*-pteridine-2,4-dione (**4**, 21 mg, white needles) and pseudoanchynazine A (**1**, 260 mg, yellow powder). Fraction 7 (CH_2Cl_2–EtOAc 3:7) was subjected to Rp-18 HPLC [eluant: CH_3CN–H_2O (50:50)] to afford pseudoanchynazine B (**2**, 71 mg, yellow powder) and pseudoanchynazine C (**3**, 5 mg, yellow powder).

3.2.1. Pseudoanchynazine A (1). UV (MeOH) λ_{max}= 248 nm (ϵ 9700); $[\alpha]_D^{25}=-60.7°$ (*c* 0.83, MeOH); IR (KBr) ν_{max} 3543, 3358, 2966, 2881, 1725, 1676, 1590, 1554, 1505, and 1462 cm^{-1}; ^{1}H and ^{13}C (Table 1); FABMS *m/z* 727 $(M+H)^+$; HRFABMS *m/z* 727.2963 $(M+H)^+$ [calcd for $C_{35}H_{39}O_8N_{10}$ $(M+H)^+$, 727.2952].

3.2.2. Pseudoanchynazine B (2). UV (MeOH) λ_{max}= 242 nm (ε 11,300); $[\alpha]_D^{25}=81.2°$ (*c* 0.42, MeOH); IR (KBr) ν_{max} 3515, 3372, 2966, 2881, 1773, 1662, 1590, 1547, 1505 and 1462 cm^{-1}; ^{1}H and ^{13}C (Table 1); FABMS *m/z* 727 $(M+H)^+$; HRFABMS *m/z* 727.2956 $(M+H)^+$ [calcd for $C_{35}H_{39}O_8N_{10}$ $(M+H)^+$, 727.2952].

3.2.3. Pseudoanchynazine C (3). UV (MeOH) λ_{max}= 253 nm (ϵ 10,900); $[\alpha]_D^{25}$=96.6° (*c* 2.21, MeOH); IR (film) ν_{max} 3342, 3055, 2963, 2876, 1720, 1670, 1616, 1586, 1548, 1499 and 1413 cm^{-1}; ^1H and ^{13}C (Table 1); FABMS *m/z* 727 $(M+H)^+$; HRFABMS *m/z* 727.2958 $(M+H)^+$ [calcd for $C_{35}H_{39}O_8N_{10}$ $(M+H)^+$, 727.2952].

3.3. Methanolysis of pseudoanchynazine A (1)

A MeOH solution (2 mL) of pseudoanchynazine A (**1**, 30 mg) was treated with conc. HCl (0.05 mL) at 80°C for 2 h. After cooling the residue was neutralized using $NaHCO_3$ and extracted with a EtOAc–H_2O mixture. The organic portion was subjected to preparative silica gel TLC (CH_2Cl_2–EtOAc 1:1) to afford the methyl ester **5a** (8.7 mg, R_f 0.8, yellow powder) and the alcohol **4** (3.2 mg, R_f 0.3, white needles).

3.3.1. Compound 5a. ^1H NMR ($CDCl_3$) δ 9.68 (1H, s, H-1), 7.40 (1H, d, *J*=7.5 Hz, H-4), 7.10 (1H, t, *J*=6.2 Hz, H-5), 7.02 (1H, t, *J*=6.8 Hz, H-6), 7.34 (1H, d, *J*=7.7 Hz, H-7), 8.75 (1H, s, H-2″), 3.68 (3H, s, H-9″), 3.54 (3H, s, H-10″), 4.44 (1H, m, H-11″), 2.20 (2H, m, H-12″), 0.88 (3H, t, *J*=7.3 Hz, H-13″), 4.56 (1H, m, α-Trp), 3.34 (2H, m, β-Trp), 5.34 (1H, d, *J*=7.6 Hz, NH-Trp), 3.67 (3H, s, methyl carbamate), 3.52 (3H, s, methyl ester); ^{13}C NMR ($CDCl_3$) δ 136.2 (C-2), 106.6 (C-3), 128.0 (C-4a), 118.3 (C-4), 122.0 (C-5), 111.5 (C-7), 136.2 (C-7a), 148.0 (C-2″), 153.6 (C-3″), 126.5 (C-5a″),160.4 (C-5″), 150.7 (C-7″), 147.1 (C-7a″), 29.9 (C-9″), 29.6 (C-10″), 42.8 (C-11″), 29.0 (C-12″), 12.5 (C-13″), 55.5 (α-Trp), 28.0 (β-Trp), 156.5 (carbamate carbonyl), 52.5 (methyl carbamate), 172.8 (CO-Trp), 60.6 (methyl ester); EIMS *m/z* 509 $M^{+\cdot}$; HREIMS *m/z* 363.1616 $(M-C_5H_7O_4N)^+$ [calcd for $C_{20}H_{21}O_2N_5$ $(M-C_5H_7O_4N)^+$, 363.1695].

3.3.2. 6-(1-Hydroxypropyl)-1,3-dimethyl-1*H*-pteridine-2,4-dione (4). $[\alpha]_D^{25}$=59.1° (*c* 0.38, MeOH); ^1H NMR ($CDCl_3$) δ 8.77 (1H, s, H-2′), 3.74 (3H, s, H-9′), 3.56 (3H, s, H-10′), 4.94 (1H, dd, *J*=7.3, 5.3 Hz, H-11′), 2.00 (2H, m, H-12′), 1.02 (3H, t, *J*=7.5 Hz, H-13′); ^{13}C NMR ($CDCl_3$) δ 145.9 (C-2′), 154.2 (C-3′), 126.0 (C-5a′), 160.1 (C-5′), 150.6 (C-7′), 147.3 (C-8a′), 29.5 (C-9′), 29.0 (C-10′), 75.1 (C-11′), 31.1 (C-12′), 9.5 (C-13′). EIMS *m/z* 250 M^+; HREIMS *m/z* 221.0638 $(M-C_2H_5)^+$ [calcd for $C_9H_9O_3N_4$ $(M-C_2H_5)^+$, 221.0675].

3.4. Crystal structure of 4

The yellow compound was recrystallized by slow evaporation of an ethanolic solution. Crystalline material in the shape of overgrown needles were obtained, from which a small piece (0.35×0.15×0.12 mm^3) suitable for X-ray diffraction could be cut out. Data collection was performed in a Siemens R3 single crystal difractometer, using the $\omega/2\theta$ scan mode and graphite-monocromatized Mo K_α radiation. The structure was conventionally solved by direct methods (SHELXS97[26]), and refined by full matrix least squares in F^2 (SHELXL97[27]). Hydrogen atoms attached to carbon were idealized, and allowed to ride during refinement. The one attached to O(14), instead, was found in the latest difference Fourier and refined with a restrained O–H distance. Due to the feeble anomalous dispersion effects present it was impossible to unambiguously determine the enanthiomeric form just from X-ray data alone. The model presented herein (Fig. 4), which yielded the lowest Flack parameter[28] of the two enanthiomeric sets refined, coincides with the one independently determined by spectroscopic data. Interatomic distances and angles do not depart significantly from commonly accepted values.

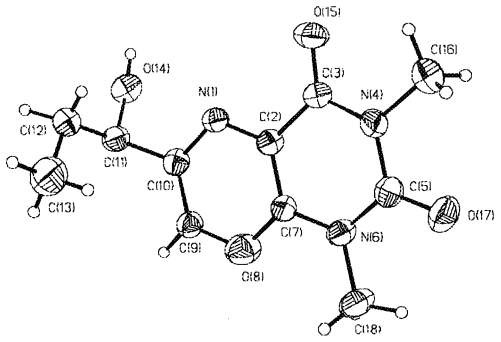

Figure 4. X-Ray structure for compound **4**.

*Crystal data for (**4**).* $C_{11}H_{14}N_4O_3$; *M*=250.26; orthorhombic, space group $P2_12_12_1$, *a*=6.007 (1), *b*=11.553 (2), *c*=16.949 (2) Å, *V*=1176.2 (3) $Å^3$, *Z*=4, D_c=1.413 g cm^{-3}, F_{000}=528, mu=0.106 mm^{-1}, *N*=1241, $N[I>2\sigma(I)]$=1038, R=0.032, *wR*2=0.079.

3.5. Ethanolysis of pseudoanchynazine A (1)

Pseudoanchynazine A (**1**, 30 mg) was treated with HCl in EtOH by the same procedure as described above to afford the ethyl ester **5b** (3.2 mg, yellow oil).

3.5.1. Compound 5b. ^1H NMR ($CDCl_3$) δ 9.37 (1H, s), 7.43 (1H, d, *J*=7.5 Hz), 7.11 (1H, t, *J*=6.2 Hz), 7.04 (1H, t, *J*=6.8 Hz), 7.35 (1H, d, *J*=7.7 Hz), 8.72 (1H, s), 3.70 (3H, s), 3.56 (3H, s), 4.44 (1H, m), 2.20 (2H, m), 0.89 (3H, t, *J*=7.3 Hz), 4.57 (1H, m), 3.33 (2H, m), 5.25 (1H, d, *J*=7.6 Hz), 3.66 (3H, s), 3.99 (2H, m, $COOCH_2CH_3$); 1.01 (3H, t, *J*=5.8 Hz, $COOCH_2CH_3$); EIMS *m/z* 221 $(M-C_2H_5)^+$; HREIMS *m/z* 221.06378 $(M-C_2H_5)^+$ [calcd for $C_9H_9O_3N_4$ $(M-C_2H_5)^+$, 221.06746].

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