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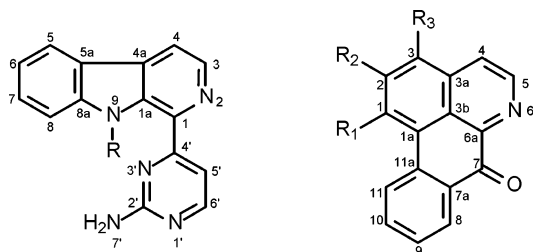
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Bioassay-guided fractionation of the bark extract of *Annona foetida* afforded a new antileishmanial pyrimidine- $\beta$ -carboline alkaloid, *N*-hydroxyannomontine (**1**), together with the previously reported annomontine (**2**), *O*-methylmoschatoline (**3**), and liriodenine (**4**). The structure of compound **1** was established on the basis of extensive 1D and 2D NMR and MS analyses. This is the third reported pyrimidine- $\beta$ -carboline-type alkaloid and is particularly important for *Annona* genus chemotaxonomy. In addition, all compounds exhibit in vitro antileishmanial activity against promastigote forms of *Leishmania braziliensis*. Compounds **2** and **4** showed better activity than compounds **1** and **3** against *L. braziliensis*. Compound **2** was not active against *L. guyanensis*.

Plants of the Annonaceae family are known as a rich source of aporphinic alkaloids. Previous chemical and pharmacological investigations on species in this family have indicated the presence of important bioactive compounds, exhibiting various pharmacological activities including antiparasitic, in particular against *Leishmania* sp.,<sup>1–5</sup> *Plasmodium falciparum*,<sup>6,7</sup> and *Trypanosoma cruzi*.<sup>1,5</sup> Therefore, in a search for novel antiparasitic natural products we have studied the bark of *Annona foetida* Martius. This annonaceous plant, known commonly as “envira-ata” and “graviola do mato”, is a tropical native tree found in the Brazilian and Peruvian Amazon.<sup>8</sup> Its seeds are traditionally used in Brazil as an insecticide and antiparasitic. The present paper reports the isolation and characterization of a new pyrimidine- $\beta$ -carboline alkaloid, named *N*-hydroxyannomontine (**1**), together with previously reported annomontine (**2**)<sup>9</sup> and the oxoaporphinic alkaloids *O*-methylmoschatoline (**3**)<sup>10</sup> and liriodenine (**4**).<sup>10</sup> The antiparasitic activity was evidenced for the crude extract and investigated for each purified compound on *Leishmania braziliensis* and *L. guyanensis*, the main causes of leishmaniasis in the Brazilian state of the Amazon.



(**1**) R = OH  
(**2**) R = H

(**3**) R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = OCH<sub>3</sub>  
(**4**) R<sub>1</sub> = R<sub>2</sub> = OCH<sub>2</sub>O, R<sub>3</sub> = H

Having discovered that the CH<sub>2</sub>Cl<sub>2</sub> and MeOH crude extracts possessed in vitro activity against *L. braziliensis* (IC<sub>50</sub> 23.0 and

**Table 1.** In Vitro Activity of *Annona foetida* Extracts and Compounds against *Leishmania* Species (promastigote forms)<sup>a</sup>

extract/fraction	IC <sub>50</sub> (μg/mL)	
	<i>L. braziliensis</i>	<i>L. guyanensis</i>
hexane	> 160	42.7 ± 5.4
CH <sub>2</sub> Cl <sub>2</sub>	23.0 ± 0.6	2.7 ± 0.4
CH <sub>2</sub> Cl <sub>2</sub> alkaloidal fraction	18.3 ± 2.5	10.3 ± 0.9
MeOH	40.4 ± 3.2	23.6 ± 3.1
MeOH alkaloidal fraction	24.3 ± 1.9	9.1 ± 0.8

compound	IC <sub>50</sub> (μM)	
	<i>L. braziliensis</i>	<i>L. guyanensis</i>
<i>N</i> -hydroxyannomontine ( <b>1</b> )	252.7 ± 2.2	437.5 ± 2.5
annomontine ( <b>2</b> )	34.8 ± 1.5	> 613.0
<i>O</i> -methylmoschatoline ( <b>3</b> )	320.8 ± 3.1	103.7 ± 3.4
liriodenine ( <b>4</b> )	58.5 ± 1.8	21.5 ± 0.4
pentamidine <sup>b</sup>	2.9 ± 0.3	0.9 ± 0.3

<sup>a</sup> The IC<sub>50</sub> values are expressed as mean ± SEM of three determinations. <sup>b</sup> Standard antileishmanial agent.

40.4 μg/mL, respectively) and *L. guyanensis* (IC<sub>50</sub> 2.7 and 23.6 μg/mL, respectively), as shown by screening tests (Table 1), bioassay-guided fractionation of the extracts was undertaken as described in the Experimental Section, which led to the isolation and identification of four alkaloids, namely, two pyrimidine- $\beta$ -carboline alkaloids (**1** and **2**) and two oxoaporphine alkaloids (**3** and **4**). The structures of these compounds were elucidated by spectroscopic methods, including 1D and 2D NMR and MS analyses.

Compound **1** was obtained as red needles and has the molecular formula C<sub>15</sub>H<sub>11</sub>N<sub>5</sub>O, as deduced from its HREIMS (observed *m/z* 277.09469) and NMR data. The IR spectrum showed broad absorption bands due to O-H/N-H groups (3420 and 3172 cm<sup>-1</sup>) and typical absorption for aromatic rings (1600–1450 cm<sup>-1</sup>). The UV spectrum revealed a conjugated aromatic chromophore with maxima at 246, 312, 398, and 428 nm. The ESIMS of **1** showed a protonated molecule at *m/z* 278.5 [M + H]<sup>+</sup>, 16 Da higher than the pyrimidine- $\beta$ -carboline alkaloid annomontine (**2**). This suggested the presence of a hydroxyl group in the structure of **1**, which was evidenced in the IR spectrum and NMR data. Additionally, the ESIMS/MS (70 eV) experiments of **1** gave informative fragment

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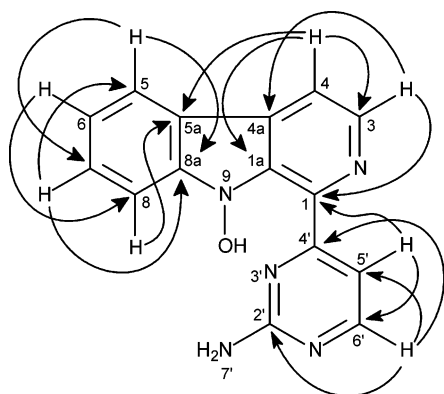
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**Table 2.**  $^1\text{H}$ ,  $^{13}\text{C}$ , and HMBC NMR Data for *N*-Hydroxyannomontine (**1**) and  $^1\text{H}$  for Annomontine (**2**)<sup>a</sup>

position	$^{13}\text{C}$ $\delta$ ( <b>1</b> )	$^1\text{H}$ $\delta$ ( <b>1</b> ) (mult., <i>J</i> in Hz)	$^1\text{H}$ $\delta$ ( <b>2</b> ) (mult., <i>J</i> in Hz)	HMBC $^1\text{H}$ – $^{13}\text{C}$ ( <b>1</b> ) <sup>b</sup>
1	136.1			
1a	132.6			
3	138.8	8.53 (1H, d; 5.0)	8.53 (1H, d; 5.0)	1, 4, and 4a
4	117.9	8.312 (1H, d; 5.0)	8.25 (1H, d; 5.0)	1a, 3, and 5a
4a	128.6			
5	122.4	8.308 (1H, ddd; 7.9; 1.1; 0.8)	8.30 (1H, ddd; 7.9; 1.2; 0.8)	4a, 7, and 8a
5a	118.2			
6	121.0	7.34 (1H, ddd; 7.9; 6.5; 1.7)	7.31 (1H, ddd; 7.9; 7.1; 1.1)	5a, 7, and 8
7	130.1	7.70 (1H, ddd; 8.3; 6.5; 1.1)	7.60 (1H, ddd; 8.3; 7.1; 1.2)	5 and 8a
8	110.5	7.67 (1H, ddd; 8.3; 1.7; 0.8)	7.70 (1H, ddd; 8.3; 1.1; 0.8)	5a and 6
8a	138.9			
9		15.08 (N-OH, br s)	11.81 (N-H, br s)	
2'	163.0			
4'	164.0			
5'	109.5	8.02 (1H, d; 5.2)	7.88 (1H, d; 5.2)	1, 4', and 6'
6'	161.9	8.57 (1H, d; 5.2)	8.48 (1H, d; 5.2)	1, 2', 4', and 5'
7'		6.83 (2H, br s)	6.40 (2H, br s)	

<sup>a</sup> The experiments were taken at 400 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}$  in  $(\text{CD}_3)_2\text{CO}$  and TMS as internal reference ( $\delta$  0.00 ppm). <sup>b</sup> Carbons atoms that showed correlation with respective hydrogen.

**Figure 1.** Key HMBC  $^1\text{H}$ – $^{13}\text{C}$  correlations for compound **1**.

peaks at  $m/z$  260  $[(M + H) - 18]^+$  and 167  $[(M + H) - 18 - 93]^+$ , corresponding to the loss of  $\text{H}_2\text{O}$  and  $\text{H}_2\text{O}$  plus the 2'-aminopyrimidine ring without a hydrogen atom, respectively.

The  $^1\text{H}$  NMR spectrum showed four adjacent aromatic hydrogens [ $\delta$  8.308 (ddd,  $J = 7.9$ ; 1.1 and 0.8 Hz, H-5), 7.67 (ddd,  $J = 8.3$ ; 1.7 and 0.8 Hz, H-8), 7.34 (ddd,  $J = 7.9$ ; 6.5 and 1.7 Hz, H-6), and 7.70 (ddd,  $J = 8.3$ ; 6.5 and 1.1 Hz, H-7)] characterizing the indole moiety. A pair of doublets at  $\delta$  8.53 and 8.312 (H-3 and H-4,  $J = 5.0$  Hz, respectively) was consistent with two hydrogens in a pyridine-like skeleton. Two other doublets at  $\delta$  8.02 and 8.57 ( $J = 5.2$  Hz) were attributed to hydrogens 5' and 6', respectively, of the 2'-aminopyrimidine ring. In fact, the  $^1\text{H}$  spectrum of **1** was very similar to that of **2** except for the absence of the N-H (indole hydrogen) signal at  $\delta$  11.81, which was replaced by the N-OH signal at  $\delta$  15.08 ppm (Table 2).

The  $^{13}\text{C}$  NMR data together with one-bond  $^1\text{H}$ – $^{13}\text{C}$  correlations observed in the gHSQC experiments of **1** indicated the presence of 15 aromatic carbons, comprising seven quaternary and eight methines (Table 2). The long-range  $^1\text{H}$ – $^{13}\text{C}$  correlations observed in the gHMBC experiment allowed assignment of carbon atoms and confirmed the harman-type moiety, the 2'-aminopyrimidine ring, and mainly the linkage of the pyrimidine- $\beta$ -carboline substructures by the coupling of H-5' and H-3 with C-1 (Figure 1). Accordingly, structure **1** was elucidated as the new alkaloid derivative *N*-hydroxyannomontine. Furthermore, 2D and 1D NMR data (Figure 1 and Table 2) supported the proposed structure. Compounds **2**–**4** were identified by spectroscopic methods and by comparison with literature data (see Experimental Section).

Pyrimidine- $\beta$ -carboline alkaloids with a harman moiety linked to 2-aminopyrimidine are unusual and interesting from a structural point of view. Only two similar compounds, annomontine and

methoxyannomontine, were previously isolated, originally from *Annona montana*<sup>9</sup> and *A. reticulada*.<sup>11</sup> However, the methoxyannomontine was also reported in *Neolitsea parvigemma* and *N. konishii*, two members of the Lauraceae family.<sup>12</sup> Compound **1** thus represents the third new member of the pyrimidine- $\beta$ -carboline alkaloid type.

The antiparasitic activity was investigated for purified compounds on *Leishmania* sp. (Table 1). Annomontine (**2**) and liriodenine (**4**) were the most effective compounds, with  $\text{IC}_{50}$  values lower than 60  $\mu\text{M}$  for *L. braziliensis*. The structure–activity relationships established by means of these compounds led to the following observations: in the oxoaporphine alkaloids, liriodenine (**4**), possessing a methylenedioxy moiety, was about 8 times more active against *L. guyanensis* and *L. braziliensis* than *O*-methylmoschatoline (**3**). In the pyrimidine- $\beta$ -carboline alkaloids, annomontine (**2**) was 6 times more active against *L. braziliensis* than *N*-hydroxyannomontine (**1**). However, **1** was active against *L. guyanensis*, while **2** was inactive. Liriodenine (**4**) was the most active against *L. guyanensis*, with an  $\text{IC}_{50}$  of 21.5  $\mu\text{M}$ , while annomontine (**2**) was the most active against *L. braziliensis*, with an  $\text{IC}_{50}$  of 34.8  $\mu\text{M}$ .

## Experimental Section

**General Experimental Procedures.** Melting points were determined on a Microquímica MQAPF-301 micromelting point apparatus. Optical rotations were measured in  $\text{CHCl}_3$  solutions at room temperature on a Bellingham + Stauly Limited BS R94041 polarimeter. UV spectra were obtained in  $\text{CHCl}_3$  on a Varian Cary 500 Scan UV–vis–NIR spectrophotometer. IR spectra were acquired on a Perkin-Elmer Spectrum 2000 spectrophotometer. All NMR data were recorded at 293 K in  $(\text{CD}_3)_2\text{CO}$  on a Bruker AVANCE DRX 400 spectrometer operating at 9.4 T, observing  $^1\text{H}$  at 400.13 and  $^{13}\text{C}$  at 100.61 MHz.

HSQC and HMBC experiments were optimized for average coupling constants  $^1J(\text{C},\text{H})$  of 140 Hz and  $^{\text{LR}}J(\text{C},\text{H})$  of 8 Hz, respectively. Chemical shifts ( $\delta$ ) are given in ppm relative to TMS ( $\delta$  0.00) as internal standard, and all pulse programs were supplied by Bruker. Low-resolution ESIMS and ESIMS/MS data were taken in the positive ion mode, on a Micromass Quattro LC mass spectrometer. HREIMS measurements were carried out on a Micromass VG-Autospec mass spectrometer, using electron impact as the ionization mode. Silica gel 60 (70–230 mesh) was used for column chromatography, precoated silica gel plates (60 F<sub>254+366</sub> Merck, 0.25 mm, aluminum) were used for analytical TLC, and precoated silica gel plates (60 GF<sub>254</sub> Merck, 1 mm, glass) were used for preparative TLC. The spots were detected by spraying with Dragendorff's reagent or 5%  $\text{H}_2\text{SO}_4$  in EtOH and then heating on a hot plate.

**Plant Material.** The bark of *A. foetida* was collected from the Adolpho Ducke Reserve, in the vicinity of Manaus, a city in the Brazilian Amazon, in August 2002 and identified by an Annonaceae specialist, Dr. Antônio Carlos Weber, from the Universidade Federal

do Amazonas (UFAM). A voucher specimen (number 7275) has been deposited at the Herbarium of the Department of Biology, UFAM, Manaus, Amazon, Brazil.

**Extraction and Isolation.** The powdered, air-dried bark of *A. foetida* (1.1 kg) was extracted successively with hexane ( $4 \times 2$  L),  $\text{CH}_2\text{Cl}_2$  ( $4 \times 2$  L), and then MeOH ( $4 \times 2$  L), at room temperature. Removal of the solvents from the extracts under reduced pressure gave 3.66 g (hexane), 6.74 g ( $\text{CH}_2\text{Cl}_2$ ), and 25.0 g (MeOH) residues.

Antileishmanial screening of the crude  $\text{CH}_2\text{Cl}_2$  and MeOH extracts of the bark showed in vitro activity against *L. braziliensis* and *L. guyanensis* (Table 1). Furthermore, TLC investigations revealed the presence of alkaloids in these extracts. The  $\text{CH}_2\text{Cl}_2$  extract of the bark (6.74 g) was redissolved in  $\text{CH}_2\text{Cl}_2$  (100 mL) and subjected to extraction with 3% aqueous HCl ( $4 \times 500$  mL). Following this, the aqueous solution was adjusted with  $\text{NH}_4\text{OH}$  to pH 9–10 and extracted with  $\text{CH}_2\text{Cl}_2$  ( $4 \times 500$  mL). The organic fractions were combined, and the solvent was evaporated under vacuum to yield the  $\text{CH}_2\text{Cl}_2$  alkaloidal fraction (300 mg).

The alkaloidal fraction [(antileishmanial activity (Table 1)] was initially subjected to silica gel column chromatography, having been previously treated with a 10%  $\text{NaHCO}_3$  solution and eluted with increasing concentrations of  $\text{CH}_2\text{Cl}_2$  in hexane followed by EtOAc in  $\text{CH}_2\text{Cl}_2$ . The eluted fractions (30 mL) were evaluated and pooled by TLC analysis. Additional chromatographic separation of the fractions 07–12 (13 mg) and 13–26 (47 mg) by preparative TLC eluted with  $\text{CHCl}_3$ –MeOH (90:05, v/v) resulted in respectively **3** (4.0 mg), **1** (7.0 mg), and **2** (7.0 mg). The same acid–base extraction applied to the MeOH extract gave an alkaloidal brownish viscous fraction (3.7 g). The MeOH alkaloidal fraction [antileishmanial activity (Table 1)] was subjected to the same chromatographic separation as described above to afford **4** (4.0 mg), **3** (4.0 mg), **1** (5.0 mg), and **2** (30 mg).

**N-Hydroxyannomontine (1):** red needles ( $\text{CHCl}_3$ ); mp 249–250 °C;  $[\alpha]_D^{25}$  0.00; UV ( $\text{CHCl}_3$ )  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 244 (4.30), 314 (3.97), and 434 (3.63) nm; IR (KBr)  $\nu_{\text{max}}$  3420, 3172, 1664, 1630, 1574, 1465, 1420, 1346, 1317, 1228, 1209, 1155, 1080, 836, and 733  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data given in Table 2; ESIMS  $m/z$  278.5  $[\text{M} + \text{H}]^+$ ; ESIMS/MS (daughter ions, 70 eV)  $m/z$  278  $[\text{M} + \text{H}]^+$  (100), 260  $[(\text{M} + \text{H}) - \text{H}_2\text{O}]^+$  (70), 167 (40), 150 (35); HREIMS  $m/z$  277.09469 (calcd for  $\text{C}_{15}\text{H}_{11}\text{N}_5\text{O}$ , 277.09636).

**Annomontine (2):** yellow needles ( $\text{CHCl}_3$ ); mp 249–250 °C (lit. 247–248 °C); UV ( $\text{CHCl}_3$ )  $\lambda_{\text{max}}$  246, 312, 398, and 428 nm; IR (KBr)  $\nu_{\text{max}}$  3346, 3169, 1652, 1630, 1576, 1551, 1467, 1457, 1419, 1384, 1324, 1288, 1225, 1071, 818, 740, 668, and 630  $\text{cm}^{-1}$ ; ESIMS  $m/z$  262.2  $[\text{M} + \text{H}]^+$ ; ESIMS/MS (daughter ions, 70 eV)  $m/z$  262  $[\text{M} + \text{H}]^+$  (100), 193 (60), 167 (20); HREIMS  $m/z$  261.10659 (calcd for  $\text{C}_{15}\text{H}_{11}\text{N}_5$ , 261.10144); identified by comparison with literature data (mp, UV, IR,  $^1\text{H}$  and  $^{13}\text{C}$  NMR).<sup>9</sup>

**O-Methylmoschatoline (3):** orange-yellow needles ( $\text{CHCl}_3$ ); mp 181–182 °C (lit. 182–184 °C), identified by comparison with literature data (mp, UV, IR,  $^1\text{H}$  and  $^{13}\text{C}$  NMR).<sup>10,13</sup>

**Liriodenine (4):** orange needles ( $\text{CHCl}_3$ ); mp 280–281 °C (lit. 279–281 °C), identified by comparison with literature data (mp, UV, IR,  $^1\text{H}$  and  $^{13}\text{C}$  NMR).<sup>10</sup>

**Antileishmanial Assay.** Cultures of *Leishmania braziliensis* (MHOM/BR/95/IOCL-2033) and *L. guyanensis* (MHOM/BR/95/IOCL-2092) promastigotes identified by isoenzyme analysis were used for in vitro

screening. Briefly, promastigotes were cultivated in Schneider's *Drosophila* medium (Hendricks et al., 1978)<sup>14</sup> supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS) and harvested from the medium in the late-log phase.

The antileishmanial screening was performed in flat-bottomed 96-well plastic-tissue trays maintained at 26 °C in an atmosphere of 95% air/5%  $\text{CO}_2$ . Promastigotes from a logarithmic phase culture were suspended to yield  $4.0 \times 10^6$  cells/mL after hemocytometer counting. Each well was filled with 100  $\mu\text{L}$  of the parasite suspension, and plates were incubated at 26 °C. The extracts, fractions, and alkaloids were dissolved in DMSO and then added to each well to obtain a concentration range of 160–0.16  $\mu\text{g/mL}$ . At up to 1.6% (v/v), DMSO had no effect on parasite growth. Each concentration was screened in triplicate. The viability of promastigotes was checked using a Neubauer's chamber. The results are expressed as the concentrations inhibiting parasite growth by 50% ( $\text{IC}_{50}$ ) after a 24 h incubation period. Pentamidine was used as a positive control.

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