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Colubricidin A, a novel macrolide antibiotic from a *Streptomyces* sp.

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Abstract

A new 34-membered macrolide antibiotic, colubricidin A (**1**), was isolated from the fermentation broth of a new streptomyces species. Its structure was elucidated on the basis of analysis of the spectroscopic data and chemical degradation. It was identified as a glycomacrolide with an unprecedented aromatic chromophore. Colubricidin A showed excellent activity against Gram-positive bacteria. © 1999 Elsevier Science Ltd. All rights reserved.

In the course of our search for novel substances from microorganisms, an extract from an unidentified *Streptomyces* culture, designated LL-C13122, was found to have nematocidal activity in an intro screen against *Caenorhabditis elegans*. Subsequently, the material also demonstrated in vivo activity in gerbils infected with *Trichostrongylus colubriformis*. The isolated nematocidal agent, colubricidin A (**1**), demonstrated potent activity against Gram-positive bacteria but only weak activity against Gram-negative isolates. It was slightly more potent than vancomycin versus staphylococci but less potent versus streptococci and enterococci.

The fermentation broth was filtered through a small Celite disk. The Celite was washed with water and extracted with methanol. The methanol extract was concentrated and then loaded onto a preparative reversed phase cartridge. The cartridge was eluted with a gradient of 30–60% acetonitrile in 0.05 M ammonium acetate buffer at pH 4.5. Fractions showing activity were combined, evaporated, and desalted to yield a complex of C13122 antibiotics, which was further purified by multiple reversed phase HPLC to give colubricidin A (**1**).

Colubricidin A (**1**) was isolated as a yellowish powder ($[\alpha]_D^{25}$=–7; MeOH, *c* 0.50). Its molecular formula was determined by high resolution FT-ICR mass spectrometry that gave a molecular weight of 2153.9140, appropriate for a molecular formula of $C_{96}H_{154}Cl_3N_5O_{42}$ (calcd: 2153.9134). The UV absorption data of colubricidin A had a maximum at 375 nm and a shoulder at 280 nm in neutral solution. Under acidic conditions, the absorption maximum was shifted to 405 nm and shoulder peak to 265 nm. An intensive analysis of 1D and 2D NMR data (Table 1) acquired on **1** coupled with chemical degradation led to the proposed structure as shown in Fig. 1.

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Table 1
^{1}H and ^{13}C NMR data of colubricidin A (**1**) in CD_3OD[a]

Position	^{1}H	J(Hz)	^{13}C	Position	^{1}H	J(Hz)	^{13}C	Position	^{1}H	J(Hz)	^{13}C
1			168.78	36	1.83	m	35.99	Sugar-B			
2	5.97	d(15.6)	124.95	37	3.46		79.80	1	4.42	d(7.5)	103.42
3	7.04	dt(15.6, 7.5)	148.46	38	1.98		41.20	2	3.60		82.20
4	2.34, 2.46		41.35	39	4.19	m	76.75	3	3.83	t(9.0)	80.97
5	3.94		70.41	40	1.14		15.95	4	3.50		76.82
6	1.63		44.57	41	0.86		13.94	5	3.42		72.91
7	4.02	m	71.38	42	0.85		9.90	6	1.28	d(6.3)	19.22
8	1.57		44.96	43	0.87		10.44	Sugar-C			
9	3.72		72.27	44	1.08	d(6.7)	14.73	1	4.53	d(7.9)	104.61
10	1.34, 1.55		37.64	45	0.85		9.58	2	3.86		74.33
11	1.01	m, 1.45	31.03	46	0.91	d(6.7)	5.02	3	3.29		78.22
12	2.21	m	39.43	47	0.79	d(6.7)	10.20	4	3.17	t(9.1)	76.61
13	3.72		82.64	Sugar-A				5	3.32		73.77
14	1.53, 2.02	dd(15.0, 8.0)	39.43	1	4.76	bd(9.8)	99.54	6	1.32	d(6.0)	18.11
15			99.79	2	1.74, 2.39		38.87	Sugar-D			
16	3.33		75.62	3	5.26	td(10.7, 5.1)	73.44	1	5.29	d(7.6)	100.78
17	3.90		69.49	4	3.98		56.88	2	1.52, 2.35		41.00
18	1.23, 2.09	bd	39.20	5	3.70		71.69	3	3.63		69.84
19	3.87		70.72	6	1.36	d(6.1)	18.91	4	2.43		60.84
20	1.44		45.29	Pyridine				5	3.44		71.90
21	4.38	bd(10.6)	67.45	2			137.7	6	1.33		18.58
22	1.20, 1.79		40.91	3			146.95	Sugar-E			
23	3.77	m	71.62	4			109.73	1	4.48	d(7.7)	102.49
24	1.42		45.79	5	7.69	d(6.9)	126.71	2	3.46		73.56
25	3.98		72.48	6	7.50	d(6.9)	128.12	3	3.37		87.21
26	1.34, 1.56		35.99	7			166.34	4	3.03	t(9.0)	75.23
27	1.51		24.00	8			166.44	5	3.34		73.77
28	1.35, 1.53		34.66	Pyrrole				6	1.29	d(6.2)	18.47
29	3.38		71.50	2			124.81	Sugar-F			
30	2.76	m	64.22	3			112.65	1	4.73	dd(9.9, 1.3)	102.37
31	2.72	bd(8.9)	60.62	4			109.47	2	1.52, 2.31		40.50
32	1.58		40.69	5			119.75	3	3.52		72.27
33	5.31	d(10.2)	75.43	6			161.78	4	2.93	t(9.0)	78.37
34	1.95		38.78	7	3.58		34.08	5	3.31		73.77
35	3.37		78.37					6	1.29	d(6.4)	18.30

[a] ^{1}H and ^{13}C chemical shifts were measured in parts per million relative to partially deuterated solvent peaks of methanol-d_4 at δ 3.30 (^{1}H) and δ 49.15 (^{13}C), respectively.

The ^{13}C and DEPT-135 data for **1** indicated the presence of six anomeric methine carbons including the one (Sugar-A) connected to the chromophore as discussed for compound **2** below. Detailed analysis of the COSY, TOCSY, and HMQC–TOCSY data led to identification of the other five sugar residues as 6-deoxyglucose (B), 6-deoxyglucose (C), 2,4,6-trideoxy-4-aminoglucose (D), 6-deoxyglucose (E), and 2,6-dideoxyglucose (F). Sugar-D appeared to be an amino sugar based on the characteristic proton and carbon chemical shifts of H4-D at δ 2.43 and C4-D at δ 60.8. HMBC data was used in the identification of the sugar–sugar linkages and all of the expected HMBC correlations between the anomeric protons/carbons and the corresponding glycosidic carbons/protons were observed.

Methanolysis[1] of colubricidin A with 0.1N HCl in methanol followed by HPLC separation generated one major product (**2**, Fig. 2) having the same UV spectrum as the parent molecule. The molecular formula of compound **2** was determined by positive ion HRFABMS, which gave a molecular ion at *m/z* 551.0519 [$(M+H)^+$, calcd: 551.0503] corresponding to a molecular formula of $C_{20}H_{21}Cl_3N_4O_8$. The isotope pattern for the $[M+H]^+$ ion was characteristic of three chlorine atoms. The ^{13}C and DEPT-135 NMR data acquired in $CDCl_3$ indicated the presence of three carbonyls, two aromatic methines, seven aromatic quaternary carbons, and eight aliphatic carbon signals. Seven of these eight aliphatic carbons

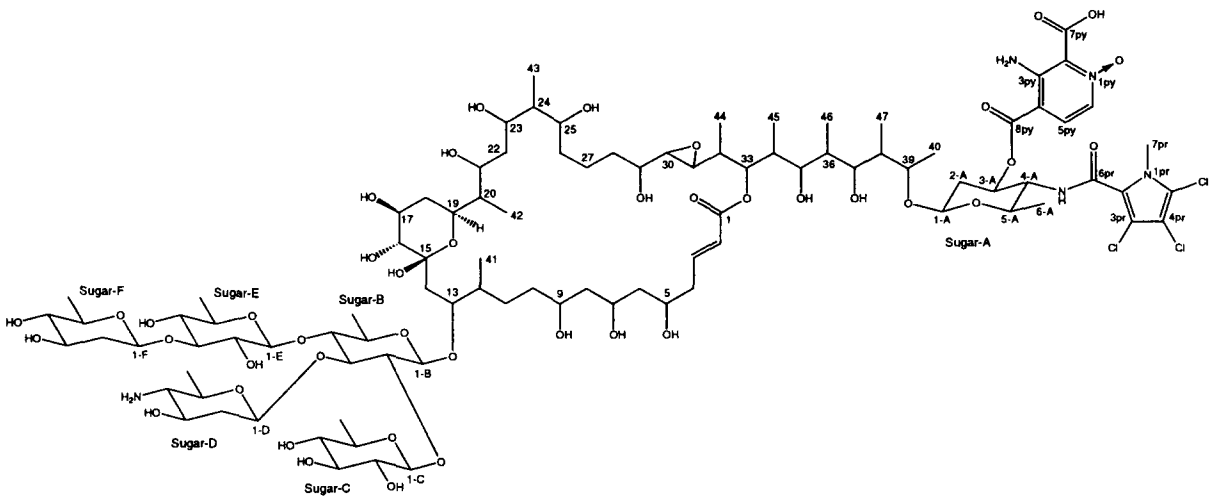


Figure 1. Structure of colubricidin A (**1**)

were assigned to a sugar moiety. A deshielded doublet at δ 4.81 with a coupling constant of 2.8 Hz was very diagnostic for the equatorial anomeric proton H1-A, which was correlated to the anomeric carbon resonance C1-A at δ 98.3 in the HMQC spectrum. With the aid of the COSY data, the sugar residue was identified as 2,4,6-trideoxy-4-aminoglucose. It was evident that the sugar moiety was methylated during the methanolysis, as there was a typical methyl singlet at δ 3.33 that correlated to the anomeric carbon C1-A in the HMBC spectrum and lack of this signal in the parent molecule. The chemical shift of C4-A at δ 55.2 indicated this carbon was not bonded to an oxygen atom. In fact, H4-A at δ 4.13 was coupled to a typical amide proton resonance at δ 6.28 in the COSY experiment. H4-A also showed an important three-bond HMBC correlation to the amide carbonyl at δ 159.7. The upfield shift of this carbonyl indicated that it was adjacent to a pyrrole moiety as found in cyclocinamide A (with a C=O at δ 160.9 and N–Me at δ 36.4).[2] The remaining aliphatic carbon signal resonated at δ 34.8 was assigned to *N*-methyl in the pyrrole moiety. Its attached protons showed HMBC correlations to two sp^2 carbon signals at δ 120.8 and 121.1. The pyrrole ring was trichloro-substituted as the MS data clearly demonstrated a fragment with characteristic isotope patterns at *m/z* 210 (100%), 212 (90%), and 214 (30%). The remaining ^1H and ^{13}C NMR signals were assigned to the pyridine moiety. A network of HMBC correlations from the two ortho-coupled aromatic proton signals H5-py at δ 7.86 and H6-py at δ 7.51 played a key part in establishing this pyridine ring substructure. H5-py showed an additional three-bond HMBC correlation to an ester carbonyl carbon resonance at δ 165.2. The presence of this ester linkage was supported by the deshielded chemical shift of H3-A at δ 5.34. Alkaline hydrolysis of **2** resulted in the pyridine and pyrrole-sugar derivatives, verifying the presence of this ester bond. The remaining carbonyl signal at δ 164.7 was assigned to the carboxylic group whose presence was indicated by a very low field signal at δ 18.6 acquired in DMSO in the proton spectrum, appropriate for a carboxylic proton. In addition, the MS data of **2** showed a loss of 44 Da, in good agreement with decarboxylation. Although this carboxylic group was not methylated during the methanolysis, its methylation in compound **2**[3] was achieved using CH_3I–K_2CO_3 in DMF to yield the methyl ester **3** that showed a gain of 14 mass units in the mass spectrum. The NMR data confirmed that compound **3**[4] contained an extra methyl singlet at δ 3.90 that showed correlation to a carbonyl at δ 162.0 in the HMBC experiment. A 3 ppm upfield shift of the carbonyl resonance was consistent with the functional group conversion from carboxylic acid to methyl ester.

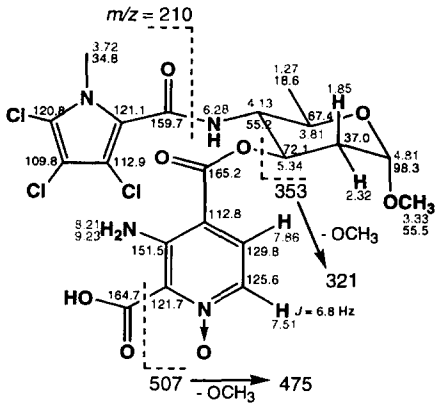
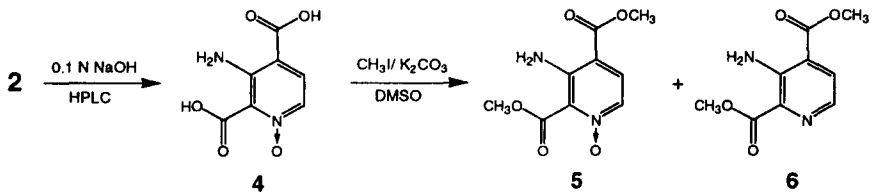


Figure 2. ^{1}H and ^{13}C NMR data assignments and MS fragmentation pattern for compound **2**

Alkaline hydrolysis of **2** with 0.1N NaOH followed by HPLC purification gave diacid **4**.[5] This compound was methylated with CH_3I–K_2CO_3 in DMSO to yield the corresponding dimethyl ester **5**[6] and a deoxy compound **6**.[7] Structures of both compounds **5** and **6** were determined by analyses of the MS and NMR data. Deoxydimethyl ester **6** showed a *J* coupling of 4.7 Hz between H5-py and H6-py, which is diagnostic for a pyridine derivative.



Once the chromophore and oligosaccharide portions of the molecule were identified, the rest of the ^{1}H and ^{13}C resonances were readily assigned to the macrolide core as shown in Fig. 1. Subtraction of the atoms present in the chromophore ($C_{19}H_{18}N_4O_7Cl_3$) and oligosaccharide portions ($C_{30}H_{52}NO_{17}$) from the molecular formula **1** revealed that the core substructure had a composition of $C_{47}H_{84}O_{18}$. By comparison to the structure of brasilinolide A that has a macrolide core composition of $C_{43}H_{76}O_{17}$,[8] the core substructure of **1** contained additional atoms of C_4H_8O. Detailed analysis of the ^{1}H, ^{13}C, DEPT-135, COSY, HMQC, HMBC, and HMQC–TOCSY data led to the proposed substructure for the macrolide core. The NMR data assignments for the macrolide of **1** were almost identical to those of brasilinolide A.[9] The most notable differences were the resonances from C19 to C26 where an extra methyl group (C42) was attached to C20 and a propionate derived C_3 unit (C23–C24–C43) was inserted for **1**. Another noticeable difference was the resonance of C13, which showed a downfield shift to δ 82.64 in **1** from δ 72.0 in brasilinolide A owing to glycosylation. Finally, mutual HMBC correlations observed between the anomeric H1-A/C1-A and H39/C39 completed the structure as shown in Fig. 1.

Acknowledgements

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3. Compound **2**: ESMS *m/z* 551.0 (100% M+H)$^+$, 553.0 (100% M+2+H)$^+$, 555.0 (35% M+4+H)$^+$; NMR (400 MHz, $CDCl_3$, ppm downfield from TMS) see Fig. 2, $H2_{ax}$-A (td, *J*=12.2, 3.0), $H2_{eq}$-A (dd, *J*=12.2, 5.1), H3-A (td, *J*=11.2, 5.1), H4-A (q, *J*=10.0), H5-A (m), H6-A (d, *J*=6.0).
4. Compound **3**: ESMS *m/z* 565.1 (100% M+H)$^+$, 567.2 (100% M+2+H)$^+$, 569.2 (30% M+4+H)$^+$; NMR [300 MHz, MeOH-d_4, δ 3.30 (^{1}H) and δ 49.0 (^{13}C)] H1-A δ 4.85, OCH_3 3.38, $H2_{ax}$-A 1.91, $H2_{eq}$-A 2.37, H3-A 5.46, H4-A 4.05, H5-A 4.01, H6-A 1.32, $H5_{py}$ 7.82 (d, *J*=6.3), $H6_{py}$ 7.54 (d, *J*=6.3), OCH_3 3.97, $H_{pr}7$ 3.70, C1-A 99.4, OCH_3 55.1, C2-A 36.6, C3-A 72.2, C4-A 56.9, C5-A 67.4, C6-A 18.6, $C_{py}2$ 129.3, OCH_3 53.6, $C_{py}3$ 147.3, $C_{py}4$ 110.4, $C_{py}5$ 129.3, $C_{py}6$ 128.5, $C_{py}7$ 163.0, $C_{py}8$ 166.0, $C_{pr}2$ 124.6, $C_{pr}3$ 112.6, $C_{pr}4$ 109.4, $C_{pr}5$ 119.6, $C_{pr}6$ 161.6, $C_{pr}7$ 33.8.
5. Compound **4**: ESMS *m/z* 199.0 (M+H)$^+$; NMR [300 MHz, DMSO-d_6, δ 2.49 (^{1}H) and δ 39.5 (^{13}C)] $H5_{py}$ 7.98 (d, *J*=6.6), $H6_{py}$ 7.91 (d, *J*=6.6), $C_{py}2$ 122, $C_{py}3$ 150.5, $C_{py}4$ 113, $C_{py}5$ 130, $C_{py}6$ 125.5, $C_{py}7$ 164.5, $C_{py}8$ 166.6.
6. Compound **5**: ESMS *m/z* 227.1 (M+H)$^+$; UV λ_{max} nm=233, 248, 288, 369; NMR [300 MHz, DMSO-d_6, δ 2.49 (^{1}H) and δ 39.5 (^{13}C)] $H5_{py}$ 7.70 (d, *J*=6.8), $H6_{py}$ 7.55 (d, *J*=6.8), C7-OCH_3 3.87, C8-OCH_3 3.83, $C_{py}2$ 129.7, $C_{py}3$ 144.7, $C_{py}4$ 106.5, $C_{py}5$ 127.3, $C_{py}6$ 127.1, $C_{py}7$ 161.4, OCH_3 53.1, $C_{py}8$ 165.4, OCH_3 52.2.
7. Compound **6**: ESMS *m/z* 211.1 (M+H)$^+$; UV λ_{max} nm=232, 267, 382; NMR [300 MHz, DMSO-d_6, δ 2.49 (^{1}H) and δ 39.5 (^{13}C)] $H5_{py}$ 7.84 (d, *J*=4.7), $H6_{py}$ 7.93 (d, *J*=4.7), C7-OCH_3 3.83, C8-OCH_3 3.86, $C_{py}2$ 129.4, $C_{py}3$ 146.8, $C_{py}4$ 118.1, $C_{py}5$ 127.2, $C_{py}6$ 134.4, $C_{py}7$ 167.0, OCH_3 51.6, $C_{py}8$ 166.4, OCH_3 52.1.
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9. Systematic slight difference of the chemical shifts between the two sets of ^{1}H and ^{13}C data is caused by the calibration of the NMR solvent references: colubricidin A, methanol-d_4 at δ 3.30 (^{1}H) and δ 49.15 (^{13}C); brasilinolide A, methanol-d_4 at δ 3.35 (^{1}H) and δ 49.8 (^{13}C).