COUMAMIDINES, NEW BROAD SPECTRUM ANTIBIOTICS OF THE CINODINE TYPE

II. ISOLATION AND STRUCTURAL ELUCIDATION

RANDAL H. CHEN, DAVID N. WHITTERN, ALEXANDER M. BUKO and JAMES B. MCALPINE

Anti-infective Research, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A.

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Two novel, isomeric compounds, coumamidines γ1 and γ2, were isolated from fermentations of an actinomycete. The structures were elucidated spectroscopically using 2D NMR correlation experiments and mass spectral data. The coumamidines were found to be close structural relatives of the cinodines (LL-BM123 γ1 and γ2).

In the course of screening microbial fermentation products for antimicrobial activity, an isomeric pair of novel antibiotics, coumamidines γ1 and γ2, were discovered. These compounds are structurally related to the cinodines γ1 and γ2[1,2]. The carbohydrate and phenylpropanoid region of both families are identical[3] (using 2D NMR techniques, a few minor spectral misassignments of the cinodines have been corrected). However, the coumamidines do not contain an acylated spermidine residue, but instead have a 3-aminopropanamidine. Both coumamidines have potent broad spectrum activity against Gram-negative and Gram-positive bacteria, but are ineffective against anaerobes. The microbiological data are presented in companion papers[4,5]. This paper describes the purification and structural elucidation of the coumamidines.

Isolation

Whole fermentation broth was passed through filter paper to remove the mycelial mass. The eluate was mixed with Diaion HP-20 resin and the active constituents were allowed to adsorb to the resin. The resin was recovered and the activity was eluted with a step gradient from water to methanol. Active fractions were pooled and evaporated to dryness. The residue was triturated with acetone followed by methanol. The remaining water soluble material was adsorbed onto a column of CM-Sephadex. After washing with water, the activity was eluted by the application of a linear gradient of 0 to 5% NaCl. The active fractions were desalted over a carbon column. A final purification over Fractogel in a buffered solution yielded two pure compounds, the acetate salts of coumamidine γ1 and γ2. The activity was monitored by the disk diffusion bioassay on agar plates which had been inoculated with *Pseudomonas aeruginosa* BMH 1.

Characterization and Structural Determination

The acetate and chloride salts of coumamidine γ1 and γ2 are white powders and were readily soluble in water or DMSO. They possessed only marginal solubility in dry methanol. The UV absorption spectra of both compounds were identical and had an absorbance maximum at 286 nm with an ε 15,400 in water.

The positive fast atom bombardment mass spectra (FAB-MS) of both γ1 and γ2 gave the same

Table 1. NMR data of coumamidine γ1.

Assignment	C shift	DEPT	H shift	COSY	LR-HETCOR
1	169.5	CNN			2.74, 3.68
2	33.2	CH_2	2.74	3.68	3.68
3	37.0	CH_2	3.68	2.74	2.74
4	169.6	CON			6.55, 7.52
5	119.0	CH=	6.55	7.52	7.52
6	141.5	CH=	7.52	6.55	7.64
7	129.8	Ar C			6.55, 7.18
8, 12	130.2	Ar CH	7.64	7.18	7.52, 7.64
8, 12	130.2	Ar CH	7.64	7.18	7.52, 7.64
9, 11	117.7	Ar CH	7.18	7.64	7.18
9, 11	117.7	Ar CH	7.18	7.64	7.18
10	157.5	Ar C			7.64, 7.18
1′	95.7	O-CH-O	5.86	3.87	
2′**	56.8	CH-N	3.87	3.98, 5.86	3.98
3′***	70.5	CH-O	3.98	3.56, 3.87	3.56, 3.87, 5.86
4′**	57.9	CH-N	3.56	3.93, 3.98	1.14, 3.98
5′***	68.8	CH-O	3.93	1.14, 3.56	1.14, 5.86
6′	17.1	CH_3	1.14	3.93	
1″	82.1	O-CH-N	4.89	3.59	3.63, 4.17
2″	55.4	CH-N	3.59	3.63, 4.89	3.63
3″	73.1	CH-O	3.63	3.59, 3.85	3.85, 4.17
4″	74.8	CH-O	3.85	3.47, 3.63, 4.17	5.34
5″	64.2	CH_2-O	3.47, 4.17	3.85	
1‴	97.9	O-CH-O	5.34	3.58	3.58, 3.62, 4.37
2‴*	60.4	CH-N	3.58	3.52, 5.34	3.52
3‴*	55.8	CH-N	3.52	3.58, 4.00	4.00
4‴	69.7	CH-O	4.00	3.52, 3.62, 4.37	3.62, 4.37
5‴	66.5	CH_2-O	3.62, 4.37	4.00	5.34
2′	158.0	C=N			3.87
4′	159.5	C=O			4.89
2″	161.8	C=O			
2‴	160.9	C=O			
3‴	156.5	C=O			

*,**,*** The reassigned carbons, indicating the values which were interchanged.

protonated molecular ion of nominal mass *m/z* 836. The exact mass was measured as *m/z* 836.3654 by a high resolution peak match and corresponds to the formula $C_{33}H_{49}N_{13}O_{13}+H^+$ (calcd 836.3655).

The NMR data are tabulated and presented in Tables 1 and 2. The carbons are listed in order of their numerical assignment. Across from each carbon are coupling data pertaining to that carbon. The chemical shift of the attached proton(s) was determined using heteronuclear correlation (HETCOR) experiments[6]. Long range (LR) proton-carbon couplings were determined by the LR-HETCOR technique[7]. The data under the distortionless enhancement by polarization transfer (DEPT) heading not only gives the information from that experiment, but also indicates the hybridization state and attached functionality, as assigned in the structure. COSY data lists the protons which couple to the protons attached to that carbon. The numerical assignments based on spectral analyses refer to the coumamidine structure in Fig. 1.

A comparison of the ^{13}C data for the coumamidines and the cinodines[3] shows that the coumaryl and carbohydrate regions are identical for coumamidine γ1 and cinodine γ1, and similarly for coumamidine γ2 and cinodine γ2. Only small insignificant differences in chemical shifts (<1 ppm) were seen

Table 2. NMR data of coumamidine γ2.

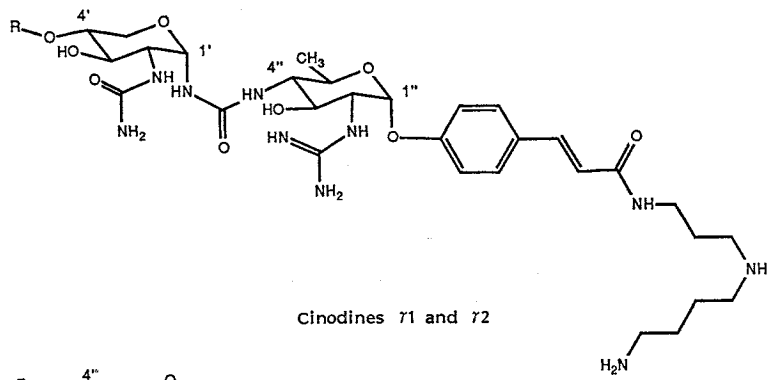
Assignment	C shift	DEPT	H shift	COSY	LR-HETCOR
1	169.5	CNN			2.77, 3.72
2	33.2	CH_2	2.77	3.72	3.72
3	37.0	CH_2	3.72	2.77	2.77
4	169.6	CON			6.63, 7.60
5	119.0	CH=	6.63	7.60	7.60
6	141.5	CH=	7.60	6.63	7.72
7	129.8	Ar C			6.63, 7.26
8, 12	130.2	Ar CH	7.72	7.26	7.72, 7.60
8, 12	130.2	Ar CH	7.72	7.26	7.72, 7.60
9, 11	117.7	Ar CH	7.26	7.72	7.26
9, 11	117.7	Ar CH	7.26	7.72	7.26
10	157.5	Ar C			7.26, 7.72
1′	95.7	O-CH-O	5.86	3.93	
2′*	56.8	CH-N	3.93	4.05, 5.86	3.62, 4.05
3′**	70.5	CH-O	4.05	3.62, 3.93	3.93, 5.86
4′*	57.9	CH-N	3.62	3.99, 4.05	1.18, 4.05
5′**	68.9	CH-O	3.99	1.18, 3.62	1.18, 5.86
6′	17.1	CH_3	1.18	3.99	
1″	82.1	O-CH-N	4.89	3.62	3.41, 3.62, 4.08
2″	55.1	CH-N	3.62	3.68, 4.89	3.68
3″	73.1	CH-O	3.68	3.62, 3.80	3.62, 4.08
4″	77.7	CH-O	3.80	3.41, 3.68, 4.08	3.68, 4.72
5″	64.2	CH_2-O	3.41, 4.08	3.80	
1‴	102.9	O-CH-O	4.72	3.83	4.35
2‴	55.4	CH-N	3.83	3.77, 4.72	3.77
3‴	60.4	CH-N	3.77	3.83, 4.37	3.90, 4.35
4‴	76.7	CH-O	4.37	3.77, 3.90, 4.35	3.77, 3.90, 4.35
5‴	64.5	CH_2-O	3.90, 4.35	4.37	
2′	157.9	C=N			3.93
4′	159.4	C=O			4.89
2″	161.7	C=O			
2‴	160.8	C=O			
3‴	162.8	C=O			

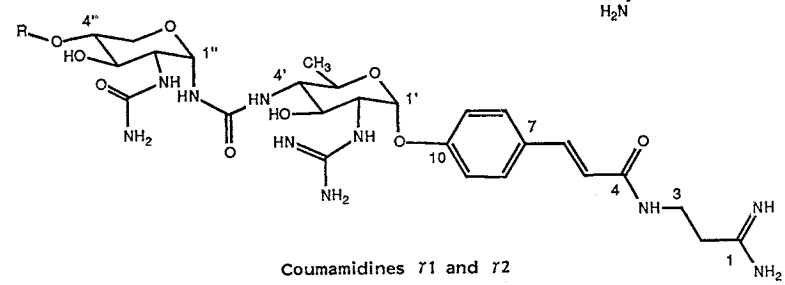
*,** The reassigned carbons, indicating the values which were interchanged.

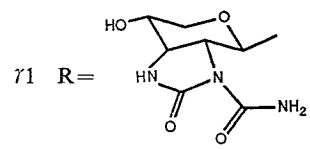
and could have been caused by the different counter ions or other experimental conditions. It was necessary to reassign the ^{13}C chemical shifts of three pairs of carbons of the cinodines which were misassigned based on degradation studies. The reassignment is based on proton-proton coupling and proton-carbon correlation data taken on the intact molecules. The reassigned carbons have been marked with asterisk pairs (see the assignment column of Tables 1 and 2), indicating the values which were interchanged.

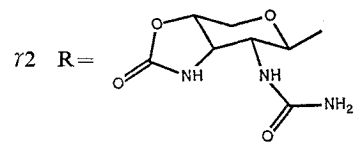
The unique feature of the coumamidines is the remaining residue, which by the molecular formula is composed of $C_3H_7N_2$. The low field carbon, C-1 at 169.5 ppm, couples to both pairs of methylene protons. The geminal protons of each methylene, C-2 and C-3, are equivalent and appear as triplets, J=7.3 Hz. Each couples to the other methylene protons and carbon. These data indicate the linear propionyl-type structure. The remaining atoms and the carbon chemical shift[8] all indicate that C-1 is an alkyl amidine. The NMR data suggests that C-3 is bonded to a nitrogen atom. The chemical shift of its protons correlate better for that of an amide nitrogen (with β effects from the amidine) than an amine. The amide to which it is bonded is the allylic amide (169.6 ppm) of the coumaryl

Fig. 1. Structures of cinodines and coumamidines.









residue. This is analogous to the attachment of the spermidine residue in the cinodines.

Fragment ions in the mass spectrum support the structural assignment. Cleavage between the anomeric carbon 1′ and the phenolic hydroxyl results in loss of the coumaryl and propanamidine moieties and gave an observable carbohydrate fragment at *m/z* 603. Scission of the C-N bond between the urea carbonyl and the glycosidic nitrogen at 1″ gave two observable fragments. The first, at *m/z* 391, contains the two terminal sugars, and the second, *m/z* 446, contains the sugar, coumaryl and propanamidine residues.

Experimental

General Methods

NMR spectra were recorded on either a General Electric GN300 or GN500 spectrometer. All samples were dissolved in D_2O. ^1H NMR correlation spectroscopy (COSY) data were acquired at 300.1 MHz. ^{13}C NMR and DEPT experiments were performed at 75.5 MHz. HETCOR and LR-

HETCOR data were recorded at 300.1 MHz. Mass spectra and high resolution peak match values were acquired on a Kratos MS-50. UV absorption spectra were measured on a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer.

Isolation

The mycelial mass was removed from the fermentation broth (18 liters) by filtration through Whatman No. 1 filter paper. The filtrate was stirred for 4 hours with 2 liters of Diaion HP-20 resin and then was allowed to stand for 12 hours at 4°C. The spent beer was decanted and the resin was washed with 2 liters of water. The active material was eluted in batch from the resin with two 2-liter aliquots of 50% methanol - water and two 2-liter aliquots of 50% acetonitrile - water. The washings were pooled and the volume was reduced on a vertical evaporator. The sample was lyophilized to yield 35.3 g of crude material. This was triturated with 200 ml of acetone and 200 ml of methanol. The insoluble material was redissolved in 100 ml of water and adsorbed onto a column of CM-Sephadex, Na^+ form (2.5×30 cm), in distilled water. The column was isocratically eluted with 500 ml of water, and then a linear gradient of 0 to 5% NaCl, over 4 liters of solvent, was applied. The active fractions were pooled (350 ml) and adsorbed onto a bed of Ambersorb XE 347 (2.5×16 cm). The column was washed with 300 ml of water, 300 ml of 50% methanol - water, and 300 ml of 50% acetone - water. The aqueous methanol and aqueous acetone fractions were pooled and the solvent was removed to yield 110 mg of material. This was dissolved in 2.5 ml of 50 mM ammonium acetate and chromatographed on a column of Fractogel TSK HW-40 (S) (2.5×100 cm) in 50 mM ammonium acetate. The flow rate was 1.5 ml/minute (0.18 kg/cm^2) and 15 ml fractions were collected. The eluate was monitored at 254 nm. Two peaks which were both UV absorbent and bioactive were collected at fractions No. 120~125 and No. 126~155. These correspond to 9.1 mg of coumamidine $\gamma 2$ and 61.6 mg of coumamidine $\gamma 1$, respectively.

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