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New Pacidamycins Produced by *Streptomyces coeruleorubidus*, NRRL 18370

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During the course of isolating gram quantities of pacidamycins from the fermentation of *Streptomyces coeruleorubidus* (NRRL 18730) for mode-of-action and chemical-modification studies, three new pacidamycins were isolated.[1~3] Here we report the production, isolation, structure elucidation, and biological activities of pacidamycins D (**1**), 4N (**2**), and 5T (**3**).

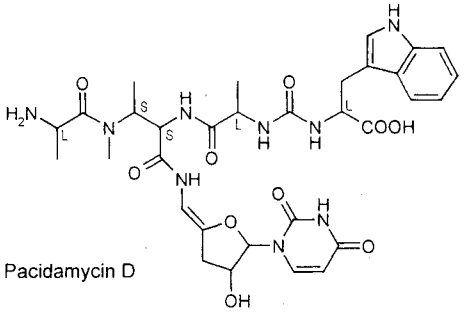
A fresh culture of strain NRRL 18730 was inoculated into four 250-ml baffled shake flasks, each containing 50 ml of seed medium consisting of glucose monohydrate 1.0%, soluble starch 1.5%, yeast extract 0.5%, casitone 0.5%, $CaCO_3$ 0.1% (pH adjusted to 7.0 before sterilization). The flasks were incubated on a rotary shaker (250 rpm) at 28°C for 36 hours to give the stage-1 seed. The stage-1 seed (200 ml) was inoculated into two 4000-ml baffled flasks, each containing 1000 ml of the same seed media. The flasks were incubated under the same condition as the above for 27 hours to give the stage-2 seed. The stage-2 seed (2000 ml) was inoculated into 22 liters of the seed media in a 28-liter fermenter. The fermenter was maintained at 28°C, 300 rpm agitation, 10 slpm aeration, and 4.0 psi backpressure for 26 hours to give the stage-3 seed. The stage-3 seed was inoculated into a 300-liter reactor, containing 230 liters of a production media consisting of 1% soytone, 1% soluble starch, 2% D-maltose and 5 ml/liter trace elements. The fermentation was allowed to proceed with 400 rpm agitation, 100 slpm aeration, 5.0 psi backpressure, 29°C, and maintained at pH 6.5 with the addition of 6 N sodium hydroxide or 30% phosphoric acid. The fermentation mash (220 liters) was harvested after 117 hours, processed through a Sharples continuous flow centrifuge, and the clarified culture broth was adjusted to pH 5.5. The antibiotic complex contained in the culture broth was batch adsorbed on to Diaion® HP20 (Mitsubishi) resin (5 liters) by stirring overnight at 5°C. The supernatant was decanted off and the resin containing the antibiotic complex was treated with 0.1% sodium azide and stored at 5°C before further processing. The production of the pacidamycins and their purification were monitored by activity against *Pseudomonas aeruginosa* (ATCC 27853).

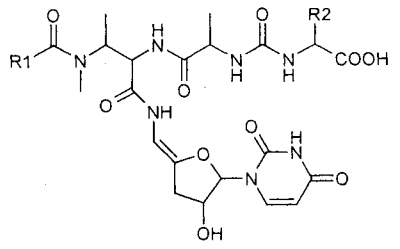
Each 2.5 liters of the above charged HP-20 resin was slurry packed into a column (8.9 cm i.d.×40 cm L, 2.5 liters) and the column was sequentially eluted with water (7.2 liters), a gradient of 0 to 15% acetone (1.2 liters), 15% acetone (4.8 liters), a gradient of 15 to 90% acetone (12.0 liters), and 90% acetone (3.2 liters). The active eluates were combined and concentrated *in vacuo* (T≤35°C) to remove acetone and the residual aqueous solution was adjusted to pH 5.0 and extracted twice with equal volumes of *n*-BuOH. The combined *n*-BuOH extract was concentrated *in vacuo* (T≤50°C) until most of the water was removed. The resultant precipitate of the antibiotic complex was collected by centrifugation, washed with EtOAc and dried under vacuum to give 8.8 g of crude pacidamycin complex.

A 2.19 g sample of the crude pacidamycin complex, dissolved in a mixture of 16 ml of buffer A (0.05 M NaOAc, pH 3.6) and 8 ml of acetonitrile, was top loaded on a Toyopearl® SP-650M (strong cation exchanger) column (2.5 cm×50 cm, 245 ml) which was pre-equilibrated in buffer A. The column was sequentially eluted, at 10 ml/minute, with buffer A (0.5 liters), a gradient of buffer A to 75% buffer B (0.05 M NaOAc pH 5.6) over 1.25 liters, 75% buffer B (2.5 liters), a gradient of 75%~100% buffer B over 2.5 liters and 100% buffer B (2.5 liters). The bioactive fractions were analyzed by HPLC (PLRP-S column, 15% CH_3CN in 0.1 M NH_4OAc pH 7.8) and the corresponding fractions obtained from three identical runs were combined to give 8 pools enriched in individual pacidamycins. New pacidamycins 5T and D eluted between pacidamycin-5 and pacidamycin-1 during the 75% buffer B elution, while new pacidamycin 4N eluted near the end of the 75%~100% buffer B gradient after pacidamycin-6 and pacidamycin-4 had eluted. Each pool was neutralized (pH 6.0~6.4) with 28% NH_4OH and the major pacidamycin component in each pool was further purified by reversed phase column chromatography on Amberchrom® as described below for pacidamycin D.

A 2.5-liter pool containing pacidamycin D was loaded on an Amberchrom® column (2.5 cm×100 cm, 490 ml) pre-equilibrated in deionized water. Upon completion of loading, the column was washed successively with 2 bed volumes of water and 2 bed volumes of 0.1 M NH_4OAc (pH 7.8). It was then eluted with a gradient of 0% to 12% CH_3CN in buffer over 100 ml, and 12% CH_3CN in buffer

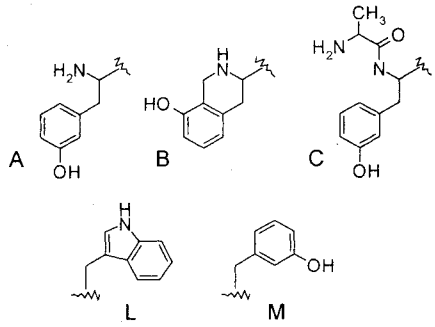
Fig. 1. Structures, molecular formula and molecular weights of new pacidamycins.





Compound Name	R1	R2	Mol. Formula	MW[a]
Pacidamycin D (**1**)			$C_{32}H_{41}N_9O_{10}$	711.7
Pacidamycin 4N (**2**)	B	L	$C_{39}H_{45}N_9O_{11}$	815.8
Pacidamycin 5T (**3**)	A	M	$C_{36}H_{44}N_8O_{12}$	780.8
Pacidamycin-3 (**4**)	C	M		
Pacidamycin-4 (**5**)	A	L		
Pacidamycin-1 (**6**)	C	L		

[a] Natural abundance



(800 ml). Fractions containing pure pacidamycin D were combined and desalted by adsorption on an Amberchrom® column. After washing the column with 5 bed volumes of water, pacidamycin D was desorbed by elution with CH_3CN-H_2O (1 : 1). After concentration and lyophilization, 1.1 g of pacidamycin D was obtained.

Following similar procedure as above, new pacidamycins 4N (0.86 g), and 5T (0.49 g) were isolated from the corresponding pooled fractions.

Close examination of the ^1H and ^{13}C NMR data of pacidamycin D suggested that it is pacidamycin-4 (**5**) with a second alanine unit in place of the *m*-tyrosine unit at the *N*-terminus. This observation was further supported by the exact mass of its $M+H^+$ ion, 712.3080 for $C_{32}H_{42}N_9O_{10}$ (calcd. 712.3054), measured in HRFAB-MS. Its chemical structure (**1**) as shown in Fig. 1 was assigned based on 2-D NMR data as shown in Table 1. The proton and the C-13 chemical shifts associated with each amino acid unit, and the pseudo nucleoside residue were unambiguously assigned by COSY, DEPT, and HMQC data. The connectivity between structural fragments separated by peptide linkages were suggested by fragmentation pattern in FAB-MS initially and was confirmed by across-the-amide-bond long-range ^1H-^{13}C coupling in HMBC between all amide carbonyl carbons and the corresponding α-proton of the adjacent amino acids as illustrated in Fig. 2. Pacidamycin D is the first member of this class of compounds,[4~7] having an alanine residue condensed with the methylamino group of the 2-amino-3-*N*-methylaminobutyric acid unit. The exact configuration of the carbons in the peptide backbone of pacidamycin D was determined to be as shown in Fig. 1 by total synthesis and HPLC analysis of pacidamycin hydrolysate using two different chiral methods. Details of this work will be reported elsewhere.[8]

The molecular weight of pacidamycins 4N ($[M+H]^+$ ions at *m/e* 816.4 in ESI-MS) and its NMR data suggested that it is pacidamycin-4 (**5**) with a isoquinoline moiety in place of the *m*-tyrosine unit, similar to that found in the napsamycins.[7] This observation was confirmed by 2-D NMR data as shown in Table 2. The structure of the isoquinoline unit was assigned to be 8-hydroxy-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid based on COSY, HMBC, and ROESY data as highlighted in Fig. 3.

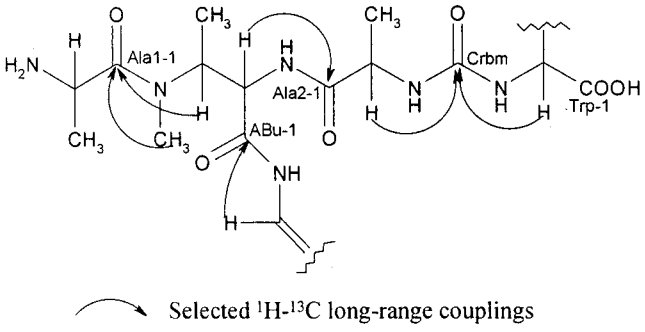
The chemical structure of pacidamycin 5T was deduced based on its molecular weight ($[M+1]^+$ at *m/e* 781.4 in ESI-MS) and comparison of its ^{13}C NMR data with that published for pacidamycins-3 (**4**) and that obtained for pacidamycin-4 (**5**) during this study. The ^{13}C NMR data of pacidamycin 5T is shown in Table 3.

Table 1. ^{1}H NMR (400 MHz, 40°C, D_2O with 0.75% 1-trimethylsilylpropionic acid-d_4) and ^{13}C NMR (100 MHz) data of pacidamycin D (**1**).

Assignment*	C-13	DEPT	H-1	COSY	HMBC
U-2	153.8	C			7.42, 6.08
U-4	168.8	C			7.42, 5.86
U-5	104.9	CH	5.86	7.42	7.42
U-6	143.7	CH	7.42	5.86	5.86, 6.08
Sug-1	96.7	CH	6.08	4.65	2.69, 3.01, 7.42
Sug-2	75.3	CH	4.65	2.69, 3.01	2.69
Sug-3	35.8	CH2	2.69	3.01, 4.65, 5.97	
		CH2	3.01	2.69, 4.65, 5.97	
Sug-4	147.1	C			5.97, 6.08, 3.01
Sug-5	99.4	CH	5.97	2.69, 3.01	3.01, 2.69
ABu-1	170.2	C			4.55, 4.93, 5.97
ABu-2	58.3	CH	4.55	4.93	4.93, 1.22
ABu-3	53.6	CH	4.93	4.55, 1.22	2.91, 1.22, 4.55
ABu-4	15.6	CH3	1.22	4.93	4.55, 4.93
ABu-3N-Me	32.4	CH3	2.91		4.93
Ala2-1	179.2	C			4.55, 4.15, 1.25
Ala2-2	52.8	CH	4.15	1.25	1.25
Ala2-3	19.6	CH3	1.25	4.15	4.15
Crbm	161.4	C			4.40, 4.15
Trp-1	182.2	C			4.40, 3.32, 3.14
Trp-2	59.1	CH	4.40	3.14, 3.32	3.32, 3.14
Trp-3	31.1	CH2	3.14	3.32, 4.40	4.40
		CH2	3.32	3.14, 4.40	
Trp-2'	127.0	CH	7.26		3.32, 3.14
Trp-3'	113.4	C			3.32, 3.14, 7.75, 7.26
Trp-3'a	130.1	C			3.32, 3.14, 7.53, 7.26
Trp-4'	121.6	CH	7.75	7.21, 7.28	7.53, 7.28
Trp-5'	122.0	CH	7.21	7.75, 7.28, 7.53	7.53
Trp-6'	124.5	CH	7.28	7.21, 7.53, 7.75	7.75
Trp-7'	114.5	CH	7.53	7.28, 7.21	7.21
Trp-7'a	138.9	C			7.75, 7.28
Ala1-1	173.7	C			4.93, 4.31, 2.91, 1.37
Ala1-2	50.2	CH	4.31	1.37	1.37
Ala1-3	18.2	CH3	1.37	4.31	4.31

* Abbreviation for the structure units are: U = uracil, Sug = the pseudo pentylfuranose sugar unit, Abu = 3-methylamino-2-aminobutyric acid, ala = alanine, Crbm = the Carbamyl unit, Trp = tryptophan. The same abbreviations are used in Tables 2 & 3.

Fig. 2. Across-the-peptide-bond long-range ^{1}H-^{13}C coupling observed for pacidamycin D.



Selected ^{1}H-^{13}C long-range couplings

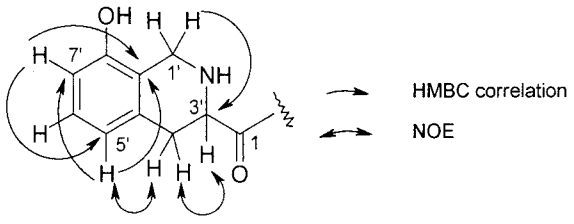
Similar to the other pacidamycins, the mureidomycins, and the napsamycins, the new pacidamycins reported here are active against *Pseudomonas aeruginosa* but not active against representatives of Gram-positive and other Gram-negative bacteria (Table 4). Pacidamycins D and 4, however, were found to be quite active against an *E. coli* strain (*E. coli* ECAcrAB in Table 4) lacking a functioning AcrAB pump. The new pacidamycins are not active against a resistant *Pseudomonas aeruginosa* strains which were selected in the presence of pacidamycin-4 (**5**). The mureidomycins were reported to be selective inhibitors of

Table 2. ^{1}H NMR (400 MHz, 40°C, D_2O), and ^{13}C NMR (100 MHz) data of pacidamycin 4N (**2**).

Assignment	C-13	H-1 (mult, J in Hz)	COSY	HMBC	ROESY
U-2	154.0 (s)			7.35	
U-4	169.0 (s)			7.35	
U-5	105.2 (d)	5.65 (d, 7.6)	7.35		7.35
U-6	143.6 (d)	7.35 (d, 8.0)	5.65	5.65	6.08, 5.65, 4.57, 2.95
Sug-1	96.9 (d)	6.08 (br s)			7.35, 4.57, 2.67
Sug-2	75.6 (d)	4.57 (*)	2.95		7.35, 6.08, 2.95
Sug-3	35.8 (t)	2.95 (*)	4.57, 2.67		7.35, 4.57
		2.67 (d, 17.2)	2.95		6.08, 5.99
Sug-4	147.5 (s)			6.08, 5.99, 2.95	
Sug-5	100.0 (d)	5.99 (s)			2.67
ABu-1	170.6 (s)				
ABu-2	58.0 (d)	4.62 (*)	4.91	4.91, 1.20	
ABu-3	54.0 (d)	4.91 (quintet, 7.6)	4.62, 1.20	2.92, 1.20	2.92, 1.20
ABu-4	15.4 (q)	1.20 (*)	4.91	4.91	4.91, 2.92
ABu-3N-Me	32.5 (q)	2.92 (s)		4.91	4.91, 1.20
Ala-1	179.5 (s)			1.24	
Ala-2	52.8 (d)	4.15 (*)	1.24		1.24
Ala-3	19.7 (q)	1.24 (*)	4.15		4.15
Crbm	161.6 (s)				
Trp-1	182.5 (s)				
Trp-2	59.1 (d)	4.37 (*)	3.25, 3.08		7.68, 7.20, 3.25, 3.08
Trp-3	31.1 (t)	3.25 (*)	4.37, 3.08		7.68, 7.20, 4.37
Trp-2'	127.0 (d)	7.20 (s)		3.08	4.37, 3.25, 3.08
		3.08 (*)	4.37, 3.25		7.68, 7.20, 4.37
Trp-3'	113.5 (s)			7.20, 3.25, 3.08	
Trp-3'a	130.2 (s)			7.41, 7.20, 7.13, 3.08	
Trp-4'	121.8 (d)	7.68 (d, 7.2)	7.13	7.2	7.13, 4.37, 3.25, 3.08
Trp-5'	122.0 (d)	7.13 (t, 6.8)	7.68	7.41	7.68
Trp-6'	124.5 (d)	7.20 (t, *)		7.68	
Trp-7'	114.7 (d)	7.41 (d, 7.2)	7.2		7.2
Trp-7'a	139.0 (s)			7.68, 7.20	
Isoq-1	172.8 (s)			2.92	
Isoq-1'	43.3 (t)	4.50 (*)	4.16		
		4.16 (*)	4.5		
Isoq-3'	56.3 (d)	4.37 (*)	3.08, 2.90	4.5	3.08, 2.90
Isoq-4'	30.9 (t)	3.08 (*)	4.37, 2.90	6.7	6.70, 4.37
		2.9	4.37, 3.08		4.37
Isoq-4'a	134.6 (s)			7.2	
Isoq-5'	123.2 (d)	6.70 (d, 7.2)	7.2	6.85	7.20, 3.08
Isoq-6'	132.0 (d)	7.20 (m)	6.85, 6.70		6.85, 6.70
Isoq-7'	116.6 (d)	6.85 (d, 8.0)	7.2	6.72	7.2
Isoq-8'	155.6 (s)			7.2	
Isoq-8'a	118.0 (s)			6.85, 6.72, 4.40, 4.16	

* Overlapped signals

Fig. 3. Determination of the isoquinoline moiety in pacidamycin 4N as 8-hydroxy-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid.



the bacterial translocase reaction in peptidoglycan synthesis with an IC_{50} of 0.05 μg/ml against the *P. aeruginosa* enzyme.[9] Pacidamycins D, 4N, 1, and 4 exhibited IC_{50}s of 0.08, 0.71. 0.15, and 0.09 μg/ml, respectively, against the translocase of *E. coli*. Details of the enzymatic studies will be reported elsewhere.

Acknowledgements

The authors wish to thank Dr. OLGA LOMOVSKAYA for providing the *E. coli* ECAcrAB strain.

Table 3. ^{13}C NMR (100 MHz, 40°C, D_2O with 0.75% 1-trimethylsilylpropionic acid-d_4) data of pacidamycin 5T (**4**).

Assignment	DEPT	C13	Assignment	DEPT	C13
U-2	C	154.1	Tyr-1	C	181.6
U-4	C	168.6			170.6
U-5	CH	105.1	Tyr-2	CH	59.4
U-6	CH	143.8			56.4
Sug-1	CH	96.2	Tyr-3	CH2	41.1
Sug-2	CH	75.3			39.4
Sug-3	CH2	34.1	Tyr-1'	C	138.9
Sug-4	C	142.6			138.8
Sug-5	CH	99.7	Tyr-2'	CH	119.0
ABu-1	C	169.0			118.9
ABu-2	CH	57.9	Tyr-3'	CO	158.9
ABu-3	CH	54.5			158.3
ABu-4	CH3	15.9	Tyr-4'	CH	117.9
Abu-3NMe	CH3	32.9			116.5
Ala-1	C	179.2	Tyr-5'	CH	133.6
Ala-2	CH	52.8			132.7
Ala-3	CH3	19.7	Tyr-6'	CH	124.4
Crbm	C	161.0			124.0

Table 4. *In vitro* antibacterial activities of the pacidamycins.

	Minimal Inhibitory Concentration, MIC (μg/ml)				
Test Organism	**D (1)**	**4N (2)**	**5T (3)**	**4 (5)**	**1 (6)**
P. aeruginosa ATCC 27853	16	64	>500	16	4
P. aeruginosa 799/61	4	4	125	2	1
P. aeruginosa UPA res.	>256	>256	>500	>250	>250
E. coli ATCC 25922	>256	>256	>500	64	>250
E. coli ECAcrAB	4			2	
S. aureus ATCC 29213	>256	>256	>500	>250	>250

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