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Isolation and Characterization of Antibiotic NC0604, a New Analogue of Bleomycin

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Abstract NC0604, a new analogue of bleomycin, was isolated from fermentation broth of *Streptomyces verticillus* var. *pingyangensis* n.var. The structure of NC0604 was elucidated by spectroscopic analyses. NC0604 had the same kernel structure as bleomycin, but a different terminal amine moiety determined as amidepropyl spermidine. NC0604 exhibited antibacterial activity against a wide range of bacterial species and showed cytotoxicity *in vitro* against human $HepG_2$, KB, MCF-7, HCT116, BGC-823 and MCF-7/DOX cells with IC_{50} values of 1.18, 1.21, 1.41, 1.83, 2.02, 1.45 μM, respectively. The antitumor activity of NC0604 against these cells was 3～9 times higher than that of bleomycin; and the pulmonary toxicity of NC0604 was much lower than that of bleomycin.

Keywords NC0604, structure elucidation, biological activity, pulmonary toxicity, bleomycin

NC0604, a new analogue of bleomycin, was found in the culture broth of *Streptomyces verticillus* var. *pingyangensis* n. var. [1], a bleomycins producing strain, which was isolated from a soil sample collected from Pingyang Area, Zhejiang Province, P.R. China. The present paper reports the fermentation, isolation, physico-chemical properties and structure elucidation of NC0604. The biological activities and pulmonary toxicity are also presented and compared with those of bleomycin.

A stock culture of the strain *Streptomyces verticillus* var. *pingyangensis* n.var. was grown and maintained on an agar slant consisting of glucose 1.0%, soluble starch 1.0%, peptone 0.5%, agar 2.0%, and NaCl 0.5% (pH 7.2～7.5) at 28°C. The stock culture was transferred into 250-ml Erlenmeyer flasks containing 50 ml of the seed medium consisting of soluble starch 2.5%, glucose 0.5%, soybean meal 3.5%, KH_2PO_4 0.1%, $ZnSO_4$ 0.05%, and $CuSO_4$ 0.01% (pH 6.0～6.5). The culture was incubated on a rotary shaker (agitated at 220 rpm) at 28°C for 48 hours. Ten milliliters of the seed culture was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the producing medium with the same components as the seed medium. The fermentation was carried out at 29°C for 7～8 days on a rotary shaker at 220 rpm. Antibiotic activity in the fermentation broth was determined by paper-disk agar diffusion assay using *Bacillus subtilis*.

The fermentation broth (25 liters) was adjusted to pH 2～3 with oxalic acid solution and filtered. The filtrate was charged on a column of 122 resins (H^+ form, 1.0 liter) which was washed with distilled water (2.0 liters) and then eluted with 0.3 M HCl (4.0 liters). The active fractions were combined, adjusted to pH 7.0, and 0.2% (m/v) $CuSO_4$ was added to the solution. The resulting solution was passed through a column of x-5 (2.0 liters) for desalting. Elution was carried out with 10% Me_2CO soln (containing 0.01 M HCl) and the eluates were combined and removed the Me_2CO *in vacuo*. The active fractions (1.0 liter) were chromatographed on a column of CM-Sephadex C-25 (NH_4^+ form, 1.0 liter) which was then eluted with NH_4Cl solution with a concentration gradient from 0.1 to 0.5 M. The copper-chelated NC0604 was eluted with 0.2 M NH_4Cl

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Table 1 Physico-chemical properties of NC0604

Appearance	White powder
Elemental composition reported by Q-TOF2	$C_{60}H_{94}N_{20}O_{22}S_2$
ESI-Q-TOF2-MS Found	1511.6379 $[M+H]^+$
	Calcd 1511.6371
UV λ_{max}^{MeOH} nm (ε)	243～245 (sh.) (24,882), 292～295 (16,479)
IR ν_{max} (KBr) cm^{-1}	3385, 1718, 1653, 1549, 1054
Solubility	H_2O, MeOH
Color reaction	
Guanidino	Negative
Ninhydrin	Non-typical

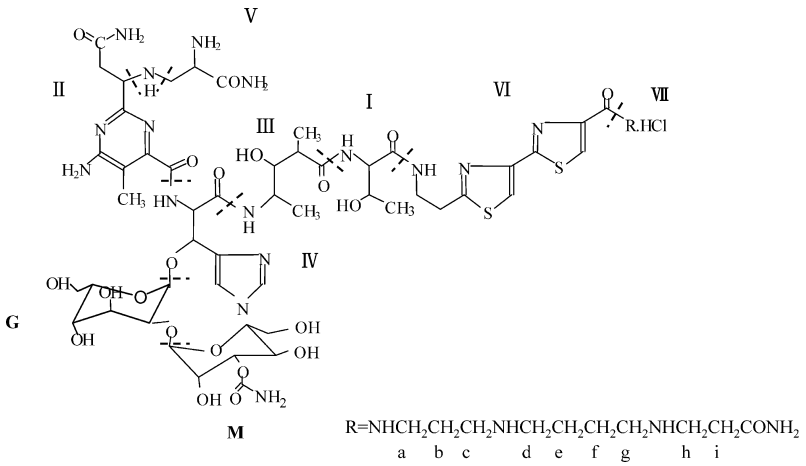

Fig. 1 Structure of NC0604

solution.

The fraction containing copper-chelated NC0604 was desalted with a column of x-5, and the column then eluted with 10% Me_2CO soln (containing 0.01 M HCl). The active eluate was concentrated *in vacuo* and lyophilized to afford a blue powder of copper-chelated NC0604 (313 mg). Finally, copper-free NC0604 was prepared by treatments with dithizone (120 mg) in MeOH (10 ml). The solution was filtered, and the filtrate was precipitated with 4 times volume of Me_2CO. After washed with acetone for three times and filtered, the precipitate was dissolved in distilled water, concentrated *in vacuo* and lyophilized to give pure copper-free compound NC0604 (266 mg).

The physico-chemical properties of NC0604 are summarized in Table 1.

The copper-chelated NC0604 showed UV absorption maximum at 243～245 nm and at 292～295 nm, respectively, and the intensity ratio of the two absorption maximum is 1.23. As for copper-free preparation, absorption at 243～245 nm turned into a shoulder peak. NC0604 showed a typical UV spectrum similar to that of bleomycin.

The IR spectrum indicated the presence of OH/NH groups (3385 cm^{-1}), C=O group (1718 cm^{-1}), CONH (1653 and 1553 cm^{-1}) and OH group (1050 cm^{-1}). These absorptions suggested that there was a glycopeptide structure in the molecule of the antibiotic.

The shift data of ^{1}H-NMR spectrum of copper-free NC0604 at δ_H 7～9 ppm indicated four aromatic protons, which belonged to the thiazole ring and imidazole ring of the kernel structure of bleomycin molecule. They are valuable characteristics for identification of bleomycins.

We assigned the ^{13}C signals of NC0604 by comparison with ^{13}C-NMR data of bleomycins reported previously [2, 3] and by analysis of ^{1}H-^{13}C COSY, heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra of NC0604. The ^{13}C-NMR spectral data are tabulated in Table 2 in comparison with those of bleomycin A_2 presented in the previous paper [3]. The numbering of the parts in the NC0604 molecule

Table 2 ^{13}C-NMR data for NC0604 (150 MHz, D_2O, pD 6.2)

	Assignment	^{13}C shift (ppm)			Assignment	^{13}C shift (ppm)	
		NC0604	BLM A_2[a]			NC0604	BLM A_2
I	CO	175.9	172.5	G	1	100.3	98.3
	β-CH	69.8	68.0		2	73.1	71.0
	α-CH	61.9	59.9		3	70.7	68.6
	CH_3	21.7	20.0		4	71.9	70.0
II	S-CO	179.0	176.7		5	69.8	68.0
	R-CO	170.5	168.3		6	63.1	61.2
	2	168.2	165.9	M	CO	160.7	158.5
	4	167.4	165.2		1	101.0	98.8
	6	155.0	152.9		3	77.1	75.2
	5	115.1	113.0		5	76.4	74.4
	α-CH	62.6	60.5		2	71.1	69.2
	β-CH_2	43.0	41.0		4	67.6	65.6
	CH_3	13.7	11.9		6	63.7	61.8
III	CO	180.3	178.1	VII (R)	(CO)	177.5	—
	β-CH	77.1	75.2		d	49.8	—
	γ-CH	50.7	48.5		g	49.7	—
	α-CH	45.4	43.5		h	46.1	—
	γ-CH_3	17.5	15.8		c	48.0	—
	α-CH_3	14.8	12.9		a	39.1	—
IV	CO	171.9	169.4		b	28.6	—
	2	139.8	137.4		i	33.3	—
	4	137.7	134.7		e	25.7	—
	5	120.5	118.6		f	25.5	
	β-CH	75.9	73.6		α-CH_2	—	41.8
	α-CH	59.8	57.7		γ-CH_2	—	38.6
V	CO	174.8	171.6		$CH_3\times2$	—	25.7×2
	α-CH	55.6	53.3		β-CH_2	—	24.6
	β-CH_2	50.3	47.8				
VI	CO	166.3	163.7				
	2	165.5	163.0				
	2′	173.4	171.0				
	4	151.6	149.4				
	4′	149.7	147.5				
	5′	121.9	119.8				
	5	127.9	125.7				
	β-CH_2	41.9	40.0				
	α-CH_2	34.8	32.9				

[a] BLM A_2: Bleomycin A_2 (pD 6, 25 MHz, ref 3).

follows the convention used in the previous paper [3] as shown in Fig. 1.

The signals of the carbons constituting the kernel structure of NC0604 are consistent with those of bleomycin A_2. Therefore, we inferred that these two compounds are different from each other only in terminal amine moiety (R). Except for the signals of the carbons that constitute the NC0604 skeleton, the remains were all assigned to the terminal amine moiety.

The ^{1}H-NMR and ^{1}H-^{1}H COSY indicated the signal positions of the R moiety: R-a (δ_H 3.58), R-b (δ_H 2.10) , R-c (δ_H 3.21), R-d (δ_H 3.17), R-e (δ_H 1.85), R-f (δ_H 1.85), R-g (δ_H 3.17), R-h (δ_H 3.36), R-i (δ_H 2.79). The HSQC spectrum indicated that R-a (δ_H 3.58) corresponded to a

Table 3 Antimicrobial activity of NC0604

Test bacteria	MIC (μg/ml)	Test bacteria	MIC (μg/ml)
Staphylococcus aureus ATCC29213	2	*Klebsiella pneumoniae* 14	0.25
Staphylococcus aureus 15	32	*Aerobacter cloacae* 45031	1
Staphylococcus aureus 05-3 (MRSA)	64	*Enterobacter aerogenes* 45102	1
Staphylococcus epidermidis ATCC 12228	16	*Acinetobacter calcoaceticus* 25001	>128
Staphylococcus epidermidis 04-5 (MRSE)	>128	*Bacillus proteus* 49027	1
Enterococcus faecalis ATCC 29212	>128	*Shigella sonnei* 51592	1
Enterococcus faecalis 775	64	*Shigella dysenteriae*	0.25
Escherichia coli ATCC 25922	0.5	*Shigella flexneri*	0.25
Escherichia coli 26	1	*Bacillus typhi murium*	0.25
Pseudomonas aeruginosa ATCC 27853	>128	*Bacillus typhi* H901	0.25
Pseudomonas aeruginosa 17	>128	*Bacillus subtilis* 6633	0.03
Klebsiella pneumoniae ATCC 700603	2		

signal at δ_C 39.1, R-b (δ_H 2.10) to δ_C 28.6, R-c (δ_H 3.21) to δ_C 48.0, R-d (δ_H 3.17) to δ_C 49.8, R-e (δ_H 1.85) to δ_C 25.7, R-f (δ_H 1.85) to δ_C 25.5, R-g (δ_H 3.17) to δ_C 49.7, R-h (δ_H 3.36) to δ_C 46.1, and R-i (δ_H 2.79) to δ_C 33.3, respectively.

In the HMBC spectrum, the signal at δ_C 39.1 (R-a) showed a cross peak with a proton at δ_H 2.10 (R-b), δ_C 28.6 (R-b) showed coupling to δ_H 3.21 (R-c) and δ_H 3.58 (R-a), δ_C 48.0 (R-c) to δ_H 3.58 (R-a) and δ_H 2.10 (R-b), δ_C 49.8 (R-d) to δ_H 3.21 (R-c) and δ_H 3.17 (R-g), δ_C 25.7 (R-e) to δ_H 3.17 (R-d), δ_H 1.85 (R-f) and δ_H 3.17 (R-g), δ_C 25.5 (R-f) to δ_H 1.85 (R-e), δ_H 3.17 (R-g) and δ_H 3.17 (R-d), δ_C 49.7 (R-g) to δ_H 3.36 (R-h), δ_C 46.1 (R-h) to δ_H 2.79 (R-i), respectively. Moreover, the methylene of R-a (δ_H 3.58) showed a cross peak with a signal at δ_C 166.3 (VI-CO) and the methylene signal at δ_H 2.79 (R-i-CH_2) showed a cross peak with the carbonyl signal at δ_C 177.5 (R-CO). Therefore, the assignments for carbons of R moiety were determined as shown in Table 2 and the ^{13}C-connectivities revealed by analysis of the HMBC spectrum are indicated with a bold line in Fig. 1.

NC0604 exhibited antibacterial activity against members of a wide range of bacterial species. The antibacterial activity of NC0604 is shown in Table 3. Better antibacterial activities were observed against members of *Bacillus* species and some Gram-negative bacteria.

Table 4 shows the growth-inhibitory activity *in vitro* of NC0604 toward human tumour cells. In comparison with that of bleomycin, NC0604 had a 3 to 9 times higher activity against tumour cells such as HepG2, KB, MCF-7, HCT116, BGC-823 and MCF-7/DOX.

The pulmonary toxicity for mice was determined in male Kunming mice. 10 mg/kg of the test compounds were injected intraperitoneally daily for 10 days. The pulmonary toxicity was evaluated by determining the content of hydroxyproline (HYP) and observing the histopathological changes of the lung tissues at day 28 after administration of NC0604 and bleomycin, respectively. The content of HYP in the lung tissues treated with NC0604 was 1.4 times lower than that treated with bleomycin ($p<0.01$). According to Ashcroft's [4] method, the degree of pulmonary fibrosis by NC0604 was measured microscopically as grade 2~3, showing moderate thickening of walls without obvious damage to lung architecture, while bleomycin was measured as grade 6~7, meaning severe distortion of structure and large fibrous areas. Obviously, NC0604 had reduced pulmonary toxicity.

Table 4 Activities (IC_{50}, μM) of NC0604 in inhibiting the growth of cultured tumour cells

Cells	NC0604 (a)	Bleomycin (b)	Ratio (b/a)
HepG2	1.18	7.87	6.67
KB	1.21	4.91	4.06
MCF-7	1.41	3.66	2.60
HCT116	1.83	8.24	4.50
BGC-823	2.02	10.79	5.34
MCF7/DOX	1.45	13.30	9.17
A549	8.71	8.01	0.92
HL-60	19.67	29.69	1.51
SK-OV-3	12.01	14.14	1.18

References

1. Zhao Y, Xie M, Wang G. Studies on Zhengguangmycins. Taxonomy of *Streptomyces verticillus* var. *pingyangensis* n.var. Acta Microbiologica Sinica 19: 361 (1979)
2. Naganawa H, Kadokura Y, Muraoka Y, *et al.* Carbon-13 NMR assignment of peplomycin. J Antibiot 12: 633–636 (1989)
3. Naganawa H, Muraoka Y, Takita T, Umezawa H. Chemistry of bleomycin. XVIII. Carbon-13 NMR studies. J Antibiot 30: 388–396 (1977)
4. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. J Clin Pathol 41: 467–470 (1988)