CORORUBICIN, A NEW ANTHRACYCLINE ANTIBIOTIC GENERATING ACTIVE OXYGEN IN TUMOR CELLS

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In the course of our screening for antitumor antibiotics which show biological activities by generating active oxygen in tumor cells, *Micromonospora* sp. JY16 was found to produce a new anthracycline antibiotic designated cororubicin. The structure of cororubicin was elucidated as shown in Fig. 5 on the basis of NMR spectral analysis. Cororubicin generated superoxide radicals in tumor cells and showed cytotoxicity, which was reduced by addition of an antioxidative agent, dithiothreitol (DTT).

In oxygen-containing environments, cellular metabolism results in the production of several potentially toxic oxygen-derived species. Accordingly, all the normal cells have various defense systems against such active oxygen species. However, several tumor cells are known to have lost part of their defense systems including manganous superoxide dismutase, a mitochondrial enzyme involved in the scavenging of superoxide radicals[1]. Thus, it could be expected that substances generating active oxygen might show selective cytotoxicity against such tumor cells. Since the cytotoxicity caused by active oxygen species can be prevented by antioxidants, we screened for cytotoxic microbial products inactivated by an antioxidative agent, dithiothreitol (DTT). The assay was carried out in the presence or absence of 250 μM DTT by using N18-RE-105 neuronal cell line[2], which is known to be vulnerable to oxygen stress. As a result, *Micromonospora* sp. JY16 which produced quinolidomicins[3~5] was found to produce a new active substance, cororubicin, belonging to the anthracycline group. In this paper, we report the fermentation, isolation, physico-chemical properties, structural elucidation and biological activity of cororubicin.

Fermentation

The seed medium consisted of soluble starch 1.0%, molasses 1.0%, meat extract 1.0% and Polypepton 1.0% (pH 7.2). Seed tubes containing 15 ml of the medium were inoculated with a stock culture of *Micromonospora* sp. JY16 maintained on a BENNET's agar slant and were incubated on a reciprocal shaker at 27°C for 2 days. The seed culture at 2% was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the seed medium. The flasks were incubated on a rotary shaker at 27°C for 2 days. A 600-ml portion of the culture was inoculated into a 50-liter jar fermenter containing 30 liters of a medium composed of soluble starch 2.5%, fish meal 1%, soybean meal 0.5% and calcium carbonate 0.2% (pH 7.3). The fermentation was carried out at 27°C for 7 days under agitation of 300 rpm and aeration of 30 liters/minute.

Isolation

The fermentation broth (60 liters) was centrifuged to give a mycelial cake, which was extracted with 18 liters of acetone. The extract was concentrated and the residue was extracted twice with one liter of chloroform-methanol (10:1) at pH 8.5. The organic layer was extracted with one liter of 0.2 N H_3PO_4, and the aqueous layer was back-extracted twice with 500 ml of chloroform-methanol (10:1) at pH 8.5.

The solvent layer was evaporated and applied to a silica gel column, which was developed with chloroform - methanol (10 : 1). The active eluate was subjected to preparative silica gel TLC with chloroform - methanol (200 : 23). The active orange-colored fraction was chromatographed on a Sephadex LH-20 column with chloroform - methanol (1 : 1) and further purified by HPLC using a YMC-pack D-ODS-7 column (YMC Co.) with methanol - 0.2 N H_3PO_4 (3 : 2). The active fraction was evaporated and then extracted with chloroform - methanol (10 : 1) at pH 8.5. The extract was concentrated to dryness to yield an orange powder of cororubicin (39 mg).

Physico-chemical Properties

The physico-chemical properties of cororubicin are summarized in Table 1. The molecular formula was determined to be $C_{48}H_{62}N_2O_{21}$ by high-resolution FAB-MS. The UV and visible spectra revealed the presence of an anthraquinone chromophore. The IR spectrum exhibited absorption peaks due to hydroxyls (3450 cm^{-1}), an ester carbonyl (1729 cm^{-1}), quinone carbonyls (1663, 1623 cm^{-1}) and a nitro group (1545 cm^{-1}). The ^1H NMR spectrum of cororubicin in $CDCl_3$ - CD_3OD (10 : 1) is as shown in Fig. 1.

Structural Elucidation

The ^{13}C NMR spectrum of cororubicin revealed the presence of 48 carbons, which were assigned to 9 methyls, 5 methylenes, 18 methines and 16 quaternary carbons by the heteronuclear single-quantum coherency (HSQC)[6] spectrum (Table 2).

Four sugar moieties were elucidated by the ^1H-^1H COSY spectrum as shown in Fig. 2. Connectivities from C-1 to C-5 of each sugar were confirmed by ^1H-^{13}C long-range couplings observed in the heteronuclear multiple-bond correlation

Table 1. Physico-chemical properties of cororubicin.

Appearance	Orange powder
MP (°C)	192～194
Molecular formula	$C_{48}H_{62}N_2O_{21}$
HRFAB-MS (*m/z*)	
Found:	1003.3970 $(M+H)^+$
Calcd:	1003.3924
$[\alpha]_D^{21}$	+630° (*c* 0.11, MeOH)
UV λ_{max}^{MeOH} nm (ε)	208 (22,900), 237 (45,200), 261 (25,600), 294 (8,600), 476 (15,500)
$\lambda_{max}^{MeOH+NaOH}$ nm (ε)	207 (84,200), 244 (41,800), 292 (8,300), 539 (14,600)
IR ν_{max} (KBr) cm^{-1}	3450, 1729, 1663, 1623, 1545

Fig. 1. ^1H NMR spectrum of cororubicin in $CDCl_3$ - CD_3OD (10 : 1).

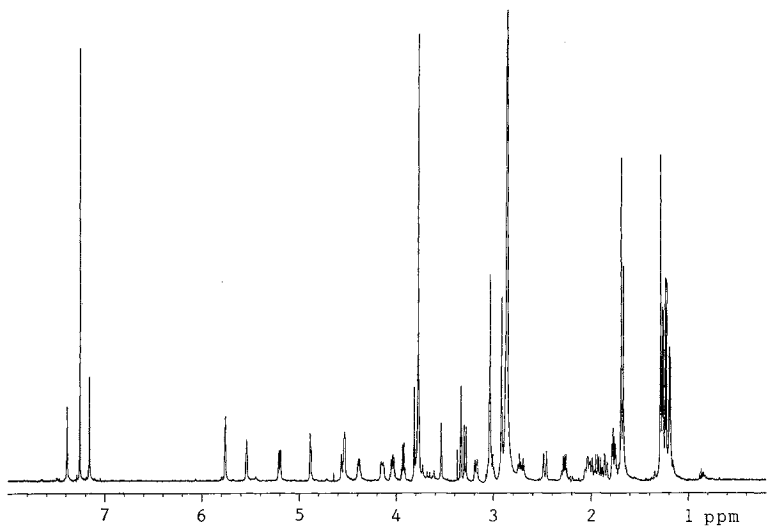


Table 2. ^{13}C and ^{1}H NMR data summary for cororubicin in $CDCl_3$-CD_3OD (10:1).

	δ_C	δ_H		δ_C	δ_H
1	147.7		S-1		
2	139.3		1	98.9	5.54
3	122.1	7.16	2	33.3	2.05
4	155.9				1.99
4a	114.6		3	64.4	4.15
5	191.2		4	82.1	3.77
5a	112.5		5	66.8	4.39
6	159.9		6	16.5	1.19
6a	132.8		S-2		
7	20.7	3.03	1	99.4	5.21
		2.72	2	41.4	2.47
8	32.2	2.28			1.93
		1.76	3	88.5	
9	68.7		4	83.5	3.29
10	56.1	3.82	5	70.2	4.04
10a	142.2		6	18.2	1.26
11	119.6	7.39	3-Me	24.8	1.69
11a	131.2		S-3		
12	180.0		1	101.6	4.89
12a	116.7		2	32.4	1.86
13	27.9	1.29			1.76
COO–	172.8		3	65.0	3.79
–OMe	52.4	3.78	4	70.5	3.54
1′	97.2	5.76	5	67.4	3.93
2′	68.5	4.56	6	16.4	1.23
3′	61.0	3.18			
4′	81.2	4.53			
5′	76.6				
6′	23.5	1.67			
N-Me_2	44.0	3.04			
		2.92			

(HMBC)[7] spectrum. These data show that cororubicin contains two 2-deoxyfucose[8] (S-1 and S-3) and one decilonitrose[9] (S-2) residues. The coupling constants observed on their anomeric protons (Fig. 2) established an α-configuration for each 2-deoxyfucose and a β-configuration for decilonitrose. The absolute configurations of these sugars were established by methanolysis of cororubicin, which gave methyl 2-deoxy-α-L-fucoside and methyl β-L-decilonitroside. In the remaining sugar moiety, ^{1}H-^{13}C long-range couplings were observed from 6′-H to C-4′ and C-5′, and from N-Me_2 to C-3′, indicating that the fourth sugar is a dimethylamino sugar as shown in Fig. 2.

This amino sugar moiety was elucidated to be attached to an aromatic ring by a C-C bond in addition with a glycosidic linkage by ^{1}H-^{13}C long-range correlations from 1′-H to C-1, from 3-H to C-5′, and from 6′-H to C-2 (Fig. 3). Long-range couplings from 4′-H to C-1 of S-1, from 1-H of S-2 to C-4 of S-1, from 4-H of S-2 to C-1 of S-3, and from 1-H of S-3 to C-4 of S-2 arranged a trisaccharide moiety composed of S-1, S-2 and S-3 on C-4′. These data established partial structure I as shown in Fig. 3.

Another partial structure contained a six-membered ring, which was constructed by ^{1}H-^{13}C long-range couplings from 7-H to C-6a, C-9 and C-10a, from 8-H to C-6a and C-9, and from 10-H to C-9 and C-10a (Fig. 4). Long-range correlations from 13-H to C-8, C-9 and C-10, and from 10-H and a methoxy proton (3.78 ppm) to an ester carbonyl carbon (172.8 ppm) revealed the presence of a methyl group (C-13) at C-9 and a carbomethoxy group at C-10. Furthermore, long-range couplings from 7-H to a phenolic carbon

Fig. 2. Sugar components of cororubicin.

Numbers accompanied with dashed arrows indicate coupling constants (Hz).

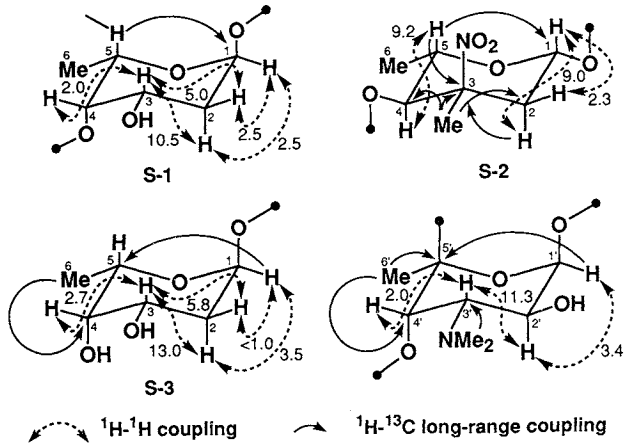
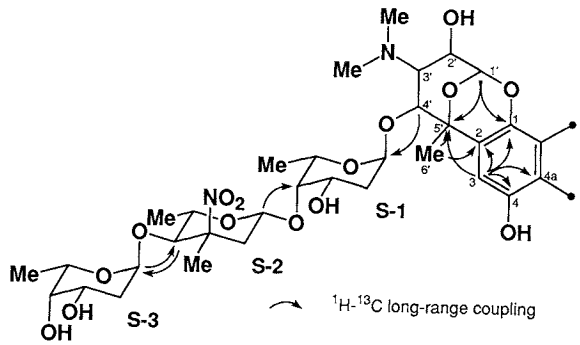
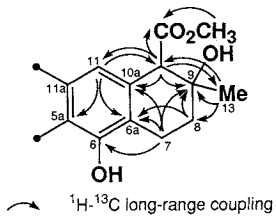


Fig. 3. Partial structure I.



(C-6) and from an aromatic proton (11-H) to its *meta*-positions (C-5a and C-6a) and C-10 expanded the ring to a bicyclic system to establish partial structure II as shown in Fig. 4.

Fig. 4. Partial structure II.



The remaining units were only two quinone carbonyls (191.2 ppm and 180.0 ppm), one of which showed a significant down-field shift. Since hydrogen-bonded quinone carbonyls show a down-field shift[10], the connection between partial structures I and II should be made to form a quinone moiety with one carbonyl (C-5) hydrogen-bonded with the two phenolic hydroxyl groups (4-OH and 6-OH).

The structure of cororubicin thus obtained is as shown in Fig. 5 and seems to be related to that of arugomycin[10,11], an anthracycline antibiotic comprising arugorol as the aglycone with a tetrasaccharide chain at C-7 and a trisaccharide chain consisting of 2-deoxyfucose (S-1), decilonitrose (S-2) and diginose (S-3) at C-4′. The aglycone of cororubicin was identified as 7-deoxyarugorol based on the ^{13}C chemical shift similarity to a hydrogenolysis product of arugomycin[11].

Fig. 5. Structure of cororubicin.

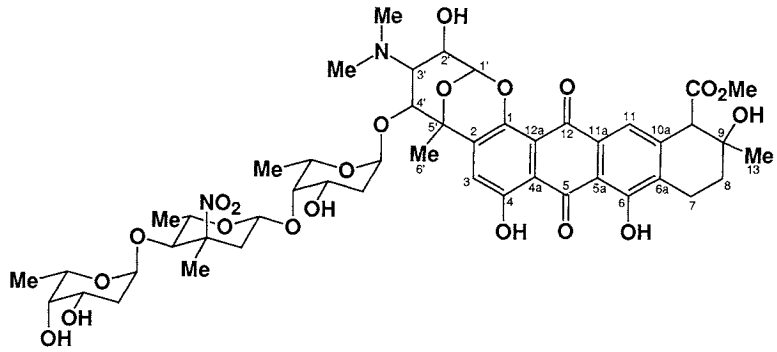
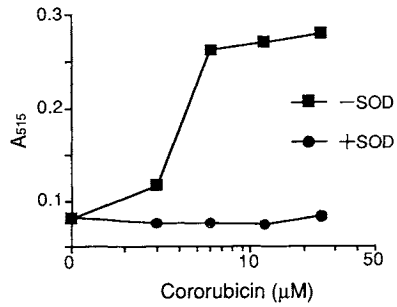


Table 3. Effect of dithiothreitol (DTT) on cytotoxicity of cororubicin (IC_{50}, μM).

Cell line	−DTT	+DTT (250 μM)
KB	4.3	10
N18-RE-105	0.84	2.9

Fig. 6. Generation of superoxide radicals in N18-RE-105 cell lysate by cororubicin.



Biological Activity

Cororubicin showed cytotoxicity against KB human epidermoid cancer cells and N18-RE-105 neuronal hybridoma cells (rat retina cells × mouse neuroblastoma cells)[2], and the cytotoxic activity was reduced by addition of 250 μM DTT as shown in Table 3. The generation of superoxide radicals in N18-RE-105 cell lysate by cororubicin was examined by measuring reduction of nitro blue tetrazolium (NBT)[12]. As shown in Fig. 6, cororubicin dose-dependently generated superoxide radicals, which were not observed in the presence of 130 μg/ml of superoxide dismutase (SOD). Further studies on the antitumor activity of cororubicin are in progress.

Experimental

Microorganism

A culture designated JY16 was isolated from a soil sample collected at Yoshii-machi, Tano-gun, Gunma Prefecture, Japan. The culture has been deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the name *Micromonospora* sp. JY16 with the accession number FERM BP-3940. Taxonomic studies on strain JY16 has been reported in the previous paper[4].

Spectral Analysis

Specific rotations were obtained on a Jasco DIP-140 spectropolarimeter at 589.6 nm and 21°C. Mass spectra were measured on a JEOL HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. UV and visible spectra were recorded on a Shimadzu UV-160 spectrophotometer. NMR spectra were obtained on a JEOL JNM-A500 spectrometer at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. Chemical shifts are given in ppm using TMS as internal standard.

Methanolysis of Cororubicin

A solution of cororubicin (50 mg) in 5% HCl-MeOH was heated at reflux for 2 hours. The reaction mixture was subjected to silica gel column chromatography with toluene-EtOAc (10:1) to give methyl β-L-decilonitroside (1.2 mg): $[\alpha]_D^{21}$ −10° (*c* 0.06, MeOH) (literature 9, $[\alpha]_D^{23}$ −13° (*c* 0.2, MeOH)); ^{1}H NMR ($CDCl_3$) δ 4.49 (dd *J*=9.6, 2.1 Hz, 1-H), 3.68 (dq *J*=9.8, 6.0 Hz, 5-H), 3.47 (3H, s, OMe), 3.30 (dd, *J*=11.3, 9.8Hz, 4-H), 3.08 (d, *J*=11.3 Hz, 4-OH), 2.75 (dd, *J*=14.7, 2.1 Hz, 2-Heq), 1.77 (dd, *J*=14.7, 9.6 Hz, 2-Hax), 1.73 (3H, s, 3-Me), 1.39 (3H, d, *J*=6.0 Hz, 6-H). Development of the column with EtOAc gave another methyl glycoside fraction, which was purified by silica gel column chromatography with $CHCl_3$-MeOH (10:1) to yield methyl 2-deoxy-α-L-fucoside (9.6 mg): $[\alpha]_D^{21}$ −120° (*c* 0.48, $CHCl_3$) (literature 8, $[\alpha]_D$ −119° (*c* 0.68, $CHCl_3$)); ^{1}H NMR ($CDCl_3$) δ 4.77 (br d, *J*=3.9 Hz, 1-H), 3.99 (ddd, *J*=12.0, 5.1, 3.0 Hz, 3-H), 3.90 (br q, *J*=6.8 Hz, 5-H), 3.62 (br d, *J*=3.0 Hz, 4-H), 3.32 (3H, s, OMe), 1.90 (br dd, *J*=13.0, 5.1 Hz, 2-Heq), 1.77 (ddd, *J*=13.0, 12.0, 3.9 Hz, 2-Hax), 1.28 (3H, d, *J*=6.8 Hz, 6-H).

Cells and Cell Culture

KB cells were grown in EAGLE's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% Bacto-peptone. N18-RE-105 cells were grown in DULBECCO's modified EAGLE medium supplemented with 0.1 mM hypoxanthine, 40 μM aminopterin, 0.14 mM thymidine and 10% heat-inactivated fetal calf serum. These cells were cultured in humidified atmosphere of 5% CO_2 in air at 37°C. KB cells at 5×10^4 cells/ml and N18-RE-105 cells at 2×10^4 cells/ml were incubated for 2 days with various concentrations of cororubicin in the presence or absence of 250 μM DTT, and then the viable cells were counted to determine IC_{50}s after trypan-blue staining.

Determination of Superoxide Radicals

Superoxide radical generation was determined by the nitro blue tetrazolium (NBT) assay[12]. N18-RE-105 cells (5×10^7) were homogenized in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.15 M potassium chloride and 1 mM EDTA, and centrifuged at $1,000\times g$ for 20 minutes. The supernatant was used as cell lysate. The reaction mixture (1 ml) containing the cell lysate (0.7 mg protein/ml), 0.05% NBT and various concentrations of cororubicin was incubated in the presence or absence of superoxide dismutase (SOD, Sigma, from bovine erythrocytes; 130 μg/ml) at 37°C for 30 minutes. The residue was washed with 1 N HCl and dissolved in 1 ml of hot pyridine. The absorbance of supernatant was measured at 515 nm.

Acknowledgments

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