NEW ANTHRACYCLINE GLYCOSIDES FROM *MICROMONOSPORA*
II. ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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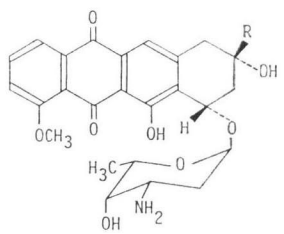
Four glycosides, designated A, B, C and D, are the main components of the anthracycline complex produced by cultures of *Micromonospora* sp. nov. They were extracted by solvent partition, separated by column chromatography and characterized by chemical and physical methods as 11-deoxy analogues of daunorubicin. Among these new anthracyclines, displaying antibacterial and cytotoxic activity *in vitro*, 11-deoxydaunorubicin and 11-deoxydoxorubicin are also active against P388 leukemia in mice.

During our continuing search for new biosynthetic[1] and semisynthetic antitumor antibiotics[2], the crude extracts of the cultural broths of *Micromonospora* sp. nov.[3], showed some activity against P388 leukemia in mice. The microorganism produced an antibiotic complex which was extracted and separated into four main yellow components, designed as glycosides A, B, C and D, which on the basis of their chemical and physical properties were recognized as components of a novel structural class within the group of the daunorubicin related anthracyclines. Recent studies[4] assigned to glycosides A, B, C and D, respectively, the structures of 11-deoxydoxorubicin (**I**), 11-deoxy-13-dihydrodaunorubicin (**II**), 11-deoxydaunorubicin (**III**) and 11-deoxy-13-deoxodaunorubicin (**IV**), as shown in Fig. 1.

This paper describes the isolation and purification, and the physicochemical and biological properties of these four new anthracyclines.

Fig. 1. Structure of glycosides A, B, C, and D.



I: R=–CO–CH_2OH
II: R=–CHOH–CH_3
III: R=–CO–CH_3
IV: R=–CH_2CH_3

Isolation and Purification

The fermentation broth of *Micromonospora*[3], was filtered with filter aid at pH 4 and the yellow pigments were extracted from the mycelium with an acetone - 0.1 N hydrochloric acid mixture (4: 1, v/v). The extract, adjusted to pH 4.5, was concentrated under reduced pressure and the aqueous concentrate

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combined with the filtered broth. The resulting solution was adjusted to pH 8.5 with 1 N aqueous sodium hydroxide and exhaustively extracted with a chloroform - methanol mixture (9 : 1, v/v). The combined organic extracts, concentrated and adjusted to pH 5 with hydrochloric acid, gave the anthracycline complex as a brown yellow powder by a five-fold addition of *n*-hexane.

The thin-layer and paper chromatographic analysis of the crude complex indicated the presence of four main components designated as glycosides A, B, C and D, as shown in Table 1. By partition column chromatography on cellulose powder buffered at pH 5.4 (phosphate buffer), and using *n*-butanol previously saturated with M/15 phosphate buffer at the same pH as eluent, the components D, C, B and A were successively eluted. The glycosides D and A were recovered by transfer into acidic water (pH 3.5) followed by reextraction into chloroform at pH 7.5, concentration and isolation of the hydrochlorides upon addition of an equivalent of methanolic hydrogen chloride. The purification of glycosides B and C was accomplished by column chromatography on buffered silica gel (pH 7) with a chloroform - methanol - water mixture gradient. Glycoside C was eluted with a 94.8 : 5 : 0.2 mixture while the elution of glycoside B required a 89.5 : 10 : 0.5 mixture. The pooled fractions containing a single component were washed with water and concentrated to a small volume. Addition of an equivalent of hydrochloric acid gave pure glycoside B and C as the hydrochlorides. By this procedure, as summarized in Fig. 2, glycosides A, B, C and D were obtained, respectively, with typical yields of

Table 1. Rf Values of glycosides A, B, C and D.

Glycosides	PC	TLC
A	0.30	0.50
B	0.50	0.55
C	0.55	0.65
D	0.60	0.70

PC: on paper Whatman No. 1 buffered with M/15 phosphate buffer at pH 5.4, descending system: *n*-propanol - ethylacetate - water, 7 : 1 : 2.

TLC: on Merck 60-F-254 pre-coated plate; elution with chloroform - methanol - water - acetic acid, 80 : 20 : 6 : 14.

Fig. 2. Isolation and purification of glycosides A, B, C and D.

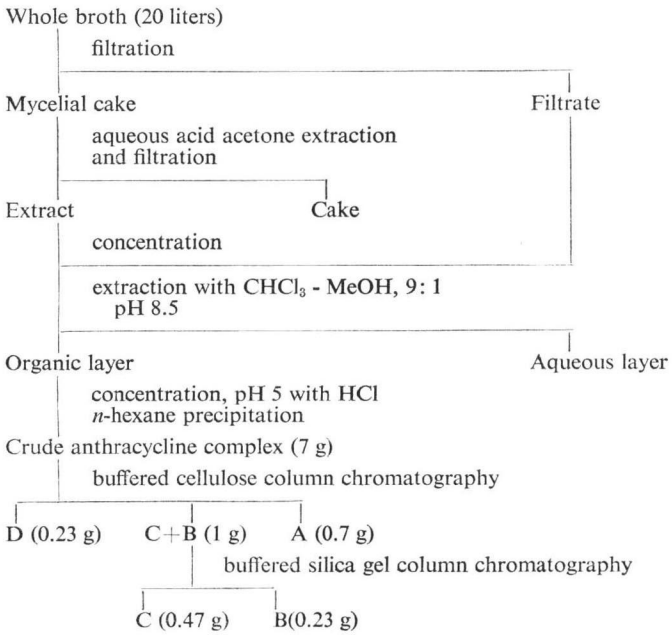

Table 2. Chemical and physical properties of glycosides A, B, C and D as hydrochlorides.

Properties	Glycoside			
	A	B	C	D
Melting point	171~173°C (dec.)	163~164°C (dec.)	175~176°C (dec.)	140~150°C (dec.)
$[\alpha]_D^{23}$ (*c* 0.2, CH_3OH)	+111°	+107°	+139°	+122°
UV and VIS spectra:				
λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	228, 260, 418 (645, 420, 193)	228, 260, 418 (640, 410, 179)	228, 260, 418 (713, 450, 199)	228, 260, 418 (610, 395, 171)
IR spectrum (KBr) cm^{-1}	3,700~2,400; 1,725; 1,670; 1,625; 1,585	3,700~2,400; 1,665; 1,625; 1,585	3,700~2,400; 1,710; 1,670; 1,625, 1,585	3,700~2,400; 1,665; 1,625; 1,585
Empirical formula	$C_{27}H_{29}NO_{10}\cdot HCl$	$C_{27}H_{31}NO_9\cdot HCl$	$C_{27}H_{29}NO_9\cdot HCl$	$C_{27}H_{31}NO_8\cdot HCl$
m/z in FD equivalent to the free base	527 (M^+)	513 (M^+)	511 (M^+)	497 (M^+)

Table 3. Chemical and physical properties of aglycones of glycosides A, B, C and D.

Properties	Aglycones of glycosides			
	A	B	C	D
Melting point	220°C	175~180°C (dec.)	213~215°C	175~180°C (dec.)
$[\alpha]_D^{23}$ (*c* 0.1)	+161° (dioxane)		+144° (methanol)	+164° (methanol)
UV and VIS spectra:				
λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	227, 259, 418 (895, 640, 268)	227, 258, 418	227, 258, 418 (930, 660, 317)	227, 258, 418 (900, 640, 280)
Empirical formula	$C_{21}H_{18}O_8$	$C_{21}H_{20}O_7$	$C_{21}H_{18}O_7$	$C_{21}H_{20}O_6$
Molecular weight (M^+)	398	384	382	368

35, 11, 23 and 11 μg/ml of fermentation broth.

Chemical and Physical Properties

The four new anthracyclines show some common properties, but they can be distinguished on the basis of their chemical and physical properties; as free bases they are soluble in polar organic solvent and aqueous alcohols, while their hydrochlorides are soluble in water and lower alcohols but insoluble in organic solvent. Some properties of their hydrochlorides, obtained in the form of crystals or microcrystalline powders, are summarized in Table 2. These new anthracyclines show also characteristic PMR spectra. The spectrum of glycoside A·HCl in DMSO-d_6 showed significant signals at δ 1.14 (d, CH_3-C-5′), 3.93 (s, CH_3O-), 4.60 (broad s, C-14-H_2), 4.89 (broad s, C-7-H), 5.27 (broad s, C-1′-H), 7.23 (s, C-11-H), 7.4~7.9 (m, 3-Ar-H) and 13.61 (s, C-6-OH), while the spectrum of glycoside C·HCl (DMSO-d_6) showed signals at δ 1.15 (d, CH_3-C-5′), 2.26 (s, CH_3CO), 3.92 (s, CH_3O), 4.90 (broad s, C-7-H), 5.26 (broad s, C-1′-H), 7.31 (s, C-11-H), 7.4~7.9 (m, 3-Ar-H) and 13.65 (s, C-6-OH). Field desorption mass spectroscopy confirmed the assigned molecular formulae, as shown in Table 2.

Aqueous acid hydrolysis (0.2 N HCl, 1 hour, 90°C) of the four glycosides gave different water insoluble aglycones, whose chemical and physical properties are summarized in Table 3, and the same reducing aminosugar which was identified as daunosamine[5], previously found in daunorubicin and doxorubicin. Further structural studies[4] assigned to the aglycones of glycosides A, B, C and D the

structures of 11-deoxyadriamycinone, 11-deoxy-13-dihydrodaunomycinone, 11-deoxydaunomycinone, and 11-deoxy-13-deoxodaunomycinone, respectively.

Biological Activity Data

Antibacterial Activity

The new anthracyclines displayed a low antibacterial activity. Their *in vitro* minimal inhibitory concentration (MIC) values on some microorganisms, using the standard tube dilution procedure, are reported in Table 4.

Table 4. Antibacterial activity of glycosides A, B, C and D.

Test organism	MIC in μg/ml; glycosides			
	A	B	C	D
Staph. aureus FDA 209P	125	1,000	62	250
Staph. aureus 153	500	1,000	250	1,000
Sarcina lutea ATCC 9341	100	100	12.5	25
B. subtilis ATCC 6633	100	100	50	100
E. coli B	50	100	25	50

Table 5. Effect on HeLa cells viability *in vitro*.

Compound	Dose* (ng/ml)	No. of colonies (% of controls)	ID_{50} (ng/ml)
Daunorubicin**	12.5	35, 15	
	6.25	48, 66	6.5
	3.12	71, 80	
11-Deoxydaunorubicin (**III**)	200	3	
	100	44	70
	50	54	
11-Deoxy-13-deoxodaunorubicin (**IV**)	400	0	
	200	53	220
	100	109	
11-Deoxy-13-dihydrodaunorubicin (**II**)	1,600	0	
	800	20	500
	400	66	
Doxorubicin	12.5	38	
	6.25	52	10
	3.12	76	
11-Deoxydoxorubicin (**I**)	200	1	
	100	63	100
	50	73	
	25	74	

* HeLa cells were exposed to the drugs for 24 hours, then plated. Colonies number was evaluated 5 days later.

** Data of 2 experiments.

Table 6. Activity against P 388 leukemia.*

Compound	Dose+ (mg/kg)	T/C++ (%)	No. of toxic deaths / No. of mice treated
Daunorubicin	2.9	183 (171~190)	0/29
	4.4	193 (180~200)	3/29
	6.6	188 (165~223)	19/30
11-Deoxydaunorubicin (III)	29	161	0/10
	44	142	0/10
	66	155 (120~190)	5/19
	100	115 (181~ 50)	13/18
	150	45	9/9
Doxorubicin	4.4	190	0/9
	6.6	209	0/10
	10.0	233	0/9
11-Deoxydoxorubicin (I)	66	232 (219~245)	0/18
	100	238 (223~245)	2/18
	150	61	9/10

* BDF1 mice received 10^6 ascites cells on day 0, i.p.

\+ Single treatment i.p. on day 1 after tumor transplantation.

++ (Median survival time of treated mice/median survival time of control mice) ×100. Average data of 3 experiments. In brackets, range of individual experiments.

Antitumor Activity

The cytotoxic activity of 11-deoxydoxorubicin (**I**), 11-deoxydaunorubicin (**III**) and its analogues substituted in position 13 was compared with that of doxorubicin and daunorubicin on HeLa cells *in vitro* and the results are reported in Table 5. All the 11-deoxy derivatives tested were markedly less effective than the parent antibiotics on HeLa cells cloning efficiency.

11-Deoxydaunorubicin and 11-deoxydoxorubicin were also tested against P388 ascitic leukemia in mice. The results, reported in Table 6, show that the new anthracyclines were markedly less potent than the parent compounds: 11-deoxydaunorubicin was 15-times less potent than daunorubicin and 11-deoxydoxorubicin 60-times less potent than doxorubicin (ratio between optimal non toxic doses). At the optimal doses, however, the 11-deoxy derivatives maintained an antitumor activity of the same order of magnitude as that of the parent compounds. In conclusion, the absence of the hydroxy group in position 11 of daunorubicin and doxorubicin caused a remarkable reduction of the potency *in vitro* and *in vivo*, but did not hamper the antitumor activity. Whether the reduced potency is due to decreased cell uptake or to reduced ability of binding to DNA remains to be determined.

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