NEW ANTHRACYCLINE GLYCOSIDES: 4-*O*-DEMETHYL-11-DEOXYDOXORUBICIN AND ANALOGUES FROM *STREPTOMYCES PEUCETIUS* VAR. *AUREUS*

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(Received for publication October 13, 1981)

The new anthracyclines 4-*O*-demethyl-11-deoxydoxorubicin, 4-*O*-demethyl-11-deoxydaunorubicin along with its 13-dihydro and 13-deoxo analogues are the main components of the anthracycline complex produced by cultures of *Streptomyces peucetius* var. *aureus*. They were isolated by solvent partition, separated by column chromatography and characterized by chemical and physical methods. Among these new anthracyclines, displaying antibacterial and cytotoxic activity "*in vitro*", 4-*O*-demethyl-11-deoxydoxorubicin and the corresponding daunorubicin analogue were also active against experimental tumors.

In our continuing search for new biosynthetic antitumor antibiotics[1,2], the crude extracts of the cultured broths of *Streptomyces peucetius* var. *aureus* (a biochemical mutant of *Streptomyces peucetius* var. *caesius*[3], the doxorubicin producing microorganism) showed a marked inhibiting effect on P388 leukemia in mice. The strain also designed 416 F. I., produced a new anthracycline complex, which was extracted from the culture broths and separated into four main yellow components, designed as glycosides W, X, Y and Z, which on the base of their chemical and physical properties were recognized as components of a novel class within the group of 11-deoxydaunorubicin related anthracyclines[4]. Structural studies assigned to glycosides W, X, Y and Z, respectively, the structures of 4-*O*-demethyl-11-deoxydoxorubicin (**I**), 4-*O*-demethyl-11-deoxy-13-dihydrodaunorubicin (**II**), 4-*O*-demethyl-11-deoxydaunorubicin (**III**, synonym 11-deoxycarminomycin I) and 4-*O*-demethyl-11-deoxy-13-deoxodaunorubicin (**IV**), as shown in Fig. 1.

This paper deals with the taxonomy of the producing organism, production, isolation, structural determination, physico-chemical and biological properties of the new anthracyclines.

Taxonomy

Strain 416 F. I. has been obtained among the surviving population of a mutagenic treatment with

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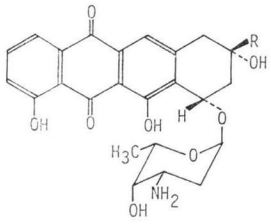
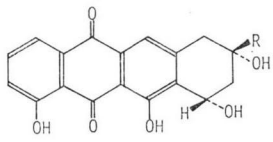
Fig. 1. Structure of glycosides X, W, Y and Z.



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

Fig. 2. Structure of aglycones.



V : R = -$COCH_2OH$
VI : R = -CHOH-CH_3
VII : R = -CO-CH_3
VIII : R = -CH_2CH_3

N-methyl-*N'*-nitro-*N*-nitrosoguanidine[5] carried out on a spore suspension of *Streptomyces peucetius* var. *caesius*.

The microscopic examination of the aerial mycelium showed a morphological identity of this mutant with its parent culture[3] and with the original *Streptomyces peucetius* culture[6]. The cultural characteristics of mutant 416 F. I. can be summarized as follows: growth is generally good on organic as well as on synthetic media; on the former ones the color of the substrate mycelium ranges from straw-yellow to lemon-yellow, becoming ochre on aging. A soluble pigment showing the same yellow tonalities is produced. The parent cultures notoriously form a red substrate mycelium and a bright red soluble pigment. About the aerial mycelium which is more abundantly formed on synthetic media, its color is very similar to that of the parent culture, *i.e.* gray-blue-green, or gray-green. Concerning the physiological and biochemical properties, the mutant 416 F. I. differs from its parent culture in as much as it grows on L-arabinose and on esculin while it does not grow neither on mannitol nor on raffinose; furthermore this mutant differs from its parent culture because it produces a new anthracycline complex.

Because of all the differences cited above and owing to its characteristic yellow colored substrate mycelium and soluble pigment, strain 416 F. I. has to be considered a variety of the species *S. peucetius* to which the designation *Streptomyces peucetius* var. *aureus* (ATCC 31428; DMS 1367; FRI 4622) has been given.

Fermentation

Fermentation was carried out in 300-ml Erlenmeyer shaken flasks as well as in 500-liter stainless-steel fermenters containing the following medium (g/liter): glucose 60, brewer's dry yeast 30, NaCl 2, KH_2PO_4 1, $CaCO_3$ 2, $MgSO_4 \cdot 7H_2O$ 0.1, $FeSO_4 \cdot 7H_2O$ 0.001, $ZnSO_4 \cdot 7H_2O$ 0.001, $CuSO_4 \cdot 5H_2O$ 0.001, tap water, up to one liter. At the 24th and 48th hour of fermentation, 0.5 g/liter of sulphanilamide was added each time.

The maximum concentration of the glycosidic components was reached between the sixth and seventh days of fermentation with a production of 50~70 mcg/ml, consisting of the glycoside Y as the main component. When samples of fermentation broths or crude preparations are subjected to paper chromatography as reported in Table 1, followed by bioautography using *Bacillus subtilis* ATCC 6633 as test organism, several active components are found to occur. The major components were designed glycosides W, X, Y and Z, while the two minor ones, designed glycosides A and C, were found to be identical with 11-deoxydoxorubicin and 11-deoxydaunorubicin[2] respectively. The amount of individual anthracycline glycoside was determined on crude extracts by HPLC[7] and/or by spectrophotometry after separation on paper chromatography.

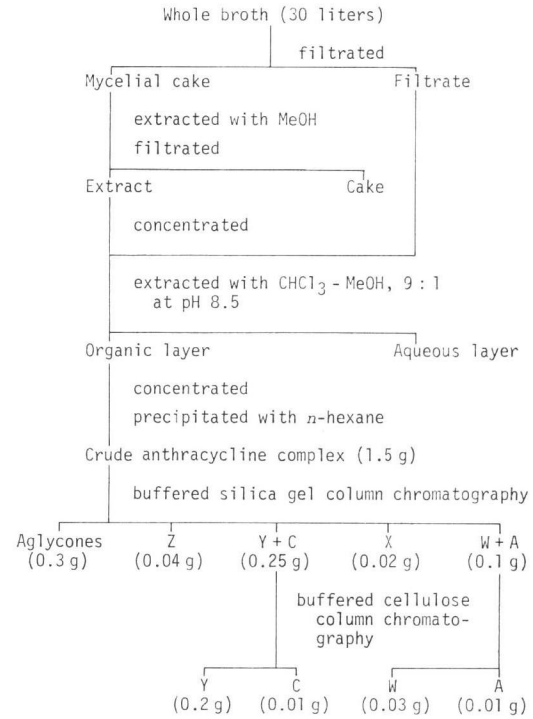
Table 1. Rf Values of glycosides W, X, Y and Z.

Glycosides	PC	TLC
W (I)	0.45	0.50
X (II)	0.60	0.55
Y (III)	0.64	0.65
Z (IV)	0.70	0.74
A (11-Deoxydoxorubicin)	0.30	0.50
C (11-Deoxydaunorubicin)	0.55	0.65

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

TLC on Merck 60-F254 pre-coated plate: elution with chloroform - methanol - water - acetic acid, 80: 20: 14: 6 (v/v/v/v).

Fig. 3. Isolation and purification of glycosidic components.



Isolation and Purification

The harvested broth was filtered with filter aid at pH 4 and the pigments were extracted from the mycelium with methanol and the extract was concentrated *in vacuo*, then it was combined with the filtered broth and exhaustively extracted at pH 8.5 with chloroform. The combined organic extracts, washed with water and dried on anhydrous sodium sulfate, were concentrated and the crude anthracycline complex was precipitated by addition of *n*-hexane. The crude orange-brown powder was dissolved in chloroform and subjected to column chromatography on buffered silica gel (pH 7) with a chloroform - methanol - water mixture gradient. By using a 92.2: 3.5: 0.2 (v/v/v) mixture some yellow colored aglycones were eluted, with the mixing ratio of 92.2: 7.5: 0.3 (v/v/v) to 83: 15: 2 (v/v/v) the glycosides Z, Y with minor amounts of C, X and finally W with minor amounts of A were successively eluted. The pooled fractions were washed with water and concentrated to a small volume. Addition of an equivalent of hydrochloric acid gave pure glycosides Z and X as the hydrochlorides. The purification of glycosides Y and W was accomplished by partition column chromatography on cellulose powder buffered with M/15 phosphate buffer at pH 5.4 using as eluent 1-butanol buffered with the same buffer. The glycosides Y, C, W and A successively eluted, were recovered by transfer into acidic water (pH 3.5) reextracted into chloroform at pH 7.5, concentrated and isolated as the hydrochlorides. The minor constituents, glycosides A and C were identified respectively as 11-deoxydoxorubicin and 11-deoxydaunorubicin after comparison with authentic samples[2,4]. The isolation and purification procedures along with typical yield of the single glycoside are summarized in Fig. 3.

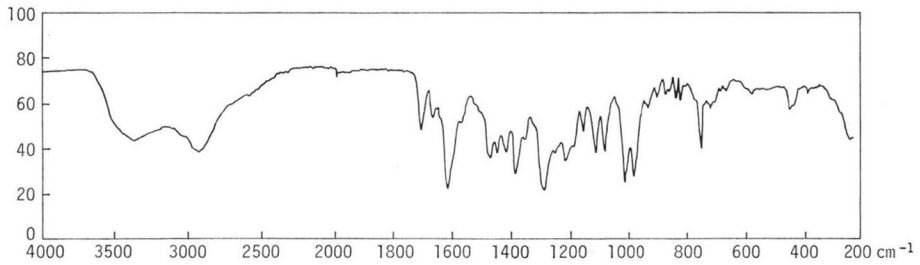
Physical and Chemical Properties

The four new glycosides as free bases are generally soluble in polar organic solvents and aqueous alcohols, while their hydrochlorides are soluble in water and lower alcohols but insoluble in organic solvents. Some properties of their hydrochlorides are summarized in Table 2. Their UV and visible spectra show maxima in neutral and acidic solutions around 228, 258 and 430 nm, the last one being

Table 2. Chemical and physical properties of glycosides W, X, Y and Z as hydrochlorides.

Properties	Glycoside			
	W (I)	X (II)	Y (III)	Z (IV)
Melting point (°C)	208~210 (dec)	200~205 (dec)	195~196 (dec)	200~201 (dec)
$[\alpha]_D^{23}$ (*c* 0.1, MeOH)	+130°	+110°	+150°	+134°
UV and VIS spectra:				
λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	228(650), 258(435), 290sh(175), 430(217)	228(640), 258(425), 290sh(170), 430(205)	228(730), 258(435), 290sh(172), 430(218)	228(660), 258(430), 290sh(180), 430(216)
IR spectra (KBr) cm^{-1}	3700~2400, 1720, 1670, 1615, 1470, 1450, 1415	3700~2550, 1660, 1620, 1470, 1450, 1415	3700~2600, 1710, 1667, 1620, 1518, 1475, 1450, 1415	3650~2500, 1665, 1620, 1470, 1450, 1420
Molecular formula	$C_{26}H_{27}NO_{10}\cdot HCl$	$C_{26}H_{29}NO_9\cdot HCl$	$C_{26}H_{27}NO_9\cdot HCl$	$C_{26}H_{29}NO_8\cdot HCl$
m/z in FD corresponding to the free base	514(M+1)	500(M+1)	498(M+1)	484(M+1)
Identification	4-*O*-Demethyl-11-deoxydoxorubicin	4-*O*-Demethyl-11-deoxy-13-dihydro-daunorubicin	4-*O*-Demethyl-11-deoxydaunorubicin	4-*O*-Demethyl-11-deoxy-13-deoxo-daunorubicin

Fig. 4. Infrared absorption spectrum of 4-*O*-demethyl-11-deoxydaunorubicin·HCl (KBr).



shifted to 520 nm in alkaline solutions. The IR spectra (KBr) indicate the presence of nonhydrogen bonded (1670 cm^{-1}) and hydrogen bonded (1620 cm^{-1}) quinone carbonyl groups in all the glycosides, while an additional carbonyl function is present in the glycosides W and Y (Fig. 4) respectively at 1720 and 1710 cm^{-1}.

Mild acid hydrolysis (0.2 N HCl, 80°C, 30 minutes) of the four glycosides affords the same amino sugar, identified as daunosamine (3-amino-2,3,6-trideoxy-L-*lyxo*-hexose)[8] by direct comparison with an authentic sample, and four different yellow aglycones (**V**~**VIII**), whose chemical and physical properties are summarized in Table 3. These data along with the spectroscopical data indicate that the aglycones (**V**~**VIII**) are anthracyclinones with a common hydroxyanthraquinone chromophore, bearing two phenolic hydroxyl groups in position "*peri*" to the same quinone carbonyl group, but with different side-chains on the alicyclic ring. Compound **VII**, the aglycone of the glycoside Y (**III**), was easily identified as 4-*O*-demethyl-11-deoxydaunomycinone (synonym 11-deoxycarminomycinone) by comparison with an authentic sample previously prepared by 4-*O*-demethylation of 11-deoxydaunomycinone[4]. The lower m.p. value of **VII** previously reported (140~142°C)[4] was affected by a small contamination with the starting material and the metal complex.

The 4-*O*-demethylation products ($AlCl_3$, CH_2Cl_2, 40°C, 2 hours) of the known anthracyclinones[2], 11-deoxyadriamycinone, 11-deoxy-13-dihydrodaunomycinone and 11-deoxy-13-deoxodaunomycinone

Table 3. Chemical and physical properties of aglycones of glycosides W, X, Y and Z.

Properties	Aglycones of glycosides			
	W (V)	X (VI)	Y (VII)	Z (VIII)
Melting point (°C)	138~140	210	167~168	178~180
$[\alpha]_D^{23}$ (*c* 0.1, $CHCl_3$ - MeOH, 1: 1)	+125°	+153°	+160°	+164°
UV and VIS spectra:				
λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	228(800), 260(585), 290sh(222), 431(265)	228(900), 260(605), 290sh(255), 431(300)	228(1040), 260(690), 290sh(272), 431(335)	262(650), 280sh(264), 291(255), 431(312)
Molecular formula	$C_{20}H_{16}O_8$	$C_{20}H_{18}O_7$	$C_{20}H_{16}O_7$	$C_{20}H_{18}O_6$
MW: *m/z* FD (M^+)	384	370	368	354
Identification	4-*O*-Demethyl-11-deoxyadriamycinone	4-*O*-Demethyl-11-deoxy-13-dihydro-daunomycinone	4-*O*-Demethyl-11-deoxydaunomycinone	4-*O*-Demethyl-11-deoxy-13-deoxo-daunomycinone

were found to be indistinguishable from the aglycones **V**, **VI** and **VIII** respectively. Moreover the physical and chemical properties of compound **VIII** are comparable to those reported for 10-demethoxycarbonylaklavinone[9].

The general appearance of the ^1H and ^{13}C NMR spectra of the new aglycones and glycosides indicated a close similarity with those of the corresponding analogues in the 11-deoxydaunomycinone and 11-deoxydaunorubicin series[2,4], the main difference being the absence of the methoxy signal and the presence of an additional phenolic hydroxyl signal. The ^1H NMR spectrum of the glycoside Y (**III**, 4-*O*-demethyl-11-deoxydaunorubicin), as the hydrochloride determined at 80 MHz with a Varian CFT 20 spectrometer in DMSO-d_6, showed significant signals at 1.16 (d, CH_3–C-5′), 2.25 (s, CH_3CO), 5.00 (broad s, C-7–H), 5.30 (broad s, C-1′–H), 7.30~8.0 (m, four aromatic protons), 11.90 (s, C-4–OH) and 12.60 (s, C-6–OH).

Direct chemical transformation of **III** into **I**, **II** and **IV** completed the chemical structure work. The doxorubicin analogue **I** was obtained from **III** *via* the 14-bromoderivative, following a procedure already described for the chemical transformation of daunorubicin to doxorubicin[10]. Reduction ($NaBH_4$, H_2O) of **III** of its 13-tosylhydrazone ($NaBH_4$, AcOH)[11] gave **II** and **IV** respectively. Thus, from all the data presented, the structures of the glycosides X, Y, W and Z are proposed to be respectively 4-*O*-demethyl-11-deoxydoxorubicin (**I**), 4-*O*-demethyl-11-deoxy-13-dihydrodaunorubicin (**II**), 4-*O*-demethyl-11-deoxydaunorubicin (**III**, synonym 11-deoxycarminomycin I) and 4-*O*-demethyl-11-deoxy-13-deoxodaunorubicin (**IV**) as shown in Fig. 1. The structures of the corresponding aglycones (**V**~**VIII**) are represented in Fig. 2.

Biological Activity Data

Antibacterial Activity

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

Antitumor Activity

The cytotoxic activity of the new anthracyclines has been compared to that of daunorubicin, doxorubicin and carminomycin on HeLa cells *in vitro*, and the results are reported in Table 5. As previously

Table 4. Antibacterial activity of glycosides X, Y, W and Z.

Test organism	MIC in μg/ml; glycosides			
	W (I)	X (II)	Y (III)	Z (IV)
Staph. aureus FDA 209 P	25	100	25	100
Micrococcus flavus ATCC 9341	12.5	25	3.12	6.25
B. subtilis ATCC 6633	50	50	12.5	25
E. coli B	12.5	25	6.25	25

observed[12], carminomycin was markedly more cytotoxic than daunorubicin and doxorubicin; it was also more cytotoxic than all the new anthracyclines here investigated. 4-*O*-Demethyl-11-deoxydoxorubicin (I) was found more cytotoxic than daunorubicin and doxorubicin. These data confirm that in the daunorubicin-carminomycin related anthracyclines the corresponding 11-deoxy derivatives are about ten times less cytotoxic than the parent compounds[2] and that the 4-*O*-demethylation increases the cytotoxicity[12].

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 6.25 3.12	38, 31, 22, 24, 6 83, 80, 51, 96, 52 99, 110, 72, 120, 111	7.8
Doxorubicin	12.5 6.25 3.12	42 53 71	9
Carminomycin	12.5 6.25 3.12 1.56	0 0 20 48	1.5
4-*O*-Demethyl-11-deoxy-daunorubicin (III, 11-Deoxy-carminomycin)	100 25 12.5 6.25	0, 0 50, 33, 3 67 89, 80, 75	14
4-*O*-Demethyl-11-deoxy-13-dihydro-daunorubicin (II)	100 25 12.5 6.25	0, 0 0, 1, 35 80, 40 99, 140	13
4-*O*-Demethyl-11-deoxy-doxorubicin (I)	25 12.5 6.25 3.12	0, 0, 2 4, 24 78, 38, 66 85, 98	6.5

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

** Calculated on dose-effect lines.

Table 6. Activity against ascitic leukemias.

Compound***	L1210*			P388**		
	Dose (mg/kg)	T / C (%)	Toxicity	Dose (mg/kg)	T / C (%)	Toxicity
Daunorubicin	2.9 4.4 6.6	144, 150 140, 162 144, 162	0 / 20 1 / 20 3 / 19			
Doxorubicin				6.6 10	231 355	0 / 10 1 / 10
4-*O*-Demethyl-11-deoxydoxorubicin (I)				1 1.5 2.25 3.4 5	159 181, 163 159, 168 190 204	0 / 10 0 / 20 0 / 20 0 / 10 1 / 9
4-*O*-Demethyl-11-deoxy-13-dihydrodaunorubicin (II)	2.9 4.4 6.6 10	125 131 137 109	0 / 8 2 / 10 5 / 10 10 / 10	1 2 4	140 140 100	0 / 8 0 / 8 7 / 8
4-*O*-Demethyl-11-deoxy-daunorubicin (III)	0.8 1.2 1.9 2.9	111 128 122 111	0 / 10 0 / 10 0 / 10 8 / 10	1.2 1.9	145 159	0 / 10 0 / 10

* BDF1 mice received 10^5 ascites cells on day 0, i.p.

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

*** Single treatment i.p. on day 1 after tumor transplantation.

Compounds **I** ~ **III** were also tested against L1210 leukemia, because of its natural relative resistance to anthracyclines and against the P388 leukemia, because of its high sensitivity to anthracyclines, and particularly to doxorubicin[13]. The results are reported in Table 6. 4-*O*-Demethyl-11-deoxydaunorubicin (**III**) and its 13-dihydroderivative (**II**) administered i.p. to L1210 leukemic mice were more toxic and less active than daunorubicin. Contrary to what observed almost constantly during our studies on the relationship between structure and biological activity of several semisynthetic and natural anthracyclines, including the 11-deoxydaunorubicin analogues[2], we did not observe for compounds **II** and **III** a correlation between cytotoxicity *in vitro* and toxicity in experimental animals. 4-*O*-Demethyl-11-deoxydaunorubicin (**III**) and its 13-dihydro derivative (**II**) were found more toxic in mice than what was expected from the *in vitro* data, suggesting either a different pharmacokinetic behavior *in vivo* or a different mechanism of action in respect to daunorubicin and doxorubicin. It is interesting to notice that carminomycin and its 11-*O*-methyl derivative were found to be more cytotoxic *in vitro* on Novikoff hepatoma ascite cells but much less active than doxorubicin as regard to their ability of binding to DNA and inhibiting DNA and RNA synthesis[14].

On the P388 leukemia the new anthracyclines were found less active than doxorubicin, however 4-*O*-demethyl-11-deoxydoxorubicin (**I**) displayed a high potency with a good effectiveness. Its higher activity, when compared to those of the daunorubicin analogues **II** and **III**, confirms the importance of the C-14 hydroxyl group within the daunorubicin-doxorubicin related anthracyclines as regard to the antitumor activity. Compound **I** was also tested against gross leukemia, and the results, reported in Table 7, show that 4-*O*-demethyl-11-deoxydoxorubicin (**I**) was more active than daunorubicin and justify further investigations on the antitumor activity of this new biosynthetic anthracycline.

Table 7. Activity against gross leukemia*.

Compound	Dose** (mg/kg)	T/C (%)	No. of toxic deaths / No. of mice treated
Daunorubicin	10	150	0/10
	15	100	6/10
	22.5	91	7/10
4-*O*-Demethyl-11-deoxydoxorubicin (**I**)	6.6	183	0/10
	10	208	3/10
	15	83	8/10
	22.5	83	10/10

* C3H mice were injected with 2×10^6 leukemia cells i.v.

** Treatment i.v. on day 1 after tumor inoculum.

Acknowledgment

We thank G. D'Amico and G. Ferni for their collaboration in the fermentation and isolation process, E. Arlandini, M. Ballabio, B. Gioia, E. Gandini and A. Vigevani for NMR and mass spectral determination and interpretation, A. Sanfilippo for antibacterial activity data, C. Soranzo and C. Geroni for cytotoxic activity data and Istituto Mobiliare Italiano (I.M.I.) for financial support.

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