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13-DEOXYCARMINOMYCIN, A NEW BIOSYNTHETIC ANTHRACYCLINE

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ABSTRACT.—A new antitumor antibiotic, 13-deoxycarminomycin (**3**), has been isolated from the anthracycline complex produced by *Streptomyces peucetius* var. *carminatus* (ATCC 31502), a biochemical mutant of *Streptomyces peucetius* var. *caesius*, the doxorubicin-producing microorganism. The new anthracycline (**3**), showing antibacterial and cytotoxic activity in vitro, was found active against P-388 murine leukemia.

The outstanding cancer chemotherapeutic efficacy of doxorubicin (**1**, Adriamycin®) and daunorubicin (**2**) has led to an intensive effort to discover new biologically active microbial metabolites within the group of the anthracycline glycosides in our own laboratories (1-4) and elsewhere as recently reviewed (5, 6).

In our continuing search for microorganisms producing new biosynthetic anthracyclines, *Streptomyces peucetius* var. *carminatus*, a new biochemical mutant of *Streptomyces peucetius* var. *caesius* (7, 8), the doxorubicin-producing microorganism, was isolated. The new strain was found to produce an anthracycline complex endowed with antitumor activity.

In this paper, we report the description of the new strain and the production, isolation, structure determination, and biological activity of 13-deoxycarminomycin (**3**), a new antitumor anthracycline (9).

EXPERIMENTAL

GENERAL PROCEDURES.—Melting points, determined with a Büchi SMP-20 apparatus, are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. Ir spectra were recorded on a Perkin-Elmer 457 instrument (KBr disks). ^{1}H-nmr spectra were recorded on a Varian XL-200 spectrometer (200 MHz), in $CDCl_3$, and chemical shifts are reported in ppm downfield from TMS. Mass spectra were performed with a Varian Mat 311-A spectrometer, equipped with ei/fi/fd/ion source.

TAXONOMY.—Strain DR 81 F.I. is a biochemical mutant of *Streptomyces peucetius* var. *caesius*, the doxorubicin-producing microorganism (7). It has been obtained by a mutagenic treatment carried out on a spore suspension of *S. peucetius* var. *caesius* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, according to the method described by Delic *et al.* (10), and selection for daunorubicin resistance. Strain DR 81 F.I. could be isolated among the very few survivors of the mutagenized population plated out on an agar medium supplemented with various concentrations of daunorubicin (**2**).

The microscopic examination of the aerial mycelium of strain DR 81 F.I. showed a morphological identity of this mutant with its parent culture (7) and with the original *S. peucetius* culture (11). The cultural characteristics of mutant DR 81 F.I. can be summarized as follows: growth is generally good on organic as well as on synthetic media; on the former media, the color of the substrate mycelium ranges from bright orange to deep carmine, and the soluble pigment formed shows similar hues. On synthetic media, the color of the substrate mycelium ranges from rose-violet to vinaceous, become brown on aging, while the soluble pigment ranges from vinaceous to violet. The aerial mycelium is most frequently absent, but when formed, its color is very similar to that of the parent culture, i.e., gray with blue-green hues. Con-

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cerning the physiological and biochemical properties, the mutant DR 81 F.I. differs from its parent culture in as much as it grows on L-arabinose and m-inositol and because it produces a new anthracycline glycoside.

Because of all the differences cited above, and owing to its characteristic carmine-colored substrate mycelium and soluble pigment on some media, strain DR 81 F.I. has to be considered a subspecies to the species *S. peucetius*, to which the designation *S. peucetius* var. *carminatus* (ATCC 31502; DSM 1524; FR 14929) has been given.

FERMENTATION.—For the production, strain DR 81 F.I. was grown in 300 ml shaken Erlenmeyer flasks at 28° for 6 days and in an 800-liter stainless steel fermenter on the following medium: (g/liter): glucose, 60; brewer's dry yeast, 30; NaCl, 2; KH_2PO_4, 1; $CaCO_3$, 2; $MgSO_4.7H_2O$, 0.1; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.001; $CuSO_4.5H_2O$, 0.001; tap H_2O up to one liter.

The fermentation was performed at 28°, stirred at 250 rpm. and aereated with an air flow of 0.7 liters/liter of medium per min. At the 24th and 48th hour of fermentation, 0.5 g/liter of sulfanilamide was added each time.

The maximum concentration of the anthracycline complex was reached between the sixth and seventh day of fermentation with a production of 50-70 μg/ml. When samples of fermentation broths or crude extracts were subjected to paper chromatography,[2] followed by bioautography with *Bacillus subtilis* ATCC 6633 as the test organism, a major active constituent could be distinguished from other known anthracyclines, such as carminomycin (**4**) (12) and its 13-dihydroderivative (**5**) (1), daunorubicin (**2**), and doxorubicin (**1**), which are also present as minor constituents.

ISOLATION AND PURIFICATION.—The harvested broth (5 liters) was filtered with filter aid at pH 4, and the mycelium was extracted with Me_2CO-0.1 N aqueous HCl (4:1) (2×5 liters). The extracts, adjusted with concentrated NH_4OH to pH 4, were concentrated to about 2 liters under reduced pressure, combined with the filtered broth, and exhaustively extracted at pH 8.5 with half a volume of $CHCl_3$. The combined organic extracts, washed with H_2O and dried on anhydrous Na_2SO_4, were concentrated under reduced pressure. Addition of PrOH followed by further concentration and dilution with Me_2CO precipitated sulfanilamide, while the crude anthracycline complex was obtained from the mother liquors by further addition of *n*-hexane. A $CHCl_3$ solution of the crude complex was chromatographed on a silica gel column (450×23 mm). Elution of the main constituent was achieved with a $CHCl_3$-MeOH-H_2O mixture (300:55:6). The pooled fractions were concentrated to dryness, and the residue was further purified on a column (450×30 mm) of buffered (pH 5.4, 0.06 M phosphate buffer) cellulose powder using *n*-BuOH-EtOAc(9:1) as eluent. Selected fractions were pooled and extracted with acidic H_2O; the aqueous phase, adjusted to pH 8.5, was then reextracted with $CHCl_3$. The extract, washed with H_2O and dried on anhydrous Na_2SO_4, was concentrated to a small volume.

Addition of methanolic HCl gave a red crystalline precipitate of the new anthracycline, as the hydrochloride, obtained in a yield of 100 mg from 5 liters of culture broth. The minor constituents of the crude complex were isolated from the silica gel chromatography and identified as carminomycin (**4** (12), its 13-dihydroderivative (**5** (1), daunorubicin (**2**), and doxorubicin (**1**) after comparison with authentic samples.

PHYSICAL AND CHEMICAL PROPERTIES.—The new anthracycline (**3**), as the hydrochloride, is a red crystalline compound soluble in H_2O, lower alcohols, and DMSO; mp 173-175° (dec.); $[\alpha]^{23}D$ (c 0.1 in MeOH)+275°. The calculated molecular formula $C_{26}H_{29}NO_9$.HCl (calc.: C, 58.26; H, 5.64; N, 2.61; Found: C, 58.46; H, 5.70; N, 2.54) was confirmed by fd ms *m/z* 500 (MH^+), 499 (M^+), 481 (M-H_2O), 370 (aglycone) and 334 (bisanhydroaglycone). Its uv spectrum, λ max (MeOH) 236, 256, 293, 464 (sh), 495, 512 (sh), and 529 nm ($E^{1\%}_{1\,cm}$ 620, 520, 164, 200, 260, 197, 186), was almost superimposable to that of carminomycin (**4**) (12). Also the ir spectra (KBr) of **3** and **4** were very similar, the main difference being the absence of the acetyl carbonyl peak (1710 cm^{-1}) observed in carminomycin (**4**). ^1H nmr ($CDCl_3$) δ 0.86 (t, J=7.2 Hz, CH_3-14), 1.11 (d, J=6.4 Hz, CH_3-5'), 1.49 (q, J=7.2 Hz, CH_2-13), 1.67 (dd, J=4.6, 14.0 Hz, H-8_{ax}), 1.83 (dt, J=4.1, 12.2 Hz, CH_2-2'), 2.10 (bd, J=<1, 14.0 Hz, H-8_{eq}), 2.40 and 3.00 (two d, J=19.2 Hz, CH_2-10), 3.26 (m, H-3'), 3.54 (bs, H-4'), 3.98 (m-H-5'), 4.93 (bs, H-7), 5.30 (bs, H-1'), 7.10 (d, J=8.3 Hz, H-3), 7.5 (t, J=8.3 Hz, H-2), and 7.66 (d, J=8.3 Hz, H-1). Comparison of the ^1H-nmr spectrum of **3** with that of carminomycin (**4**) indicated a close similarity, the only significant difference being the presence of ethyl protons (0.86 and 1.49 δ) in **3** instead of the acetyl protons (singlet at 2.40 δ) characteristic of the side chain of **4**.

STRUCTURE DETERMINATION.—Acid hydrolysis of **3** (0.2 N aqueous HCl, 95°, 1 h) gave an insol-

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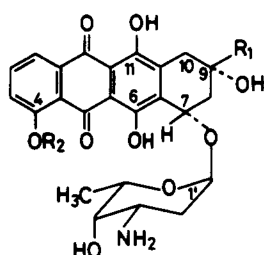
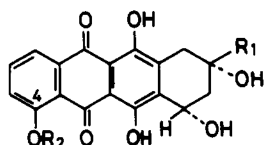
[2]On Whatman No. 1 paper, buffered with M/15 phosphate buffer at pH 5.4, descending system PrOH-EtOAc-H_2O, 7:1:2 (by volume), the new anthracycline (**3**) was found to be less polar (Rf 0.77) than **1**, **2**, **5**, and **4** which have the following Rf values: 0.30, 0.50, 0.60, and 0.65, respectively.

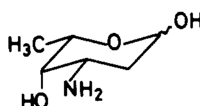
uble red aglycone (**6**) and a soluble aminosugar which was identified as daunosamine (**7**, 3-amino-2,3,6-trideoxyl-L-lyxohexose) by direct comparison with an authentic sample (13). The aglycone (**6**), mp 240° (dec.), showed ir and uv spectra very similar to those of the parent glycoside, and the analytical data indicated a molecular formula $C_{20}H_{18}O_7$, confirmed by eims *m/z* 370 (M^+). These data and its ^{1}H-nmr spectrum ($CDCl^3$), showing the presence of the side-chain ethyl protons at δ 0.92 (t, *J*=7.5 Hz, CH_3-14) and 1.59 (q, *J*=7.5 Hz, CH_2-13) instead of the acetyl protons (δ 2.42, s) of carminomycinone (**8**), allowed us to identify the aglycone as 13-deoxycarminomycinone (**6**). Further evidence was achieved by direct comparison of **6** with the 4-*O*-demethylation product ($AlBr_3$, CH_2Cl_2, 40°, 1 h) of 13-deoxydaunomycinone (**9**) (14). In the new anthracycline (**3**), the site of the glycosidic linkage to the C-7 benzylic position of the aglycone and the α-configuration of the glycosidic linkage were assigned on the basis of the close similarity of the corresponding signals in the ^{1}H-nmr spectra of **3** and **4**. Thus, from all the data presented, the new biosynthetic anthracycline was identified as 13-deoxycarminomycin (**3**).



1 R_1=-CO-CH_2OH, R_2=-CH_3 doxorubicin
2 R_1=-CO-CH_3, R_2=-CH_3 daunorubicin
3 R_1=-CH_2-CH_3 (13), R_2=-H 13-deoxycarminomycin
4 R_2=-CO-CH_3 (13), R_2=-H carminomycin
5 R_1=-CHOH-CH_3, R_2=-H 13-dihydrocarminomycin



6 R_1=-CH_2CH_3, R_2=-H
8 R_1=-$COCH_3$, R_2=-H
9 R_1=-CH_2CH_3, R_2=-CH_3



7 daunosamine

Biological activity data.—The new anthracycline (**3**) displayed antibacterial activity. The standard tube dilution procedure was used to determine the minimum inhibitory concentration (MIC) of 13-deoxycarminomycin for some microorganisms. Results are given in Table 1.

Table 1. Antibacterial Activity of 13-Deoxycarminomycin (**3**).

Test Organism	Minimal inhibitory concentration (μg/ml)
Bacillus subtilis ATCC 6633	6.25
Staphylococcus aureus 209 P (FDA) ATCC 6538-P	12.5
Staphylococcus aureus 153[a]	25.0
Escherichia coli B ATCC 11303	3.12
Sarcina lutea ATCC 9341	1.56

[a]Clinical isolate of our collection, resistant to several antibiotics and producer of β-lactamase.

Cytotoxicity and antitumor activity in vivo: The in vitro activity of 13-deoxycarminomycin (**3**) was compared with that of daunorubicin (**2**) and carminomycin (**4**) in the colony inhibition test on HeLa cells, and the results are reported in Table 2. Their cytotoxic activity against P388 leukemia cells sensitive (P 388/S) and resistant (P 388/Dx) to doxorubicin is shown in Table 3. The activity of **3** against P-388 ascitic leukemia in mice in comparison with that of daunorubicin (**2**) is reported in Table 4.

TABLE 2. Effect on HeLa Cells Colony Formation[a]

Compound	Dose (ng/ml)	%[b]	ID_{50} (ng/ml)
Daunorubicin (**2**)	25	13	
	12.5	48, 39, 47	12.5
	6.2	104, 123, 82	
Carminomycin (**4**)	12.5	0	
	6.2	3	
	3.1	33	3.1
	1.5	120	
13-Deoxycarminomycin (**3**)	25	0, 0	
	12.5	14, 36, 2	
	6.2	48, 55	6.2
	3.1	63, 90	
	1.5	126, 95	

[a]Data of three experiments; treatment for 24 h.
[b]No. of colonies/plate, percent over untreated controls.

TABLE 3. Effect on P-388 Leukemia Cells Sensitive (P-388/S) and Resistant (P-388/DX) to Doxorubicin[a]

Compound	ID_{50} (ng/ml)	
	P-388/S	P-388/Dx
Daunorubicin (**2**)	9.5	750
Carminomycin (**4**)	7.0	4
13-Deoxycarminomycin (**3**)	1.5	4

[a]Treatment for 48 h.

TABLE 4. Effect against P-388 Leukemia in Mice

Compound	Dose[a] (mg/kg)	T/C[b]	Toxic deaths
Daunorubicin (**2**)	2.9	177	0/10
	4.4	190	0/7
	6.6	168	3/9
13-Deoxycarminomycin[c] (**3**)	0.4	136	0/10
	0.6	150	0/10
	1	163, 154	1/20
	1.5	109	10/10

[a]Treatment i.p. on day one.
[b]Median survival time of treated mice/median survival time of controls, × 100.
[c]Data of two experiments.

RESULTS AND DISCUSSION

A new biochemical mutant of *S. peucetius* var. *caesius*, the doxorubicin-producing microorganism (7, 8), designated *S. peucetius* var. *carminatus* (ATCC 31502), was found to produce an anthracycline complex whose major constituent was identified as 13-deoxycarminomycxin (**3**). This new biosynthetic anthracycline, although postulated (15, 16) as an intermediate in the biosynthesis of daunorubicin (**2**) and carminomycin (**4**), has never before been isolated, as far as we know. Recently two related anthracyclines, feudomycin A (4-*O*-methyl-derivative of **3**) and akrobomycin (9,10-anhydro derivative of **3**), have been isolated from cultures of a mutant of *Streptomyces coeruleorubidus* (17) and *Actinomadura roseo-violacea* (18), respectively. The aglycone of **3**, 13-deoxycar-

minomycinone (**6**), also named 10-decarbomethoxy-ε-rhodomycinone (15) or 10-deoxy-β-rhodomycinone (16), was isolated after acid hydrolysis of an anthracycline complex produced by *Streptomyces griseoruber* 4620 (19).

The biological activity of 13-deoxycarminomycin (**3**) was compared with that of daunorbicin (**2**) and carminomycin (**4**) in several experimental systems. In the colony-inhibition test on HeLa cells in vitro, **3** was found more active than **2** but less active than **4**, as shown in Table 2. In a recently developed test for detection of cytotoxic activity in vitro against P-388 leukemia cells sensitive (P-388/S) or resistant (P-388/DX) to doxorubicin (**1**), both **3** and **4** showed high activity on P-388/DX, which is cross-resistant to most anthracyclines; on P-388/S. **3** was more cytotoxic than **2** and **4**, as shown in Table 3. Against P-388 ascitic leukemia in mice, as reported in Table 4, **3** was found about four times more toxic and more potent than **2**, even if slightly less active than **2** at the optimal dose of 1 mg/kg.

In conclusion, 13-deoxycarminomycin (**3**) is a new biosynthetic anthracycline showing increased potency against P-388 murine leukemia in respect to daunorubicin and being endowed with high cytotoxic activity against P-388 leukemia cells resistant to doxorubicin.

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LITERATURE CITED

1. G. Cassinelli, A. Grein, P. Masi, A. Suarato, L. Bernardi, F. Arcamone, A. Di Marco, A.M. Casazza, G. Pratesi, and S. Soranzo, *J. Antibiotics*, **31**, 178 (1978).
2. A. Grein, S. Merli, and C. Spalla, *J. Antibiotics*, **33**, 1462 (1980).
3. G. Cassinelli, F. Di Matteo, S. Forenza, M.C. Ripamonti, G. Rivola, F. Arcamone, A. Di Marco, A.M. Casazza, C. Soranzo, and G. Pratesi, *J. Antibiotics*, **33**, 1468 (1980).
4. G. Cassinelli, G. Rivola, D. Ruggieri, F. Arcamone, A. Grein, S. Merli, C. Spalla, A.M. Casazza, A. Di Marco, and G. Pratesi, *J. Antibiotics*, **35**, 176 (1982).
5. F. Arcamone, "Doxorubicin," in: Medicinal Chemistry Series, Vol. 17. New York: Academic Press, 1980, p. 300.
6. T. Oki, "Microbial Transformation of Anthracycline Antibiotics and Development of New Anthracyclines," in: "Anthracycline Antibiotics," Ed. by H.S. El Khadem, New York: Academic Press, 1982, p. 75.
7. F. Arcamone, G. Cassinelli, G. Fantini, A. Grein, P. Orezzi, C. Pol, and C. Spalla, *Biotechnol. Bioeng.*, **11**, 1101 (1969).
8. A. Grein, *Process Biochem.*, **16**, 34 (1981).
9. Farmitalia Carlo Erba, British Patent 2.048.245 (March 16, 1983, filed April 27, 1979).
10. V. Delic, D.A. Hopwood, and A.J. Friend, *Mutation Res.*, **9**, 167 (1970).
11. A. Grein, C. Spalla, A. Di Marco, and G. Canevazzi, *Giorn. Microbiol.*, **11**, 109 (1963).
12. M.G. Brazhnikova, V.B. Zbarsky, V.I. Ponomarenko, and N.P. Potapova, *J. Antibiotics* **27**, 254 (1974).
13. F. Arcamone, G. Cassinelli, P. Orezzi, G. Franceschi, and R. Mondelli, *J. Am. Chem. Soc.*, **86**, 5335 (1964).
14. T.H. Smith, A.N. Fujiwara, and D.W. Henry, *J. Med. Chem.*, **21**, 280 (1978).
15. A. Yoshimoto, T. Oki, and H. Umezawa, *J. Antibiotics*, **33**, 1199 (1980).
16. Z. Vanek, J. Mateju, J. Cudlin, M. Blumanerova, P. Sedmera, J. Jizba, E. Kralovcova, J. Tax, and G.F. Gauze, in: "Overproduction of Microbial Products," Ed. by V. Krumphanzl, B. Sikyta, and Z. Vanek, chap. 23, London: Academic Press, 1982, pp. 283-299.
17. T. Oki, Y. Matsuzawa, K. Kiyoshima, A. Yoshimoto, H. Naganawa, T. Takeuchi, and H. Umezawa, *J. Antibiotics*, **34**, 783 (1981).
18. K. Imamura, A. Odagawa, K. Tanabe, Y. Hayakawa, and N. Otake, *J. Antibiotics*, **37**, 83 (1984).
19. V. Prikrylova, M. Podojil, P. Sedmera, J. Vokoun, Z. Vanek, M.G. Brazhnikova, and M.K. Kidinova, *Coll. Czech. Chem. Commun.*, **45**, 1991 (1980).

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