NEW ANTHRACYCLINONE METABOLITES FROM TWO BLOCKED MUTANTS OF *STREPTOMYCES GALILAEUS* MA144-M1

HIROYASU TOBE, AKIHIRO YOSHIMOTO, TOMOYUKI ISHIKURA

Central Research Laboratories, Sanraku-Ocean Co., Ltd.
9–1 Johnan 4-chome, Fujisawa, Kanagawa 251, Japan

HIROSHI NAGANAWA, TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry, 14–23 Kamiosaki 3-chome,
Shinagawa-ku, Tokyo 141, Japan

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Two blocked mutants of the aclacinomycin-producing *Streptomyces galilaeus* MA144-M1 produced new anthraquinones and anthracyclinones. The mutant ANR-58 produced compounds 58A, 58B, 58C (7-deoxy-2-hydroxyaklavinone), 58D (2-hydroxyaklavinone) and 58WR. All these compounds have the 2-hydroxyl group. The mutant ANR-665 produced compounds 665A and 665B. The compounds 58A, 58B and 665A have an anthraquinone skeleton.

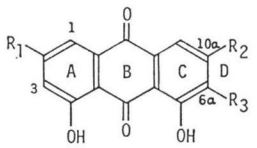
As reported previously[1], we have isolated several unique blocked mutants from *S. galilaeus* MA144-M1 by successive UV or NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) mutation. We obtained two mutants ANR-58 and ANR-665 from the mutant 3AR-33 which accumulates aklavinone. In a previous paper[2], we reported on the structures of three anthracyclinones designated as 58C, 58D and 58G produced by the mutant ANR-58. In this paper, we report the structures of 58A, 58B and 58WR produced by the mutant ANR-58, and 665A and 665B produced by the mutant ANR-665.

Results and Discussion

The structures of the-five compounds (58A, 58B, 58WR, 665A and 665B) were determined by analysis of their IR, UV, ^{1}H NMR, ^{13}C NMR and mass spectra. The structures of the compounds are shown in Table 1. The compounds 58A, 58B and 665A are anthraquinones, and 58WR and 665B are anthracyclinones. The compounds 58A, 58B, and 58WR have a hydroxyl group at C-2 (anthracycline numbering) as have 58C (7-deoxy-2-hydroxyaklavinone) and 58D (2-hydroxyaklavinone) reported in a previous paper[2]. The compound 665A was identified by ^{13}C NMR, ^{1}H NMR, IR and mass spectra as 1,8-dihydroxy-3-methoxycarbonylmethyl-2-(1-oxopropyl)-9,10-anthraquinone, which had been reported by KLALOVCOVA *et al.*[3]. On the other hand, the compound 665B had a new structure as later described.

The compound 58A had similarities to 665A in ^{13}C NMR spectra as shown in Table 2. The structure of 58A was determined by comparison of its ^{1}H NMR, ^{13}C NMR, IR and mass spectra with those of 665A. The mass spectrum of 58A suggested an additional oxygen atom compared to 665A ($C_{20}H_{16}O_7$). The ^{13}C NMR spectrum of 58A (Table 2) suggested that the additional oxygen atom was present as an hydroxyl group at C-2. In the ^{1}H NMR spectrum two of three aromatic protons in 58A molecule were determined to be in the *meta* position to each other by the presence of two doublet peaks (δ 7.07 and 6.50) with a small coupling constant (J=2.0 Hz). The other one singlet proton (δ 7.59) corresponded to C-11. We determined the structure of 58A as 1,6,8-trihydroxy-3-methoxycarbonylmethyl-2-(1-oxopropyl)-

Table 1. The structures of anthraquinones and anthracyclinones produced by the mutant strains ANR-58 and ANR-665.



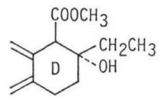
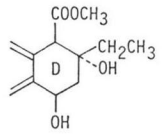
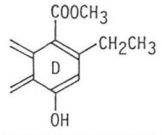
Compound	R_1	R_2	R_3
58A	OH	$^{10}-CH_2-^{15}COOCH_3$	$^{9}-CO-^{13}CH_2CH_3$
665A	H	"	"
58B	OH	"	$^{7}-CO-^{8}CH_2-^{9}CO-^{13}CH_2CH_3$
58C	OH		
58D	OH		
58WR	OH		
665B	H		

Table 2. The ^{13}C NMR* chemical shifts of the anthraquinones**.

Carbon	58A (in DMSO-d_6)	665A (in $CDCl_3$)	58B (in $CDCl_3$ & CD_3OD)
1	107.9	120.3	108.9
2	158.1	137.6	159.9
3	109.2	125.0	110.4
4	164.5	159.9	165.7
4a	108.9	115.2	110.6
5	189.3	192.7	190.4
5a	115.0	115.7	115.6
6	166.0	162.7	166.4
6a	135.7	136.5	135.4
7	—	—	195.8
8	—	—	31.9
9	205.5	205.9	183.8
10	37.8	38.7	39.6
10a	139.8	141.5	141.6
11	121.6	122.4	122.2
11a	132.7	133.4	133.9
12	180.6	180.9	181.9
12a	134.8	133.4	135.3
13	36.6	37.4	37.2
14	7.4	7.7	9.6
15	170.0	170.2	171.1
16	51.9	52.4	52.5

* Tetramethylsilane was used as internal standard.
** The numbering of the carbon atoms is based on the anthracyclinone numbering.

9,10-anthraquinone. The off-resonance multiplicities in the ^{13}C NMR spectrum and the fragmentation pattern of 58A in its mass spectrum supported this structure. The compound 58A may be biosynthesized from one acetate unit shorter than 9 acetate units of a decaketide[3].

The structure of 58B was determined by the same method as 58A. The analysis of its IR, ^1H NMR and ^{13}C NMR spectra indicated that the compound 58B had also 1,6,8-trihydroxyanthraquinone skeleton. The presence of a singlet signal (δ 3.84) of a methylene at C-10 in the ^1H NMR spectrum suggested that the anthracyclinone ring D is open. Its ^{13}C NMR spectrum contained two more carbons (31.9 and 183.8 ppm) than that of 58A. The ^1H NMR spectrum of 58B contained one more singlet methylene (δ 3.88) than that of 58A. The value of M^+ of 58B in its mass spectrum is larger than that of 58A by 42 mass units which corresponds to a $-CO-CH_2-$ fragment. The presence of absorption at 1700 cm^{-1} in its IR spectrum confirmed the presence of carbonyl groups in this moiety. This side chain was suggested to have a double bond which was connected to an ethyl methylene since the chemical shift of the ethyl methylene shifted downfield. The above data indicated that the side chain should be $-CO-CH_2-CO-CH_2CH_3$ and that the side chain was attached to C-6a. The other side chain ($-CH_2-COOCH_3$) was attached to C-10a on the ring C. Thus the structure of 58B ($C_{22}H_{18}O_9$) was proposed as shown in Table 1.

The compound 58WR had a 5,12-naphthacenequinone skeleton. In the ^1H NMR spectrum of 58-WR, the signals of four aromatic protons, a carbomethoxyl group (δ 3.98) and an ethyl methylene shifted downfield were observed. Two aromatic protons (δ 7.18 and 6.60) were *meta* coupled system (J= 2.0 Hz). The other two singlet protons at δ 7.85 and 6.96 were assigned to C-11 and C-8, respectively. Therefore the structure of 58WR was determined to be 2,7-dihydroxy-bis-anhydroaklavinone. The fragmentation pattern of the mass spectrum supported the structure.

The parent peak (m/z 392) of 665B in its mass spectrum showed that its structure lacked an oxygen atom compared that of 58WR. The ^1H NMR spectrum of 665B showed that the coupling system of three protons of the ring A coincided with that of 665A. The other two singlet protons of δ 8.38 and 6.90 were assigned to C-11 and C-8, respectively. Therefore the structure of 665B was determined to be 7-hydroxy-bis-anhydroaklavinone. The fragmentation pattern of its mass spectrum supported the structure. A tri-acetyl derivative of 665B was obtained by the treatment of 665B with pyridine and acetic anhydride. The ^1H NMR spectrum of tri-acetyl 665B showed the presence of three acetyl groups. In its IR spectrum the absorption band at 1765 cm^{-1} also showed the presence of acetyl groups. The fragmentation pattern of its mass spectrum supported the structure of tri-acetyl 665B.

We have already reported that the aklavinone skeleton is built up from nine acetate units and one propionate unit[4]. All the compounds produced by the mutant ANR-58 had a hydroxyl group at C-2. It can be said that this C-2 hydroxyl group came from the carbonyl oxygen which existed in a hypothetical decaketide[5] in aklavinone biosynthesis. Thus, the mutants ANR-58 and ANR-665 were shown to be impaired in different steps of aklavinone biosynthesis. It is possible that some of the anthracyclinones produced by these two blocked mutants ANR-58 and ANR-665 may be precursors in aklavinone biosynthesis.

Experimental

The melting points were determined by a Yanagimoto melting point apparatus. Ultraviolet and visible spectra were measured by a Hitachi model 200-20 spectrophotometer. IR spectra were measured in KBr pellets using a Hitachi 260-30 infrared spectrophotometer. ^1H NMR and ^{13}C NMR spectra were measured by a Varian XL-100A spectrometer and the chemical shifts are expressed in values (ppm) with tetramethylsilane as an internal standard. Mass spectra were measured by a Hitachi RMU-6M spectrometer. TLC was carried out on a silica gel $60F_{254}$ plate (E. Merck Co.).

Microorganisms and Cultivation

The mutant strains ANR-58 and ANR-665 were maintained on YS agar slants (0.3% yeast extract, 1.0% soluble starch, 1.5% agar, pH 7.2). The composition of fermentation medium and cultural conditions for the mutant ANR-665 in Erlenmeyer flasks were described previously[1].

The fermentation of the mutant ANR-58 was carried out using a 30-liter jar fermentor containing 15 liters of the following medium: 1.5% soluble starch, 1% glucose, 3% soy bean meal, 0.1% yeast extract, 0.3% NaCl, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0008% $MnCl_2 \cdot 4H_2O$ and 0.0002% $ZnSO_4 \cdot 7H_2O$, pH 7.4. The seed culture was prepared in a 500-ml Erlenmeyer flask containing 100 ml of the same medium. The inoculum size for the jar fermentation was 2%. The jar fermentation was done with an aeration of 30 liters per minute under agitation at 300 rpm for 66 hours at 28°C.

Isolation and Purification of Products

The cultured broth (64 liters) of the mutant ANR-58 was centrifuged and the mycelium was extracted with acetone. The acetone layer was concentrated *in vacuo* to a small volume and re-extracted with $CHCl_3$. The $CHCl_3$ layer was concentrated *in vacuo*. The residue was dissolved in MeOH and 100 g

of silica gel (E. Merck, Art. 7734) was added, mixed and dried *in vacuo*. The solid mixture thus prepared was placed on the top of a silica gel column (5×55 cm) equilibrated with benzene - acetone - AcOH (200: 30: 1, v/v), and the chromatography was developed with the same solvent system. The five anthracyclinones were eluted in the following series; 58A, 58B, 58C, 58D and 58WR. The compounds 58C (350 mg) and 58D (1,150 mg) were crystallized from acetone and $CHCl_3$ - MeOH mixture, respectively. The compounds 58A (50 mg) and 58B (30 mg) were crystallized from $CHCl_3$ - MeOH mixture. The compound 58WR was not satisfactorily purified by the chromatography. The compound 58WR was further purified by the method of preparative silica gel (E. Merck, Art. 7747) thin-layer chromatography developed with $CHCl_3$ - MeOH - NH_3 (90: 10: 1, v/v) and by Sephadex LH-20 column (2.8×41.5 cm) chromatography with MeOH as eluent. The wine-red needles of 58WR (75 mg) were obtained from $CHCl_3$ - MeOH mixture.

The cultured broth (16 liters) of the strain ANR-665 obtained by rotary flask fermentation was centrifuged and the mycelium was extracted with acetone and $CHCl_3$ in the same manner as with the mutant ANR-58. The $CHCl_3$ layer was concentrated *in vacuo* and the residue was washed with MeOH. The MeOH soluble fraction was applied to silica gel (E. Merck, Art. 7734) column (7×16 cm) chromatography which was developed with $CHCl_3$ - MeOH (100: 1, v/v). The yellow anthracyclinones were eluted. The crystals of 665A from $CHCl_3$ - MeOH mixture weighed 40 mg. On the other hand, the MeOH insoluble fraction was washed with $CHCl_3$ again and was dissolved in warmed dioxane. The dioxane solution was cooled at room temperature and 450 mg of deep-red needles (665B) were obtained.

58A: yellow needles, mp 250~252°C, ν_{max}^{KBr} cm^{-1} 3270, 1730, 1700, 1620, 1470, 1320, 1260, 1240. $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 442 (325), 290 (640), 250 (sh., 625), 230 (855). ^1H NMR (DMSO-d_6) δ in ppm 12.5~11.5 (2H, broad s, hydrogen bonded hydroxyl group ×2), 7.59 (1H, s, H-11), 7.07 (1H, d, J=2.0, H-1), 6.5 (1H, d, J=2.0, H-3), 3.8 (2H, s, H-10), 3.6 (3H, s, OCH_3), 2.92 (2H, q, J=7.0, CH_2-13), 1.0 (3H, t, J=7.0, CH_3-14). MS (% of relative intensity, assignment): m/z 384 (27.0, M^+, $C_{20}H_{16}O_8$), 353 (5.6, M^+-OCH_3), 355 (24.7, $M^+-CH_2CH_3$), 323 (100, $M^+-C_2H_5-CH_3OH$), 267 (15.7, $M^+-C_2H_5-CH_3OH-CO\times2$).

58B: yellow needles, mp 208~210°C, ν_{max}^{KBr} cm^{-1} 3270, 2950, 1700, 1620, 1600, 1470, 1420, 1330, 1260, 1240, 1160, 1100, 760. $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 440 (180), 287 (435), 235 (355), 203 (360). ^1H NMR ($CDCl_3$ and CD_3OD) δ in ppm 7.66 (1H, s, H-11), 7.20 (1H, d, J=2.0, H-1), 6.50 (1H, d, J=2.0, H-3), 3.88 (2H, s, H-8), 3.84 (2H, s, H-10), 3.72 (3H, s, OCH_3), 2.48 (2H, q, J=7.0, CH_2-13), 1.22 (3H, t, J=7.0, CH_3-14). MS m/z 426 (23.2, M^+, $C_{22}H_{18}O_9$), 408 (80.4, M^+-H_2O), 397 (42.9, $M^+-CH_2CH_3$), 393 (15.2, $M^+-H_2O-CH_3$), 377 (42.9, $M^+-H_2O-OCH_3$), 370 (42.9, $M^+-CO\times2$), 369 (42.9, $M^+-CH_2CH_3-CO$), 365 (17.9, $M^+-CH_2CH_3-CH_3OH$), 355 (29.5, $M^+-CH_2CH_3-CO-CH_2$), 348 (41.1, $M^+-H_2O-OCH_3-CH_2CH_3$), 323 (100.0, $M^+-CH_2CH_3-CH_3OH-CH_2CO$), 267 (17.9, $323-CO\times2$).

58WR: wine-red needles, mp 293~295°C (dec.), ν_{max}^{KBr} cm^{-1} 3275, 1725, 1620, 1570, 1410, 1285, 1190. $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 520 (295), 300 (sh., 275), 275 (340), 244 (380). ^1H NMR (DMSO-d_6) δ in ppm 7.85 (1H, s, H-11), 7.18 (1H, d, J=2.0, H-1), 6.96 (1H, s, H-8), 6.60 (1H, d, J=2.0, H-3), 3.98 (3H, s, OCH_3), 2.65 (2H, q, J=7.0, CH_2-13), 1.2 (3H, t, J=7.0, CH_3-14). MS m/z 408 (100, M^+, $C_{22}H_{16}O_8$), 393 (41.1, M^+-CH_3), 377 (82.2, M^+-OCH_3), 350 (23.3, $M^+-COOCH_3$).

665B: deep-red needles, mp 250°C (dec.), ν_{max}^{KBr} cm^{-1} 3400, 2970, 1720, 1675, 1620, 1575, 1460, 1440, 1375, 1270, 1250, 1195, 1150. $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 526 (24.5), 259 (99), 208 (148). ^1H NMR (pyridine-d_5) δ in ppm 8.38 (1H, s, H-11), 7.86 (1H, dd, J=2.0, 8.0, H-1), 7.42 (1H, t, J=8.0, H-2), 7.12 (1H, dd, J=2.0, 8.0, H-3), 6.90 (1H, s, H-8), 3.94 (3H, s, OCH_3), 2.70 (2H, q, J=7.0, CH_2-13), 1.20 (3H, t, J=7.0, CH_3-14). MS m/z 392 (100, M^+, $C_{22}H_{16}O_7$), 377 (29.3, M^+-CH_3), 361 (50.0, M^+-OCH_3), 333 (7.3, $M^+-COOCH_3$).

Acetylation of 665B

A solution of 665B (120 mg) in dry-pyridine (3 ml) and acetic anhydride (1 ml) was stirred overnight at room temperature. The reaction mixture was poured onto ice water and a yellow precipitate was obtained. The pale yellow crystals (100 mg) were obtained from $CHCl_3$, mp 220°C (dec.), ν_{max}^{KBr} cm^{-1} 3400, 2940, 1765, 1720, 1670, 1600, 1584, 1400, 1360, 1250, 1190. $\lambda_{max}^{90\%MeOH}$ nm ($E_{1cm}^{1\%}$) 394 (150), 284 (450), 253 (775), 238 (745), 203 (1310). ^1H NMR ($CDCl_3$) δ in ppm 8.64 (1H, s, H-11), 8.18 (1H, dd,

J=2.0, 8.0, H-1), 7.68 (1H, t, J=8.0, H-2), 7.34 (1H, dd, J=2.0, 8.0, H-3), 7.22 (1H, s, H-8), 4.11 (3H, s, OCH_3), 2.80 (2H, q, J=7.0, H-13), 2.56 (3H, s, CH_3CO), 2.43 (6H, s, $CH_3CO\times2$), 1.30 (3H, t, J=7.0, H-14). MS m/z 518 (2.1, M^+, $C_{28}H_{22}O_{10}$), 476 (21.1, M^+-CH_3CO), 434 (54.7, $M^+-CH_3CO\times2$), 392 (100.0, $M^+-CH_3CO\times3$), 377 (9.5, $M^+-CH_3CO\times3-CH_3$), 361 (20.0, $M^+-CH_3CO\times3-OCH_3$).

References

1) YOSHIMOTO, A.; Y. MATSUZAWA, T. OKI, T. TAKEUCHI & H. UMEZAWA: New anthracycline metabolites from mutant strains of *Streptomyces galilaeus* MA144-M1. I. Isolation and characterization of various blocked mutants. J. Antibiotics 34: 951~958, 1981
2) MATSUZAWA, Y.; A. YOSHIMOTO, N. SHIBAMOTO, H. TOBE, T. OKI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: New anthracycline metabolites from mutant strains of *Streptomyces galilaeus* MA144-M1. II. Structure of 2-hydroxyaklavinone and new aklavinone glycoside. J. Antibiotics 34: 959~964, 1981
3) KRALOVCOVA, E.; P. SEDMERA, J. VOKOUN & Z. VANEK: Anthraquinones related to anthracyclinones from the mutant strain *Streptomyces galilaeus* J-14. Coll. Czech. Chem. Commun. 45: 2558~2565, 1980
4) KITAMURA, I.; H. TOBE, A. YOSHIMOTO, T. OKI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Biosynthesis of aklavinone and aclacinomycin. J. Antibiotics 34: 1498~1500, 1981
5) OLLIS, W. D. & I. O. SUTHERLAND: The incorporation of propionate in the biosynthesis of ε-pyrromycinone (rutilantinone). Proc. Chem. Soc. (London) 347: 34~38, 1960