JOURNAL OF BACTERIOLOGY, Aug. 1986, p. 581–586
0021-9193/86/080581-06$02.00/0

Vol. 167, No. 2

Anthracycline Metabolites of Tetracenomycin C-Nonproducing *Streptomyces glaucescens* Mutants

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Received 27 February 1986/Accepted 9 May 1986

Mutants of *Streptomyces glaucescens* GLA.0 which are blocked in the production of tetracenomycin C (compound 1), an anthracycline antibiotic having significant antitumor activity, accumulated several new anthracycline metabolites structurally related to compound 1 and to intermediates of its biosynthetic pathway. Through chemical and spectroscopic comparisons with the known anthracycline metabolites of the wild-type strain, we identified the two regioisomers of tetracenomycin B2 (compounds 7a and 7b), 8-demethyl-tetracenomycin C (compound 12), tetracenomycin D2 (compound 11), tetracenomycin E (compound 13), and the 12-naphthacenone forms of compounds 7a, 7b, and 2 (tetracenomycin D1). A hypothetical biosynthetic pathway to compound 1 is presented that is consistent with the occurrence of compounds 7b, 13, and 5 (tetracenomycin A2) and with the cosynthetic behavior of tetracenomycin C-nonproducing mutants (H. Motamedi, E. Wendt-Pienkowski, and C. R. Hutchinson, J. Bacteriol. 167:575–580, 1986).

We have been studying the biochemistry and genetics of antibiotic production by *Streptomyces* spp. and intend to use genetic engineering methods to facilitate the acquisition and study of pathway enzymes or to create new antibiotics that are structural composites of existing bacterial metabolites (C. R. Hutchinson, Nat. Prod. Rep., in press). The use of antibiotic biosynthesis genes is one of the keystones of this approach, and their identification by complementation of mutations which specifically affect antibiotic biosynthesis is a principal method by which they can be cloned (Hutchinson, in press). Consequently, an essential requirement for such work is a set of bacterial strains whose phenotypes permit a clear distinction among the types of mutations affecting (blocking) antibiotic production by the parent microorganism.

In this paper we describe the "chemical phenotypes" of *Streptomyces glaucescens* mutants blocked in the production of tetracenomycin C (TcmC) (compound 1), an anthracycline secondary metabolite with significant antitumor properties (6, 7; J. Siebers, Ph.D. dissertation, University of Göttingen, Göttingen, Federal Republic of Germany, 1979). This information supports a hypothesis for the sequence of steps in the biosynthetic pathway to compound 1 and has been invaluable in the characterization of TcmC-nonproducing (TcmC$^-$) mutants, as described in the accompanying paper (4).

MATERIALS AND METHODS

Abbreviations. The abbreviations used for the various compounds are as follows: tetracenomycin D (TcmD) (compound 2), tetracenomycin B1 (TcmB1) (compound 3), tetracenomycin B2 (TcmB2) (compund 4), tetracenomycin A2 (TcmA2) (compound 5), elloramycin (compound 6), TcmB2 regioisomer 7a (compound 7a), TcmB2 regioisomer 7b (compund 7b), 5-deoxy-tetracenomycin B2 regioisomer 8 (compound 8), 5-deoxy-tetracenomycin D1 (compound 9), 5-deoxy-tetracenomycin B2 regioisomer 10 (compound 10), tetracenomycin D2 (TcmD2) (compound 11), 8-demethyl-tetracenomycin C (compound 12), and tetracenomycin E (TcmE) (compound 13).

General. All solvents and reagents were used as obtained from commercial sources. Solvents were evaporated on a rotary evaporator at <37°C under water aspirator vacuum. Thin (0.25 mm)- and preparative (2 mm)- layer chromatography analyses were carried out with Kieselgel 60 (Macherey and Nagel). High-performance liquid chromatography (HPLC) was done on a Waters ALC 201 chromatograph with a 5-μm analytical C-18 reversed-phase column from the same manufacturer and with acetonitrile-methanol-water (45:5:50) at a 1-ml/min flow rate. Melting points were uncorrected when they could be determined. Infrared (IR) spectra were determined in KBr pellets, and the data are given per centimeter. UV spectra were taken in methanol, methanol plus 1 drop of concentrated HCl, or methanol plus 1 drop of 4 N NaOH, and the absorbance maximum data are given in nanometers. Nuclear magnetic resonance (NMR) spectra were determined at 200 or 270 MHz in chloroform-d_1 containing just enough dimethyl sulfoxide-d_6 to dissolve the compound; the chemical shift data are given in parentheses (see below) in parts per million relative to $CHCl_3$ at δ 7.24 with s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and J = coupling constant in hertz. Mass spectra (MS) were determined at 35 eV on a Finnegan 4000 mass spectrometer (low resolution) or on an AEI MS-80 mass spectrometer (high resolution); the MS data are expressed as m/z of parent (M^+) or fragment ion, and the percent relative abundance as compared with the base peak is shown in parentheses (see below).

Bacterial strains and fermentation conditions. Bacterial strains and fermentation conditions are described in the accompanying paper (4).

Isolation and characterization of anthracycline metabolites. All of the anthracycline metabolites were isolated from 50- or 500-ml cultures at the stationary growth stage by extracting the fermentation mixture with ethyl acetate (2 equal volumes) and then drying the combined ethyl acetate ex-

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tracts over solid anhydrous Na_2SO_4 and evaporating them to dryness. The crude residue was chromatographed on silica gel, Sephadex LH20 or C-18 reversed-phase HPLC adsorbants by using the solvent(s) specified in parentheses (see below) to prepare the analytical sample. Standards of compound 1, compound 5, and 3-demethoxy-3-ethoxytetracenomycin C for comparison were obtained from A. Zeeck, University of Göttingen.

(i) Compound 7a. Compound 7a was isolated from strain GLA.4-48 (*tcm*II-22) (4) and purified by chromatography on silica gel plates (chloroform-methanol, 9:1) followed by column chromatoraphy on silica gel (chloroform-ethyl acetate, 5:1). NMR spectra: 14.65 (s, 1 H, aromatic OH), 12.20 (s, 1 H, aromatic OH), 7.90 (s, 1 H, H-6), 7.20 (d, J = 2.5, 1 H, H-4), 7.00 (s, 1 H, H-7), 6.51 (d, J = 2.5, 1 H, H-2), 3.80 (s, 6 H, C-8 and C-9 OCH_3), and 2.70 (s, 3 H, C-10 CH_3). MS: 408 (58, M^+), 393 (30, $M^+ - CH_3$), 377 (16, $M^+ - OCH_3$), 348 (38), and 44 (100).

(ii) Compound 7b. Compound 7b was isolated from strain GLA.12-41 (*tcm*IV-30) (4) and purified by chromatography on silica gel plates (chloroform-methanol, 9:1). NMR spectra: 14.67 (s, 1 H, aromatic OH), 12.35 (s, 1 H, aromatic OH), 7.85 (s, 1 H, H-6), 7.23 (d, J = 2.5, 1 H, H-4), 7.10 (s, 1 H, H-7), 6.55 (d, J = 2.5, 1 H, H-2), 3.86 (s, 3 H, OCH_3), 3.80 (s, 3 H, OCH_3), and 2.71 (s, 3 H, C-10 CH_3). MS: 408 (23, M^+), 376 (57, $M^+ - CH_3OH$), 320 (60), and 44 (100).

(iii) Compound 8. Compound 8 was isolated from strain GLA.4-48 (*tcm*II-22) (4) and purified by chromatography on silica gel plates (chloroform-methanol, 9:1) followed by column chromatography on silica gel (chloroform-ethyl acetate, 5:1). Melting point: decomposition at >200°C. IR spectra: 1700, 1630, 1600, 1430, 1340, 1223, and 1150. UV spectra: 404, 350, 276, and 246; (acid) 402, 350, 277, and 246; (base) 414, 285, 266, and 206. NMR spectra: 14.66 (s, 1 H, aromatic OH), 12.39 (s, 1 H, aromatic OH), 6.89 (t, J = 0.6, 1 H, H-4), 6.71 (s, 1 H, H-7), 6.30 to 6.26 (m, 1 H, H-6), 6.20 (d, J = 2.3, 1 H, H-2), 4.14 (broad s, 2 H, H-5), 3.82 (s, 3 H, OCH_3), 3.79 (s, 3 H, OCH_3), and 2.71 (s, 3 H, C-10 CH_3). MS: 394 (49, M^+), 379 (32, $M^+ - CH_3$), 363 (7, $M^+ - OCH_3$), and 109 (100); high resolution calculated for $C_{22}H_{18}O_7$ 394.1025, found 394.1009.

(iv) Compound 9. Compound 9 was isolated from strains GLA.12-41 (*tcm*IV-30) and GLA.4-48 (*tcm*II-22) (4) and purified by chromatography on silica gel plates (chloroform-methanol, 9:1) followed by column chromatography on Sephadex LH20 (chloroform). Melting point: decomposition at >200°C. IR spectra: 1623, 1601, 1571, 1498, and 1459. UV spectra: 412, 351, 280, and 246; (acid) 413, 350, 280, and 246; (base) 438, 372, 256, and 210. NMR spectra: 14.57 (s, 1 H, aromatic OH), 12.60 (s, 1 H, aromatic OH), 6.87 (s, 1 H, H-6), 6.75 (broad s, 2 H, H-4 and H-7), 6.30 (d, $J \cong 2$, 1 H, H-9), 6.23 (d, $J \cong 2$, 1 H, H-2), 4.17 (s, 2 H, H-5), and 2.85 (s, 3 H, C-10 CH_3). MS: 333 (24, $M^+ + 1$) and 332 (100, M^+); high resolution calculated for $C_{19}H_{14}O_5$ 322.0841, found 322.0814.

(v) Compound 10. Compound 10 was isolated from strain GLA.12-41 (*tcm*IV-30) (4) and purified by chromatography on silica gel plates (chloroform-methanol, 9:1) followed by column chromatography on silica gel (chloroform-ethyl acetate, 10:1). Melting point: decomposition at >200°C. IR spectra: 1743, 1705, 1616, 1590, and 1440. UV spectra: 410, 352, 282, and 249; (acid) 410, 352, 282, and 248; (base) 410, 320, and 213. NMR spectra: 14.73 (s, 1 H, aromatic OH), 12.58 (s, 1 H, aromatic OH), 6.86 (broad s, 2 H, H-6 and H-7), 6.37 to 6.32 (m, 1 H, H-4), 6.30 (d, J = 2.6, 1 H, H-2), 4.21 (s, 2 H, H-5), 3.93 (s, 3 H, OCH_3), 3.80 (s, 3 H, OCH_3), and 2.88 (s, 3 H, C-10 CH_3). MS: 394 (27, M^+), 362 (56, $M^+ - CH_3OH$), and 45 (100); high resolution calculated for $C_{22}H_{18}O_7$ 394.1025, found 394.1025.

(vi) Compound 11. Compound 11 was isolated from strain GLA.12-41 (*tcm*IV-30) (4) and purified by chromatography on silica gel plates (chloroform-methanol, 9:1) followed by HPLC. Melting point: decomposition at >200°C. IR spectra: 1740, 1709, 1617, and 1592. UV spectra: 424, 332, 306, 274, and 240; (acid) 425, 332, 307, 274, and 240; (base) 376, 293, and 240 (shoulder). NMR spectra: 14.47 (s, 1 H, aromatic OH), 12.00 (s 1 H, aromatic OH), 7.53 (s, 1 H, H-6), 6.87 (d, J = 2.6, 1 H, H-4), 6.83 (s, 1 H, H-7), 6.22 (d, J = 2.6, 1 H, H-2), 3.53 (s, 3 H, OCH_3), and 2.79 (s, 3 H, C-10 CH_3). MS: 394 (12, M^+), 362 (32, $M^+ - CH_3$ OH), 306 (25), and 44 (100); high resolution calculated for $C_{21}H_{14}O_7{}^{18}O$ 396.0731, found 396.0736.

(vii) Compound 12. Compound 12 was isolated from strain GLA.12-41 (*tcm*IV-30) (4) and purified by chromatography on silica gel plates (chloroform-methanol, 85:15) followed by HPLC. Melting point: 178 to 181°C. IR spectra: 1735, 1710, 1670, and 1600. UV spectra: 406, 388, 286, and 240; (acid) 406, 388, 286, and 238; (base) 428, 316, and 244. NMR spectra: 13.84 (s, 1 H, aromatic OH), 7.65 (s, 1 H, H-6), 7.07 (s, 1 H, H-7), 5.06 (d, J = 2.2, 1 H, H-2), 4.75 to 4.62 (m, 1 H, H-4), 3.81 (s, 3 H, OCH_3), 3.65 (s, 3 H, OCH_3), and 2.70 (s, 3 H, C-10 CH_3). MS: 458 (12, M^+), 424 (44, $M^+ - 2$ OH), 392 (59), 345 (79), and 313 (100); high resolution calculated for $C_{22}H_{18}O_{11}$ 458.0849, found 458.0837.

A 5-mg sample of compound 12 was refluxed in chloroform-ethanol (9:1, 10 ml) containing a catalytic amount of sulfuric acid for 3 h. After the reaction mixture was cooled to room temperature, it was neutralized with excess solid K_2CO_3. This solution was diluted with ethyl acetate (10 ml), filtered through a Celite pad, and evaporated to dryness. The crude product was purified by chromatography on silica gel plates (chloroform-methanol, 85:15) followed by column chromatography of the ethyl acetate-soluble material recovered from the silica gel on Sephadex LH20 (ethyl acetate and then chloroform). The colored material eluted from the column was finally purified by HPLC (acetonitrile-ethanol-water, 1:1:3). The resulting residue was then treated with excess diazomethane, and the methylated product was purified by HPLC (methanol-water, 3:1) to yield 3-demethoxy-3-ethoxy-tetracenomycin C, whose thin-layer chromatography R_f, NMR, and MS data were identical to those of an authentic sample.

(viii) Compound 13. Compound 13 was isolated from strain GLA.4-16 (*tcm*VII-34 *met-8 rif-567*) (4) and purified as follows. The ethyl acetate extract was evaporated without drying since some red pigment adhered to the drying agent otherwise. The residue was chromatographed on silica gel plates (chloroform-methanol, 4:1), and the crude material that was eluted with methanol from the red zone at ca. R_f 0.2 was ethylated with a large excess of diazoethane in the same solvent at room temperature. The ethereal solution of diazoethane was added every 20 min until the starting material had disappeared (thin-layer chromatography). After evaporation of the solvent, the crude residue was chromatographed on 0.5-mm silica gel plates (ethyl acetate-hexane, 1:3) followed by column chromatography on silica gel (chloroform). Final purification by HPLC on normal silica gel (ethyl acetate-hexane, 1:3, and then chloroform) yielded compound 13. NMR spectra: 14.73 (s 1 H, aromatic OH), 12.45 (s, 1 H, aromatic OH), 8.08 (s, 1 H, H-6), 7.41 (d, J = 2.5, 1 H, H-4), 7.13 (s, 1 H, H-7), 6.71 (d, J = 2.5, 1 H, H-2), 4.46 (q, J = 7.1, 2 H, C-9 CH_2), 3.97 (s, 3 H, OCH_3), 3.94 (s,

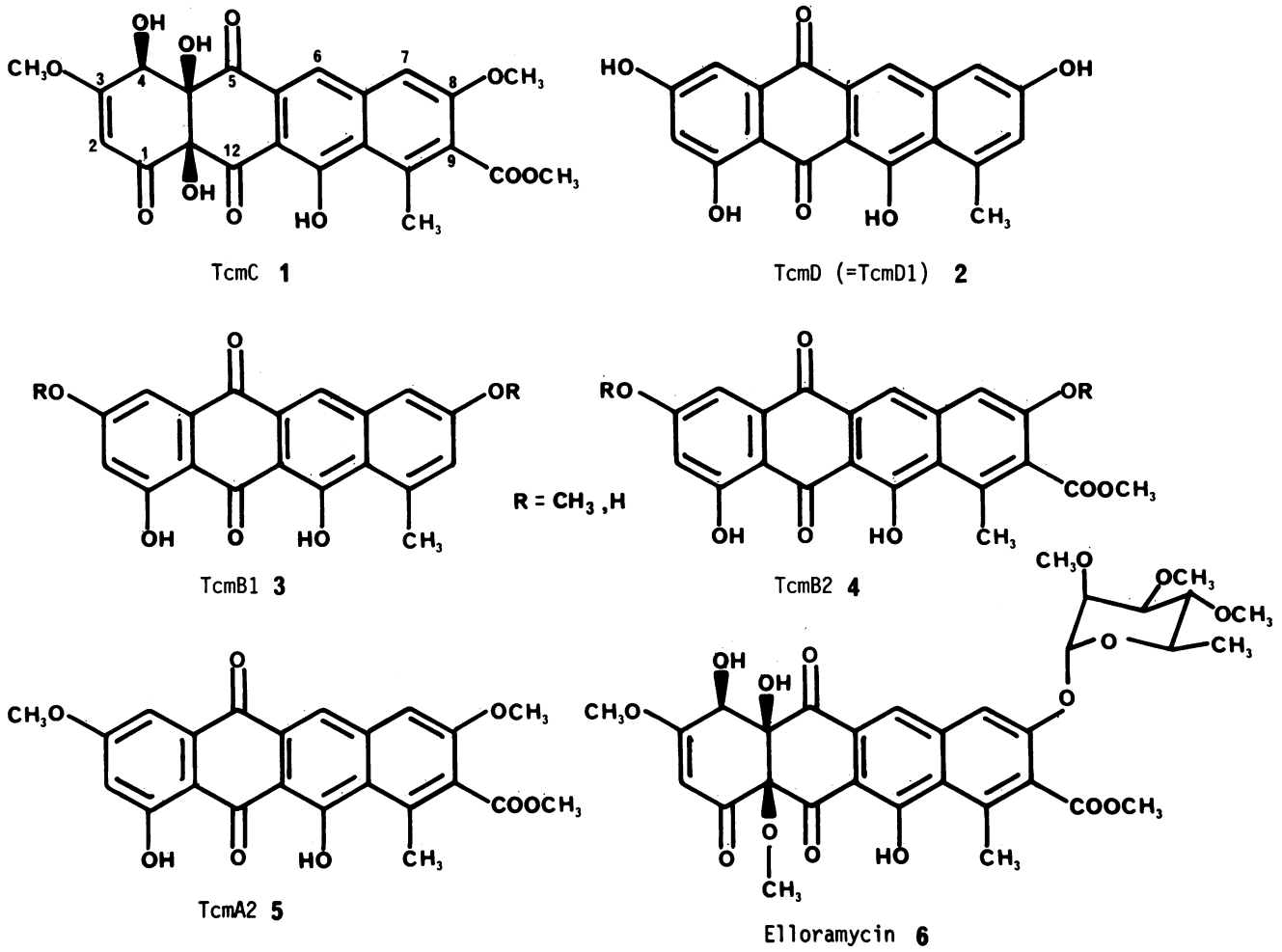

FIG. 1. Structures of the anthracycline metabolites of *S. glaucescens* GLA.0 identified by Weber and co-workers (6, 7; Siebers, Ph.D. dissertation) (see the text).

3 H, OCH_3), 2.89 (s, 3 H, C-10 CH_3), and 1.41 (t, J = 7.1, 3 H, C-9 CH_3). MS: 436 (32, M^+), 407 (24), and 41 (100); high resolution calculated for $C_{24}H_{20}O_8$ 436.1158, found 436.1157.

RESULTS AND DISCUSSION

Five anthracycline metabolites of the wild-type strain of *S. glaucescens* (GLA.0), compounds 1 through 5 (Fig. 1), were characterized by Weber and co-workers by chemical and spectroscopic methods (6, 7; Siebers, Ph.D. dissertation). They did not assign the position of the aromatic *O*-methoxy groups in compounds 3 and 4, however. The absolute stereochemistry of compound 1 also now is known through an X-ray crystallographic analysis of elloramycin (compound 6) and suitable chemical and spectroscopic correlations between compounds 1 and 6 (1; J. Rohr, Ph.D. dissertation, University of Göttingen, Göttingen, Federal Republic of Germany, 1984). Although Siebers (Ph.D. dissertation) speculated that TcmC was formed by a pathway involving the 9-methoxycarbonyl derivatives of compounds 2 and 3 plus compounds 4 and 5, this possibility was not investigated directly. Consequently, we sought new anthracycline metabolites in TcmC$^-$ mutants whose structures would solidify this thinking.

The anthracycline metabolites accumulated by type II, III, IV, and VII TcmC$^-$ mutants (4) were isolated by extraction of fermentation media with ethyl acetate and purification of the extracted substances by chromatography. Known compounds were then identified by comparison with authentic samples of TcmA2, TcmC, or the literature data. Type II mutants accumulated a TcmB2 regioisomer (compound 7a), which was converted to TcmA2 by methylation; the 12-naphthacenone derivative of compound 7a (compound 8); and the 12-naphthacenone derivative of compound 2 (compound 9) (Fig. 2). The type III mutant accumulated TcmA2 only, but the type IV mutant accumulated four new compounds: the other regioisomer of TcmB2 (compound 7b); the 12-naphthacenone derivative of compound 7b (compound 10); compound 11, which exhibited signals for the OCH_3 and two hydroxyl protons at δ 3.53, 12.0, and 14.47 ppm (chloroform-d_1–dimethyl sulfoxide-d_6), respectively, and was converted to TcmA2 by methylation; and compound 12. All three of the 12-naphthacenone derivatives exhibited a characteristic two-proton singlet at ca. δ 4.2 ppm (chloroform-d_1–dimethyl sulfoxide-d_6) which was assigned to the C-5 hydrogens by analogy to anthrone and 1,8-dihydroxy anthrone, whose methylene protons resonate at δ 4.35 and 4.3 ppm, respectively. Two of these 12-naphthacenone derivatives, compounds 9 and 10, were converted spontaneously to their 5,12-naphthacenedione forms by air oxidation. The lack of the C-8 OCH_3 group in compounds 7b, 10, 11,

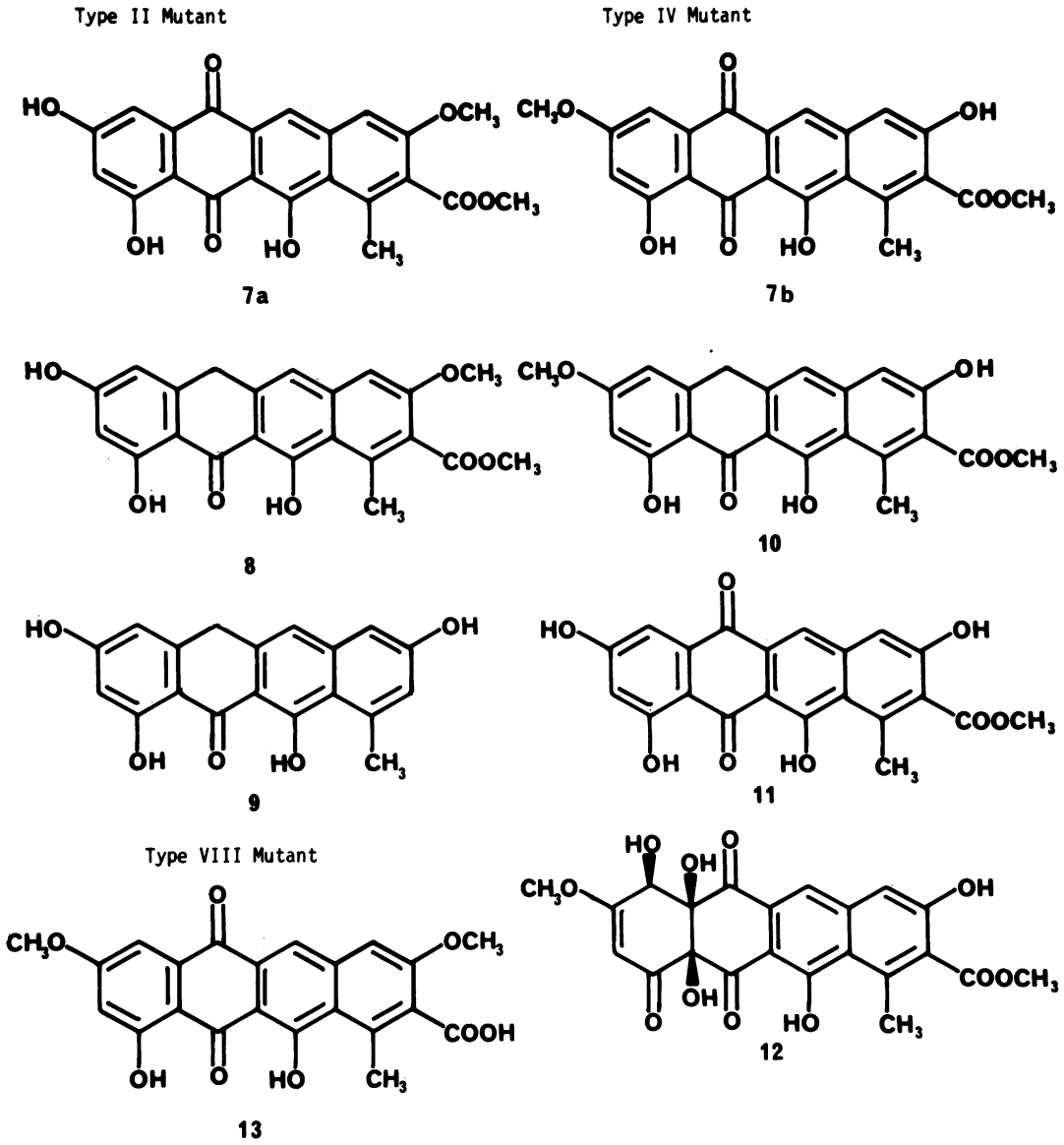

FIG. 2. Structures of the new anthracycline metabolites accumulated by three types of TmcC$^-$ mutants of *S. glaucescens* GLA.0.

and 12 is based on the absence of an $M^+ - 15$ fragment ion in their 35-eV MS which is present in the MS of compounds 5, 7a, and 8 and diagnostic for the methyl ester of an aromatic carboxylic acid having an *O*-methoxy group (5). (The MS of compounds 7b, 10, 11, and 12 exhibit an $M^+ - 32$ fragment ion because of the loss of CH_3OH rather than CH_3, as is the case with compounds 5, 7a, and 8.) On the same basis, the structures of compounds 7a and 7b can be assigned. Compound 12 was converted to 3-demethoxy-3-ethoxy-tetracenomycin C (3) by reaction with acidic ethanol followed by methylation; therefore, the *O*-methoxy group of compound 12 must be located at C-3 (3). The type VII mutant accumulated compound 2 and compound 13, which was identified by its methylation to TcmA2, its reaction with one equivalent of diazoethane to yield an ethyl ester (3 H triplet at δ 1.41 ppm and 2 H quartet at δ 4.46 ppm (chloroform), and its low R_f on silica gel thin-layer chromatography plates in comparison with the nonacidic anthracycline metabolites.

The structures of the new metabolites isolated from the *S. glaucescens* TcmC$^-$ mutants thus were assigned on the basis of the chemical conversions and spectroscopic data given above. By comparisons with the available spectral data for the known metabolites shown in Fig. 1 (6, 7; Siebers, Ph.D. dissertation) and the complete IR, UV, NMR, and MS data for the compounds shown in Fig. 2, we completed their characterization (see Materials and Methods). It is noteworthy that the isolation of 12-naphthacenone derivatives (compounds 8, 9, and 10) has not been reported previously, although they are analogous to the 6-deoxy-6-methylpretetramides, which are intermediates in the biosynthesis of the tetracycline antibiotics (2).

To simplify future discussion of the known and new anthracyclines, we propose the acronyms shown in Fig. 1 and 3. Thus, TcmD (compound 2) becomes TcmD1; its 9-methoxycarbonyl derivative (compound 11) becomes TcmD2; and 3,8-dimethyl-9-carboxy-tetracenomycin D (compound 13) becomes TcmE. This latter compound appar-

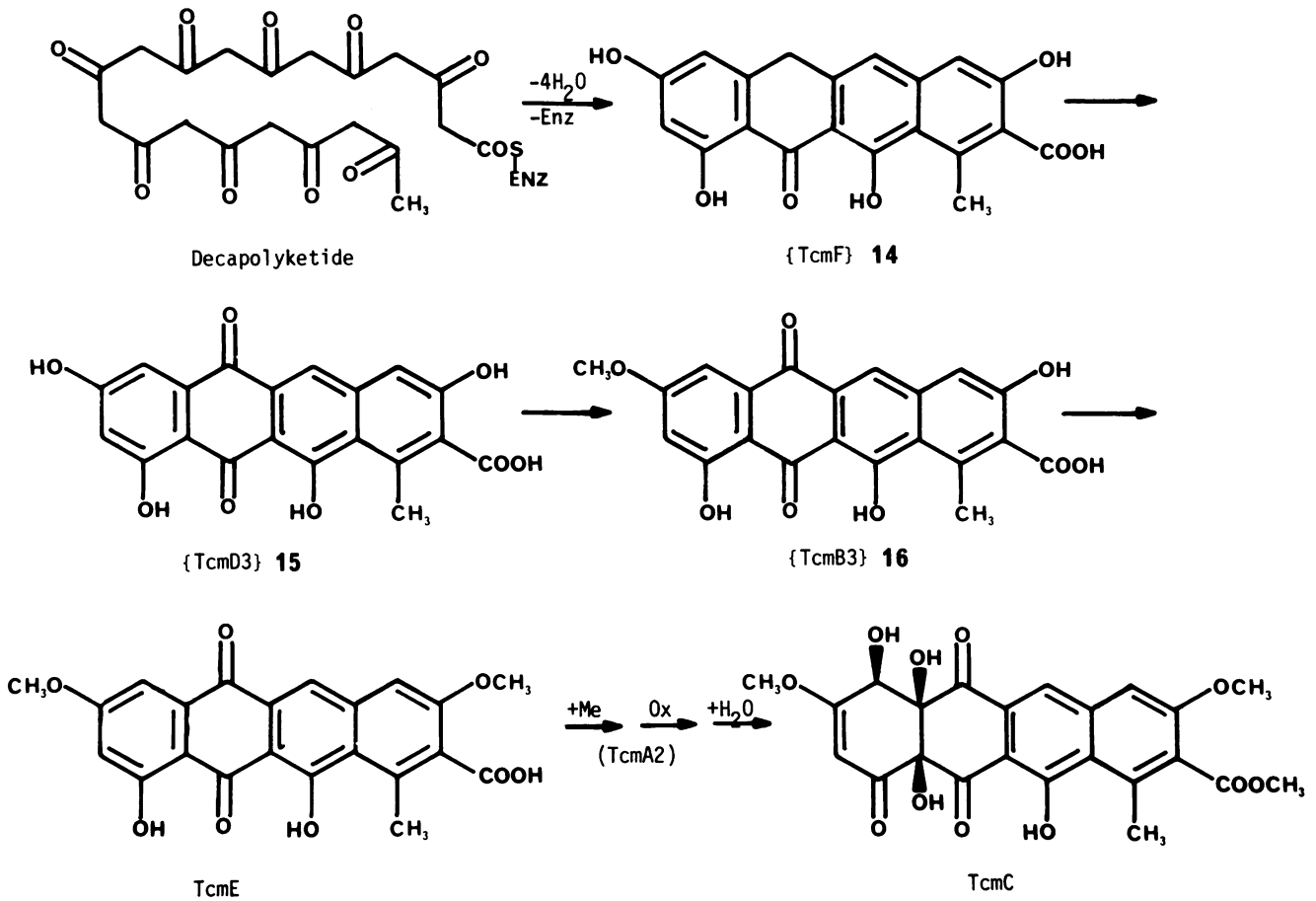

FIG. 3. Hypothetical biosynthetic pathway for TmcC. The brackets signify compounds which have not been isolated yet. $^{COS}_{ENZ}$, Attachment to an enzyme by a thioester bond; +ME, addition of a methyl group to the C-9 COOH; Ox, oxidation of TcmA2 at C-4, C-4a, and C-12a.

ently was isolated by Siebers (Ph.D. dissertation) but not characterized.

The hypothetical biosynthetic pathway to TcmC shown in Fig. 3 is consistent with the kinds of anthracycline metabolites accumulated by different TcmC$^-$ mutants and their cosynthetic behavior, as described in the accompanying paper (4). A decapolyketide constructed from acetate and malonate should be cyclized to a 9-carboxy-12-naphthacenone (compound 14) (TcmF), oxidized to the 5,12-naphthacenedione (compound 15) (TcmD3), and then methylated in sequo to compound 16 (TcmB3) and TcmE. Although alternative sequences to compound 13 cannot be ruled out at this time, the one shown agrees best with the available data. Methylation of compound 13 to TcmA2 followed by oxidation and hydration of TcmA2 would yield TcmC, but the sequence of events in this part of the pathway is less certain.

Several shunt products can be formed from the main intermediates: TcmD1 by loss of CO_2 from the aromatic *O*-hydroxy carboxylate, TcmD3; TcmB1 by the same process but starting from 3-*O*-methyl-TcmD3; and TcmD2 by C-9 O-methylation of TcmD3, thus bypassing TcmB3 and TcmE. Compound 12 could come from TcmB3 by the sequence of reactions used to convert TcmA2 to TcmC, thus bypassing TcmE. The occurrence of these shunt products and the three 12-naphthacenone metabolites, compounds 8, 9, and 10, indicates that alternate biochemical pathways can operate in the wild-type and mutant strains, but we believe that TcmC is formed mainly by the route shown. This pathway appears to involve two ring hydroxylation pattern-specific *O*-methyltransferases whose absence or malfunction results in the characteristic pattern of anthracycline metabolites accumulated by type II (3-demethyl) and type IV (8-demethyl) TcmC$^-$ mutants (4). These putative enzymes and the *O*-methyltransferases acting on the C-9 carboxyl group, however, seem to tolerate different C-5 oxidation states, as reflected in the formation of the 12-naphthacenone and 5,12-naphthacenedione forms of similar metabolites.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant CA 35381 from the National Institutes of Health.

We thank Ralf Hütter and Hans Zähner for cultures of *S. glaucescens* GLA.0, Axel Zeeck for authentic samples of metabolites and other helpful information, and Ali Shafiee for technical assistance with the HPLC analysis of metabolites.

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