Biosynthesis of anthracyclines: carminomycin 4-*O*-methyltransferase, the terminal enzymic step in the formation of daunomycin

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In the presence of *S*-adenosyl-L-methionine, cell-free extracts of *Streptomyces* sp. C5, *Streptomyces peucetius* ATCC 29050, *Streptomyces insignis* ATCC 31913 and *Streptomyces coeruleorubidus* ATCC 31276 *O*-methylated carminomycin and 13-dihydrocarminomycin to daunomycin and 13-dihydrodaunomycin, respectively. With the corresponding aglycones, carminomycinone and 13-dihydrocarminomycinone, as substrates, no methylated products were detected. Other 4-hydroxyanthracyclines such as aklavin and aclacinomycin A, and 4-hydroxyanthracyclonones such as ϵ -rhodomycinone and aklavinone, were not substrates for the 4-*O*-methyltransferase. These reaction specificities indicate that glycosylation of the anthracyclonone molecule must occur before 4-*O*-methylation, which means that 4-*O*-methylation of carminomycin is probably the terminal step in the biosynthesis of daunomycin, and that daunomycinone is not an intermediate in the pathway.

Introduction

Although daunomycin (DiMarco *et al.*, 1964), adriamycin (Arcamone *et al.*, 1969), carminomycin (Brazhnikova *et al.*, 1974) and related anthracyclines (Fujiwara & Hoshino, 1983; Strohl *et al.*, 1989; Fig. 1) are important anti-tumour drugs, the biosynthetic pathways for their formation have not been well elucidated (Strohl *et al.*, 1989). Several biosynthetic pathways have been postulated for the biosynthesis of daunomycin (Blumauerová *et al.*, 1979*b*; Eckardt & Wagner, 1988; Fujiwara & Hoshino, 1983; Yoshimoto *et al.*, 1980) but these are based on *in vivo* bioconversions and intermediates accumulated by a limited number of mutants. Recent studies by Eckardt and his colleagues (reviewed by Eckardt & Wagner, 1988), confirmed by our *in vitro* analyses (see accompanying paper: Connors *et al.*, 1990), have helped to elucidate the sequence of the early steps in daunomycin biosynthesis. On the other hand, the enzymic reactions converting ϵ -rhodomycinone to daunomycin still remain largely uncharacterized (Strohl *et al.*, 1989). A better understanding of the daunomycin biosynthesis pathway will assist investigation of the genetics of daunomycin biosynthesis and will also assist in the rational design of experiments to generate hybrid

anthracycline antibiotics via interspecies cloning of anthracycline biosynthesis genes. We describe the *S*-adenosyl-L-methionine (SAM)-dependent methylation of carminomycin and 13-dihydrocarminomycin to daunomycin and 13-dihydrodaunomycin (see Fig. 1 for structures), respectively, by several daunomycin-producing strains.

Methods

Streptomyces sp. strain C5, which produces ϵ -rhodomycinone and baumycins (anthracyclines related to daunomycin; McGuire *et al.*, 1980), was obtained from the Frederick Cancer Research Center. *Streptomyces galilaeus* (ATCC 31133), which produces aclacinomycin A (Oki *et al.*, 1979), and the daunomycin producing strains *Streptomyces peucetius* (ATCC 29050), *Streptomyces coeruleorubidus* (ATCC 31276) and *Streptomyces insignis* (ATCC 31913; Tunac *et al.*, 1985) were obtained from the American Type Culture Collection.

The growth of these streptomycetes and preparation of desalted cell extracts are described by Connors *et al.* (1990). To determine which anthracyclonone and anthracycline substrates were methylated by cell extracts of anthracycline-producing streptomycetes, assays contained, 0.25 ml: anthracycline or anthracyclonone substrate, 5 to 10 μ g; *S*-adenosyl-[methyl- 14 C]-L-methionine ([methyl- 14 C]SAM) (New England Nuclear), 2.15 nmol (46 Ci mol $^{-1}$; 1.7 TBq mol $^{-1}$); cell extract, 0.2 ml (1–2 mg protein ml $^{-1}$; prepared in 100 mM-K $_2$ HPO $_4$ /KH $_2$ PO $_4$ buffer, pH 7.5). The volume was adjusted to 0.25 ml with 100 mM-phosphate buffer (pH 7.5). The reaction mixture was incubated at 37 °C for 60 min, after which the products were extracted into 3 vols of chloroform/methanol (9:1, v/v). The extracted material was air-dried, and then redissolved in 10 μ l of chloroform (plus authentic standard for cochromatography) for application to a 0.25 mm layer of silica gel. The

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Abbreviations: SAM, *S*-adenosyl-L-methionine.

Compound*	R1	R2	R3	R4†	R5
Daunomycin (P)	-OH	-H	=O	-DN	-CH ₃
13-Dihydrodaunomycin (P)	-OH	-H	-OH	-DN	-CH ₃
Carminomycin (S)	-OH	-H	=O	-DN	-H
13-Dihydrocarminomycin (S)	-OH	-H	-OH	-DN	-H
Aclacinomycin A (S)	-H	-COOCH ₃	-H	-RN-dF-LC	-H
Aklavin (S)	-H	-COOCH ₃	-H	-RN	-H
Carminomycinone (S)	-OH	-H	=O	-H	-H
13-Dihydrocarminomycinone (S)	-OH	-H	-OH	-H	-H
ϵ -Rhodomycinone (S)	-OH	-COOCH ₃	-H	-H	-H
Aklavinone (S)	-H	-COOCH ₃	-H	-H	-H

* Letters in parentheses indicate: P, product of methyltransferase reaction; S, substrate for methyltransferase reaction.

† Sugars: DN, daunosamine; RN, rhodosamine; dF, 2-deoxy-L-fucose; LC, L-cinerulose A.

Fig. 1. Structures of the anthracyclines and anthracyclinones discussed in this paper.

thin-layer chromatography (TLC) plates (Whatman) were developed with either chloroform/heptane/methanol (5:5:1, by vol.; SS1) for anthracyclinone products, or chloroform/methanol/glacial acetic acid/H₂O (15:5:1:1, by vol.; SS2) for anthracycline products. The developed chromatograms containing radioactive products were then exposed to X-ray film (Kodak X-Omat AR) for 3 to 5 d. Reaction products were compared to authentic standards by cochromatography or chromatography in parallel. Aglycones of the methylated products were prepared by hydrolysing the radioactive anthracyclines with 0.1 M-HCl for 60 min at 90 °C. The aglycone hydrolysis products were extracted into chloroform/methanol (9:1), air-dried, and reconstituted in 10 μ l chloroform for TLC as above. Radioactive aglycones were separated using SS1 and the chromatograms were viewed under UV light and autoradiographed.

Results and Discussion

Using [methyl-¹⁴C]SAM as the methyl donor, desalted cell extracts of *Streptomyces* sp. C5 methylated carminomycin and 13-dihydrocarminomycin to [¹⁴C]daunomycin and 13-[¹⁴C]dihydrodaunomycin, respectively (Fig. 2a, b). None of the other 4-hydroxyanthracyclines (aklavin, aclacinomycin A) or 4-hydroxyanthracyclinones (aklavinone, ϵ -rhodomycinone, carminomycinone, 13-dihydrocarminomycinone) tested were suitable substrates for the 4-O-methyltransferase reaction (data not shown). The two methylation products were identified by cochromatography with authentic standards in TLC

solvent system SS2 (Fig. 2b). Moreover, the radiolabelled methylation products were hydrolysed to their aglycones, and these (daunomycinone and 13-dihydrodaunomycinone) were separated and identified by TLC using SS1 (Fig. 2c). Since the reactions gave daunomycin, daunomycinone and their 13-hydroxy derivatives rather than unknown products, the methylation took place at the 4-O-position as expected.

Desalted cell extracts or other daunomycin-producing strains tested, i.e. *S. peucetius*, *S. insignis* and *S. coeruleorubidus*, also methylated carminomycin and 13-dihydrocarminomycin to [¹⁴C]daunomycin and 13-[¹⁴C]dihydrodaunomycin, respectively (data not shown). Aklavin, aclacinomycin A, aklavinone, ϵ -rhodomycinone, carminomycinone and 13-dihydrocarminomycinone were not substrates for the 4-O-methyltransferases of these strains, as also observed with *Streptomyces* sp. C5. This suggests that late enzymic reactions in the daunomycin biosynthesis pathways of the different strains that produce daunomycin are highly conserved. We found similar conservation in early reactions of the daunomycin pathway (Connors *et al.*, 1990). On the other hand, cell extracts of *S. galilaeus* 31133, which produces aclacinomycin A (Oki *et al.*, 1979), did not methylate any of the substrates tested (data not shown). This is consistent with the lack of a 4-O-methyl group in

aclacinomycin A as well as other products of this strain (Strohl *et al.*, 1989; Fig. 1).

The enzymic reactions leading to the formation of ϵ -rhodomycinone, an important intermediate in the biosynthesis of daunomycin (McGuire *et al.*, 1980; Strohl *et al.*, 1989), have recently been clarified (Eckardt & Wagner, 1988; Connors *et al.*, 1990). On the other hand, the biosynthetic steps required to convert ϵ -rhodomycinone to daunomycin are virtually uncharacterized, although it is obvious that the following reactions must take place: (i) decarbomethoxylation at C-10; (ii) two-step oxidation from a methylene to a keto group at C-13; (iii) *O*-methylation of the C-4 hydroxyl group; and (iv) glycosylation with daunosamine of the C-7 hydroxyl group (Strohl *et al.*, 1989). This study provides the first information about the order of these reactions. Since the 4-*O*-methyltransferases of every daunomycin-producing strain tested required glycosides as substrates, glycosylation must take place before 4-*O*-methylation. Similarly, since aklavin and aclacinomycin A were not substrates for the 4-*O*-methyltransferases, it is possible that compounds containing 10-carbomethoxyl groups cannot be methylated (Fig. 1). ϵ -Rhodomycinone contains the carbomethoxyl group at C-10 (Fig. 1), suggesting that decarbomethoxylation must take place before 4-*O*-methylation. On the other hand, since aklavin and aclacinomycin A have sugars other than daunosamine attached at C-7 (Fig. 1), the reaction specificity may lie in the sugar residue and not in the absence of the carbomethoxyl group.

Both 13-dihydrocarminomycin and carminomycin were good substrates for the 4-*O*-methyltransferase, so the oxidation state of C-13 apparently is of little consequence to the 4-*O*-methyltransferase *in vitro*. It is not clear from our results which of these is the preferred substrate *in vivo*. However, 13-dihydrodaunomycin has been shown to be a product of daunomycin metabolism, and not an intermediate in daunomycin biosynthesis (Crespi-Perellino *et al.*, 1982). Since the product of the

Fig. 2. (a) Methylation of 13-dihydrocarminomycin (R = -OH) and carminomycin (R = =O) to 13-dihydrodaunomycin (1) and daunomycin (2), respectively, with subsequent acid hydrolysis to 13-dihydrodaunomycinone (3) and daunomycinone (4), respectively. (b) Autoradiogram of products after separation by TLC using SS2; the products were formed by methylation of 13-dihydrocarminomycin and carminomycin with [methyl-¹⁴C]SAM and cell extracts of *Streptomyces* C5. Lanes: A, No substrate added (background control); B 13-dihydrocarminomycin added as substrate; major radioactive products are 13-dihydrodaunomycin (1) and 13-dihydrodaunomycinone (3); C, carminomycin added as substrate; major radioactive products are daunomycin (2) and daunomycinone (4). (c) Autoradiogram of products after separation by TLC using SS1; the products were from acid hydrolysis of compounds shown in (b). Lanes: A, No substrate added (background control); B, hydrolysis of 13-dihydrodaunomycin yielded 13-dihydrodaunomycinone (3) as the major product; hydrolysis of daunomycin yielded daunomycinone (4) as the major product.

4-*O*-methylation of 13-dihydrocarminomycin is 13-dihydrodaunomycin, which was shown not to be a precursor to daunomycin (Crespi-Perellino *et al.*, 1982), this would rule out 13-dihydrocarminomycin as a substrate for the 4-*O*-methyltransferase *in vivo*. Thus, combining our data and those of Crespi-Perellino *et al.* (1982), the pathway for biosynthesis of 13-dihydrodaunomycin from 13-dihydrocarminomycin would most probably be: 13-dihydrocarminomycin → carminomycin → daunomycin → 13-dihydrodaunomycin. In support of this pathway is the finding that daunomycin can be reduced to 13-dihydrodaunomycin by a wide variety of micro-organisms (Marshall, 1985), suggesting that 13-dihydrodaunomycin is a degradation product rather than a biosynthetic intermediate.

Our data indicate that organisms accumulating the anti-tumour drug carminomycin (Brazhnikova *et al.*, 1974) may differ from daunomycin-producing strains simply by lacking 4-*O*-methyltransferase activity. This is supported by the finding that addition of DL-ethionine, an inhibitor of methylation, to a daunomycin-producing culture of *S. coeruleorubidus* caused the culture to accumulate carminomycin (Blumauerová *et al.*, 1979*a*). This would place the carminomycin 4-*O*-methyltransferase reaction at the last step in daunomycin biosynthesis.

Whether daunomycinone, the aglycone equivalent of daunomycin, is a precursor to daunomycin has been disputed for some time (Blumauerová *et al.*, 1979*b, c*; Fujiwara & Hoshino, 1983; Yoshimoto *et al.*, 1980). Because our data indicate that glycosylation must take place before 4-*O*-methylation, daunomycinone cannot be a precursor. In the *in vitro* reactions in which 13-dihydrocarminomycin and carminomycin were methylated to 13-dihydrodaunomycin and daunomycin, respectively, the corresponding aglycones (13-dihydrodaunomycinone and daunomycinone) were usually obtained as minor products (Fig. 2*b*). Thus, the cell extracts were able to hydrolyse the glycosides to the respective aglycones, further indicating that daunomycinone and 13-dihydrodaunomycinone are degradation products rather than biosynthetic intermediates. Similarly, Yoshimoto *et al.* (1980) demonstrated that carminomycinone but not daunomycinone could be converted *in vivo* to baumycins and daunomycin by a blocked mutant of *S. coeruleorubidus* ME130-A4. On the other hand, Blumauerová *et al.* (1979*c*) showed that mutants of *S. coeruleorubidus* JA10092 could apparently glycosylate exogenously added daunomycinone. Their data would suggest that *S. coeruleorubidus* JA10092 may utilize a different pathway in the formation of daunomycin, or, on the other hand, the daunomycinone used as a substrate in the *in vivo* conversion studies by Blumauerová *et al.* (1979*c*) may have been converted to a different aglycone before conversion to daunomycin.

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