CONVERSION OF TRIOSTINS TO QUINOMYCINS BY PROTOPLASTS OF *STREPTOMYCES ECHINATUS*

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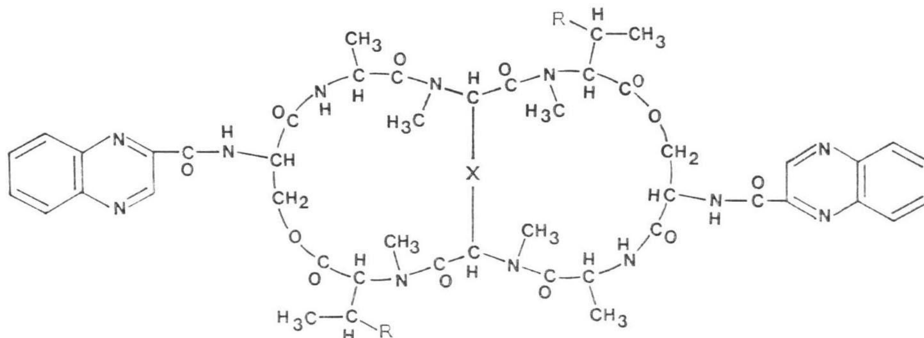
Protoplasts of *Streptomyces echinatus* have been used to investigate the biosynthesis of echinomycin (quinomycin A). It has been shown that this organism has the capacity to convert a series of triostins to the corresponding quinomycins by a mechanism involving methylation. Evidence is presented which suggests that triostin A is the natural precursor of echinomycin. Conversion of tetra-*N*-demethyl analogues of triostin A to corresponding analogues of echinomycin was not detected.

Quinoxaline antibiotics are heterodetic cyclic octa-depsipeptides produced by several *Streptomyces* sp.[1,2,3]. They all exhibit antimicrobial and antitumour activity[4] which may be attributed to their capacity to bind to the DNA of susceptible cells by a mechanism involving bifunctional intercalation[5]. Quinoxaline-producers may conveniently be divided into two groups — those making triostins, which contain a disulfide cross-bridge[6], and those which produce quinomycins where the cross-bridge is a thioacetal[7,8] (Fig. 1). Generally, members of each group make a mixture of triostins or quinomycins which differ in the nature of the branched-chain amino acid residues of the peptide ring, although *Streptomyces echinatus* produces only echinomycin, which is identical to quinomycin A (Fig. 1).

Fig. 1. Structures of naturally-occurring quinoxaline antibiotics.
Echinomycin (quinomycin A): X=$CH_2SCH(SCH_3)$, R=CH_3
Quinomycin C: X=$CH_2SCH(SCH_3)$, R=$CH(CH_3)_2$
Triostin A: X=CH_2SSCH_2, R=CH_3
Triostin C: X=CH_2SSCH_2, R=$CH(CH_3)_2$



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On structural grounds DELL *et al.*[7] speculated that triostins might be precursors of quinomycins *in vivo*, and suggested that the thioacetal linkage of quinomycins could be formed by monomethylation and rearrangement of the triostin disulfide. *S*-Adenosylmethionine was suggested as a possible donor of the methyl group. It is known that methionine supplies the four *N*-methyl groups present in triostin A, the amino acid residues of the octapeptide ring being all derived from the corresponding natural L-amino acids[4]. As yet it has not been established whether quinomycin-producers are able to effect the conversion of triostins to quinomycins. However, it is significant that biosynthesis of novel derivatives of both echinomycin and triostin A has been detected in cultures of *S. echinatus* supplemented with certain analogues of quinoxaline-2-carboxylic acid, the precursor of the chromophore moieties[9,10].

In the present study protoplasts of *S. echinatus* have been employed to investigate whether this organism does indeed have the capacity to convert triostins to quinomycins by a mechanism involving methylation.

Materials and Methods

Materials

All solvents and general reagents were purchased from Fisons Ltd., Loughborough, Leicestershire, England. Lysozyme (Grade I, 40,000 units/mg protein) was obtained from the Sigma Chemical Co., Poole, Dorset, England. Echinomycin and quinomycin C were gifts respectively from Drs. H. BICKEL and K. SCHEIBLI, CIBA-Geigy Ltd., Basel, Switzerland, and Dr. D. G. MARTIN, The Upjohn Co., Kalamazoo, MI, U.S.A. Triostin A, triostin C, and chromophore-substituted derivatives of triostin A (having two quinoline or 6-bromoquinoxaline moieties) were purified from cultures of *Streptomyces triostinicus* ATCC 21043 using methods described previously[11]. *Bis*-quinoline-substituted echinomycin was prepared according to the method of FOX *et al.*[12] and purified by standard procedures[10]. The characterization of these compounds by means of 400 MHz ^{1}H NMR and mass spectrometry has been described elsewhere[9,11,13,14]. Tetra-*N*-demethyl analogues of triostin A were gifts from Dr. R. K. OLSEN, Dept. of Chemistry and Biochemistry, Utah State University, Logan, UT, U.S.A.

Radiochemicals

L-[*methyl*-^{14}C]Methionine (56.7 mCi/mmol), was purchased from the Radiochemical Centre, Amersham, England. [^{35}S]Triostin A (initial activity 21.8×10^6 dpm/μmol) was prepared biosynthetically as described previously[15].

Organism and Culture Conditions

S. echinatus A8331 (a gift from Drs. J. NÜESCH and K. SCHEIBLI of CIBA-Geigy Ltd., Basel, Switzerland) was maintained on sterile soil. Approximately 250 mg soil were used to inoculate 500 ml of the seed medium described by YOSHIDA and KATAGIRI[16]. After 48 hours incubation a 20-ml volume of this seed culture was transferred to 500 ml of another medium which consisted of: D-glucose 10 g, Bactopeptone 5 g, yeast extract 5 g, NaCl 1.5 g, $CaCO_3$ 1.5 g and 500 ml deionised water, pH 7.0. After growth on this medium for 24 hours the mycelium from a single 500-ml culture was used for the preparation of protoplasts. All culture incubations were carried out in 2-liter Erlenmeyer flasks in a New Brunswick G-25 orbital incubator operating at 28°C and 240 cycles/minute.

Preparation of Protoplasts

Protoplasts were prepared according to the method of KELLER and KLEINKAUF[17]. The mycelium was harvested, washed once with 500 ml 10% (w/v) sucrose, collected on a tared filter paper using a Büchner funnel, and the wet weight determined (usually 8~10 g mycelium per 500 ml culture). The mycelium was then resuspended in a protoplast buffer [*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) 25 mM, $CaCl_2$ 25 mM, $MgCl_2$ 10 mM, sucrose 0.3 M, D-galactose 10 g/liter, K_2HPO_4 50 mg/liter and deionised water, pH 7.6] to a density of 1 g cells per 5 ml buffer. Solid lysozyme was added to a final concentration of 2.5 mg/ml and the mycelial suspension was incubated for 2 hours in a New Brunswick Aquatherm water bath operating at 28°C and 100 cycles/minute. After this time intact

mycelium was removed by filtering the suspension through two layers of tightly packed glass wool and the filtrate was centrifuged at 5,000 × *g* for 25 minutes at ambient temperature. The protoplast pellet was resuspended in 4 ~ 5 ml of fresh buffer and filtered twice more through tightly packed glass wool. The absorbance of the protoplast suspension, measured at 650 nm, was adjusted by the addition of buffer to give a value of 8.0. Protoplasts were then used immediately for biosynthetic studies. Examination of suspensions by means of phase-contrast microscopy showed them to be free from mycelial contamination.

As a further check on the purity of protoplast suspensions prepared in this way we examined the extent to which echinomycin synthesis was dependent on the sucrose composition of the protoplast buffer. Samples (0.8 ml) of protoplast suspensions were centrifuged at 12,000 × *g* for 5 minutes in an Eppendorf minifuge. The protoplast pellets were resuspended in 0.8 ml volumes of protoplast buffer containing different sucrose concentrations (0 ~ 0.3 M), supplemented with 0.5 μCi of L-[*methyl*-^{14}C]-*methionine*, and the incorporation of radiolabel into echinomycin was measured over a 120-minute incubation period as described below. On reducing the sucrose concentration of the buffer from 0.3 M to 0.1 M the capacity of protoplast suspensions to produce labelled echinomycin was lowered by not less than 96%, showing that practically all the measured echinomycin synthesis was carried out by osmotically labile structures.

Conversion of Triostins to Quinomycins

All protoplast incubations were set up in 10 cm × 2.5 cm flat-bottomed tubes shaken vertically in a New Brunswick Aquatherm water bath operating at 28°C and 110 cycles/minute. Incubations were started by adding 0.4 ml protoplast suspension to 0.4 ml protoplast buffer containing either 18.4 nmol of [^{35}S]triostin A or 20 nmol of unlabelled triostins (or triostin analogues) together with 0.5 μCi of L-[*methyl*-^{14}C]methionine. Owing to their low aqueous solubility all triostin compounds were added in 20 μl of dimethyl sulfoxide, and the same volume of this solvent was added to control samples. Incubations were stopped at appropriate times by the addition of 5 ml ice-cold deionised water. Quinomycin products were extracted into 5 ml ethyl acetate along with the remainder of the added triostins. Extracts were evaporated to dryness, redissolved in 100 μl chloroform containing 50 μg of carrier echinomycin (and, where appropriate, 50 μg of other quinoxaline antibiotics) and loaded on to plastic-backed TLC sheets (Merck kieselgel 60 F_{254}, 0.2 mm thickness) which were developed in ethyl methyl ketone. Radiolabelled products were located using autoradiography; carrier materials were detected by illuminating with UV light at 254 nm. Quinomycin products were identified by comparing their chromatographic mobilities with those of authentic materials.

Measurement of Radioactivity

Samples were counted in a Nuclear Enterprise 8312 spectrometer. Regions of TLC sheets containing labelled products were cut out and immersed for counting in 10 ml toluene containing 2-(4-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (0.4% w/v). Counting efficiencies in this system were 90% for ^{35}S and 85% for ^{14}C.

Results

Conversion of [^{35}S]Triostin A to [^{35}S]Echinomycin

Protoplasts of *S. echinatus* readily converted [^{35}S]triostin A to a radioactive product having identical chromatographic mobility to echinomycin. A time course for this reaction is shown in Fig. 2. After an initial burst of echinomycin formation, which lasted approximately 30 minutes, triostin A was converted to echinomycin at a constant rate of 0.69 nmol (0.75 μg) per ml protoplast suspension per hour. Over a period of 5 hours, 3.8 nmol of echinomycin were produced per milliliter of suspension, representing 17.3% utilization of the [^{35}S]triostin A originally added. Triostin A was recovered unchanged from incubations in which the protoplast suspension had been replaced by buffer.

Methylation of Triostins

Protoplasts of *S. echinatus* were competent in echinomycin synthesis *de novo* as evidenced by their capacity to produce radiolabelled antibiotic in the presence of L-[*methyl*-^{14}C]methionine (Fig. 3a, track 1). Suspensions of protoplasts supplemented with triostin C and *bis*-quinoline triostin A produced additional labelled materials which co-migrated with quinomycin C (Rf 0.32) and *bis*-quinoline echinomycin (Rf 0.35) respectively (Figs. 3a and 3b, tracks 2 and 3). A new component (Rf 0.41) was also produced by suspensions containing *bis*-6-bromoquinoxaline triostin A (Fig. 3a, track 4). This was probably *bis*-6-bromoquinoxaline echinomycin, although this could not be unequivocally established because no authentic material was available for comparison of chromatographic mobility. These experiments were repeated using a different TLC solvent system (butanol - acetic acid - water, 3: 1: 1 by volume) with substantially the same results; in each case the new components were distinct, though sometimes poorly resolved from the natural antibiotic spots.

From these observations it may be concluded

Fig. 3a. Autoradiograph showing L-[*methyl*-^{14}C]-methionine-labelled products made by *S. echinatus* protoplasts.

Protoplast suspensions (0.8 ml) were incubated for 120 minutes with 0.5 μCi L-[*methyl*-^{14}C]methionine plus, where appropriate, 20 nmol of different triostin compounds. Products were extracted into ethyl acetate and separated using TLC.

Track 1: control (no added triostin); track 2: triostin C; track 3: *bis*-quinoline triostin A; track 4: *bis*-6-bromoquinoxaline triostin A.

The distance of migration corresponding to Rf 0.5 is indicated.

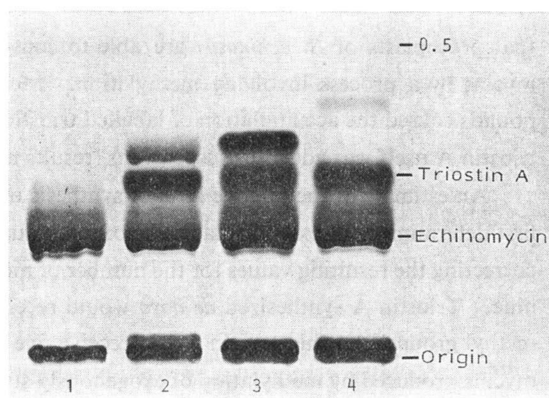


Fig. 2. Time course of the conversion of [^{35}S]triostin A into [^{35}S]echinomycin by protoplasts of *S. echinatus*.

Protoplast suspensions (0.8 ml) were incubated with 18.4 nmol [^{35}S]triostin A (specific activity 13.6×10^{3} dpm/nmol). Incubations were stopped at the times shown by the addition of 5 ml deionised water. Antibiotics were then extracted into ethyl acetate and separated using TLC. The incorporation of ^{35}S-label into the echinomycin band was determined as a means of calculating the extent of reaction.

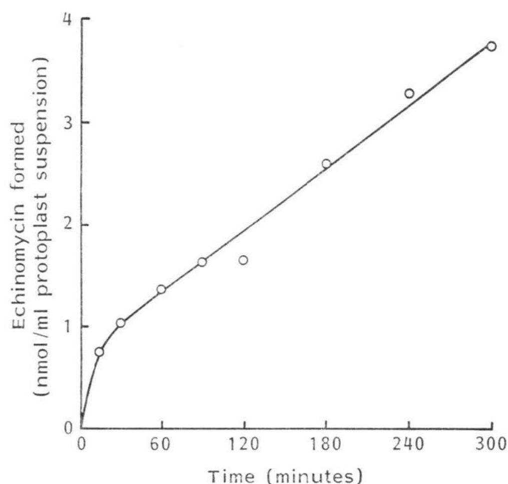


Fig. 3b. Location of authentic carrier materials, detected using UV illumination, on the chromatogram from which the autoradiograph in Fig. 3a was prepared.

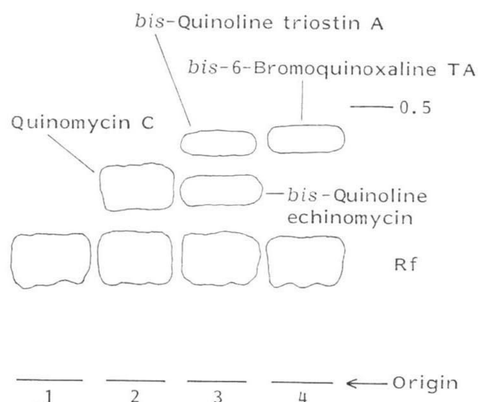


Table 1. Effects of triostin compounds on the levels of synthesis of echinomycin and triostin A by protoplasts of *S. echinatus*.
Experimental conditions were as described in the legend to Fig. 3.

Triostin compound added to protoplast suspensions	Labelled antibiotics detected	Number of methyl groups derived from L-[*methyl*-^{14}C]-methionine	Radioactivity (dpm) recovered in antibiotic divided by number of methyl groups derived from methionine
None	Triostin A	4	600
	Echinomycin	5	4,790
Triostin C	Quinomycin C	1	4,240
	Triostin A	4	2,120
	Echinomycin	5	3,940
bis-Quinoline triostin A	*bis*-Quinoline echinomycin	1	6,800
	Triostin A	4	1,800
	Echinomycin	5	4,230
bis-6-Bromo-quinoxaline triostin A	*bis*-6-Bromoquinoxaline echinomycin	1	250
	Triostin A	4	1,670
	Echinomycin	5	3,110

that protoplasts of *S. echinatus* are able to convert a series of triostins to the corresponding quinomycins by a process involving methylation. Moreover, adding any one of the three triostin compounds caused the accumulation of labelled triostin A (Fig. 3a) and this was also found to occur when triostin A itself was added to suspensions (results not shown).

An estimate of the relative level of synthesis of labelled antibiotics seen in Fig. 3a was obtained by first determining the extent of labelling of individual components by L-[*methyl*-^{14}C]methionine and then correcting the resulting values for the number of methyl groups expected to be contributed from methionine. Triostin A synthesized *de novo* would receive four methyl groups from this source (its four *N*-methyl groups[18]), echinomycin would receive five (four *N*-methyl groups and an *S*-methyl) and quinomycins produced by methylation of exogenously-supplied triostins would receive only one (the *S*-methyl group of the thioacetal linkage). These calculations are based on the assumption that label from L-[*methyl*-^{14}C]methionine enters *N*-methyl groups and *S*-methyl groups to the same extent. From the data given in Table 1 it can be seen that addition of all three triostin compounds inhibited echinomycin synthesis as well as causing the accumulation of triostin A. In separate experiments, carried out with three different protoplast preparations, the extent of labelling of echinomycin by L-[*methyl*-^{14}C]methionine was depressed 15~23% by addition of unlabelled triostin A to incubations. Addition of unlabelled triostin C reduced it by 18~26%.

The data in Table 1 also reveal that *bis*-6-bromoquinoxaline triostin A was methylated at least an order of magnitude less efficiently than either triostin C or *bis*-quinoline triostin A.

By contrast, we failed to observe the formation of any new labelled products when protoplast suspensions were supplemented with either tetra-*N*-demethyltriostin A (TANDEM) or naphthyl-TANDEM, a derivative having 2-naphthoic acid moieties in place of quinoxaline rings. Furthermore, neither of these tetra-*N*-demethyl analogues inhibited echinomycin synthesis or provoked the accumulation of triostin A.

Discussion

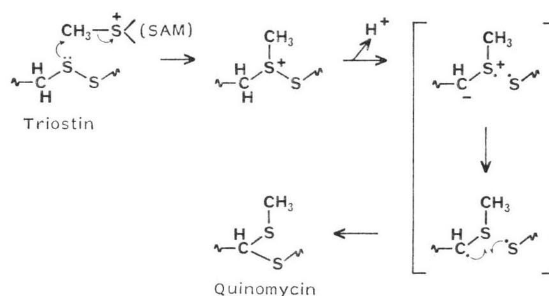
The present study has served to establish that protoplasts of *S. echinatus* are capable of converting a series of triostin compounds to the corresponding quinomycins with concomitant incorporation of methyl groups from L-[*methyl*-^{14}C]methionine. It has also been shown that triostins, while serving as substrates for methylation, reduce the level of echinomycin synthesis by protoplasts and cause the apparent accumulation of triostin A synthesized *de novo*. These findings establish that *S. echinatus* has the capacity to produce triostin A. They also suggest that triostin A is subsequently converted to echinomycin under normal circumstances since it accumulates only in the presence of exogenously-supplied triostins which presumably compete with it for the enzyme (or enzymes) which effect the triostin-quinomycin conversion. Details of the enzymology of this transformation remain to be established. Nevertheless, certain properties of the methylase(s) are apparent from the results presented here. The system in *S. echinatus* clearly has broad substrate specificity since it is able to transform a variety of triostins not normally encountered in this organism. On these grounds it seems reasonable to speculate that streptomycetes which produce both echinomycin and quinomycin C (*e.g. Streptomyces* sp. 1752 and *Streptomyces* sp. 732)[2] synthesize triostin A and triostin C which are then methylated by a common enzyme-system.

Our failure to detect conversion of tetra-*N*-demethyltriostin A (TANDEM) to tetra-*N*-demethylechinomycin suggests that the four *N*-methyl groups of triostin A may be required for enzymic recognition. This is not altogether unexpected in the light of recent crystallographic evidence which shows that the three-dimensional structures of TANDEM and triostin A differ significantly[19,20]. Alternatively, it could be argued that *S. echinatus* produces TANDEM as the precursor of triostin A. This seems unlikely because we also failed to detect novel products or indeed any other effect with *bis*-naphthyl-TANDEM (results not presented). Furthermore, available evidence suggests that *N*-methyl groups of cyclic peptide antibiotics are attached during the synthesis of the peptide rings and not after their formation[17].

The finding that *bis*-6-bromoquinoxaline triostin A is methylated with appreciably lower efficiency than triostin C or *bis*-quinoline triostin A shows that substituents on the benzenoid portion of the quinoxaline chromophores can affect enzymic recognition. This may explain why *S. echinatus* was found to incorporate 6-methylquinoline-2-carboxylic acid and 7-chloroquinoxaline-2-carboxylic acid into novel derivatives of both triostin A and echinomycin, yet produced only analogues of echinomycin when supplemented with quinoline-2-carboxylic acid[9,10].

A plausible mechanism for triostin-quinomycin interconversion involving a free radical intermediate is described in Fig. 4. It should be noted that echinomycin contains an asymmetric center as a result of this rearrangement. This center has been shown to have *S*-chirality using NMR measurements[21], proving that only one of the two chemically equivalent cysteine β-C–H protons is expelled. Whether this is due solely to enzyme stereospecificity is not known. However, it is well established that triostins can adopt different conformations as a result of the flexibility of the disulphide cross-bridge[22] and it may be that the enzyme(s) which effect the conversion recognize a particular conformation of the antibiotic in which only one of the cysteine β-C–H protons is favourably disposed for elimination.

Fig. 4. Possible mechanism for the conversion of the triostin disulphide to the quinomycin thioacetal. SAM = *S*-adenosylmethionine.



Finally, on the basis of the results reported here we suggest that protoplasts of *Streptomyces* sp. may represent a useful alternative to whole-cell or cell-free systems for elucidating whether compounds of high molecular weight are involved as intermediates in the biosynthesis of particular antibiotics.

Acknowledgments

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