STUDIES ON THE CELL-FREE SYNTHESIS OF ECHINOMYCIN AND AN ECHINOMYCIN ANALOGUE†

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ABSTRACT

Echinomycin producing *Streptomyces* cultures (X-53 or X-63) grown in the presence of quinazol-4-one-3-acetic acid yield an analogue in which one of the two quinoxaline-2-carboxyl residues is replaced by a quinazol-4-one-3-acetyl moiety. This analogue has been designated quinazomycin.

Cell-free extracts of culture X-63 synthesize echinomycin in the presence of precursors, ATP, Mg^{2+} and mercaptoethanol. Replacement of quinoxaline-2-carboxylic acid in this solution with quinazol-4-one-3-acetic acid yields an echinomycin analogue with two quinazol-4-one-3-acetyl residues, designated biquinazomycin.

The studies reported‡ owe their origin to a hypothesis that the group of antibiotics having heterocyclic or other moieties attached to a peptide sequence, might owe their origin to detoxication of the heterocyclic or other moiety by peptidation by the antibiotic producing organism. If this is so, introduction of analogues of the heterocycle or other non-peptide portion of the antibiotic into the antibiotic synthesising system should lead to the formation of analogues of the antibiotic. As echinomycin producing cultures were available to us (X-53[1] and X-63[2]) studies with these cultures were undertaken.

Echinomycin is a quinoxaline antibiotic isolated from several *Streptomyces* cultures. Although it possesses antitumour, antiviral and antibacterial activities[3, 4], its low therapeutic index has precluded any utility. The structure (I, R_1 R_2 II) of this antibiotic was established by Prelog and his associates[5]. This symmetrical dimeric molecule has an unusual cyclic octapeptide ring made up of two residues each of Ser, Ala, *N*-methylhomocysteine and *N*-methylvaline. The homocysteine residues are joined to form a dithiapyran ring and the octapeptide to two quinoxaline-2-carboxylic acid (II) residues through amide bonds.

A further reason for attempting to obtain analogues of echinomycin was the further report[6] that an echinomycin producing culture produced analogues in the presence of quinaldinic acid.

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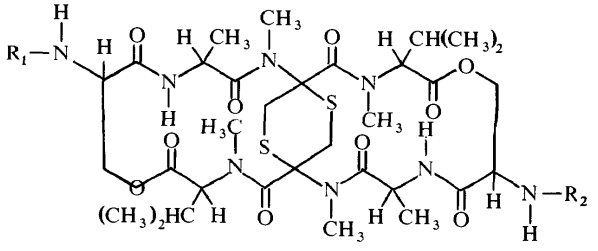
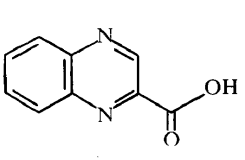
‡ These studies have been reported. For further experimental details see references 7 and 8.



I



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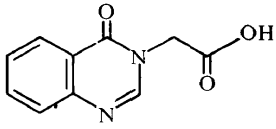

III

QUINAZOMYCIN[7]

For the microbiological synthesis of quinazomycin (I, R_1 II, R_2 III), an echinomycin analogue, *Streptomyces* culture X-53 was grown in a glycerol–soya bean–salt medium containing quinazol-4-one-3-acetic acid (III) (0·2 g/l.) in shake flasks at 27° ± 1° for 5 days. The mycelium was separated from the broth and extracted with ethanol and the broth extracted with *n*-butanol after its pH had been adjusted to 6. After removal of solvent from the combined extracts, the residue was partitioned in a Craig 100 tube counter-current apparatus with methanol–benzene–carbon tetrachloride–water (4:2.5:2.5:1) as the solvent system. The contents of every fourth tube were evaporated to dryness, their activity against *Staphylococcus aureus* tested and their UV spectra determined. The contents of tubes 16–35 were identified as echinomycin on the basis of biological activity, its movement under these conditions of counter-current separation and its UV absorption (λ_{max} 242 and 320 nm). A second antibiotic (λ_{max} 225, 244, 290 and 328 nm) was located in tubes 40–55 and was purified by preparative TLC on silica gel plates with methanol–water (1:1) as the solvent and crystallization from methanol. This product, designated quinazomycin, did not melt below 300° and was characterized by parallel hydrolytic experiment as had been used for the characterization of echinomycin.

6N Hydrochloric acid hydrolysis at 100° for 24 h of echinomycin and of quinazomycin yielded the same three ninhydrin sensitive zones, whereas earlier desulphurization with Raney nickel yielded the expected four amino acids.

6N Hydrochloric acid hydrolysis at 40° over 4 days, extraction, after removal of hydrochloric acid, with carbon tetrachloride and silica gel column chromatography enabled the isolation of both quinazol-4-one-3-acetic acid (λ_{max} 225 and 290 nm) and quinoxaline-2-carboxylic acid (λ_{max} 242 nm) in crystalline form.

These data indicate that quinazomycin possesses a quinazol-4-one-3-acetyl

residue as well as a quinoxaline-2-carboxyl one, contains the same amino acids as echinomycin and in all probability also a dithiapyran ring. It does not, however, establish that both antibiotics have the same amino acid sequences.

CELL-FREE SYNTHESIS OF ECHINOMYCIN[8]

In order to determine the optimum time for harvesting the *Streptomyces* X-63 culture, cell-free extracts from cells obtained after varying periods of fermentation were tested for their ability to bring about antibiotic synthesis. It was found that the echinomycin biosynthesizing system made its appearance after 25 h in shake flasks at 28°. It was also found that echinomycin could be detected in the cells after 25–30 h of fermentation and in the culture filtrate after 24 h. Harvesting was, therefore, done after 30 h. By this time the biosynthesizing system becomes available and only a small amount of echinomycin is present in the cells.

The nutrient medium for this fermentation contained glucose, peptone, yeast extract and sodium chloride. The cells were collected by slow speed centrifugation, washed thrice with 0.05 M phosphate buffer (pH 7.2) containing 0.02 M magnesium acetate and 0.02 M mercaptoethanol. They were then macerated with twice their weight of sand with a pestle and mortar and portion-wise addition of 0.1 M phosphate buffer (pH 7.2) containing 0.02 M magnesium acetate and 0.02 M mercaptoethanol. The final slurry, which contained about 1 g of cell per 1 ml of buffer, was centrifuged (1000 *g*) free of debris and unbroken cells and the supernatant then centrifuged at 10 550 *g* for 15 min. The 10 550 *g* supernatant was further centrifuged at 105 000 *g* for 45 min. After passing through sintered filters, these two solutions constituted the enzyme solutions. They contained 6.0 and 3.5 mg/ml of protein respectively and had similar enzymatic activities. The 10 550 *g* supernatant was used for preparative experiments and the 105 000 *g* supernatant for the other tests. After twofold dilution these solutions gave inhibition zones of 9.0 mm on *S. aureus* plates and incubation of the cell-free solutions for periods up to 24 h did not give larger inhibition zones, indicating that no further antibiotics synthesis occurred.

The composition of the cell-free echinomycin synthesizing system was as follows (in μmoles except when stated):—

ATP, 10; creatine phosphate, 5; creatine kinase, 20 μg; DL-Ser, 2; Ala, 1; Cys, 1; Val, 1; Met, 1 and quinoxaline-2-carboxylic acid, 10 in 0.6 ml water. To this was added 0.4 ml of 0.1 M phosphate buffer containing 0.02 M mercaptoethanol and 0.02 M magnesium acetate and 1.0 ml of enzyme solution added to make the total volume 2.0 ml. ATP, creatine phosphate and creatine kinase could be replaced with mitochondria from 250 mg of fresh rat liver along with 200 μmoles each of sodium succinate, sodium citrate and glucose.

Incubation of the complete cell-free system resulted in rapid increase in antibiotic activity for 4 h which then gradually tapered off. After 4 h incubation, inhibition zones of 18 mm were obtained and after 12 h, the inhibition zones were 22 mm.

To establish that the antibiotic synthesized was echinomycin, a preparative scale experiment (100 ml enzyme solution) was carried out in the presence of (^{14}C-1)-alanine (25μC). After incubation at 30° for 4h, the pH of the reaction

mixture was adjusted to 4 and the antibiotic extracted out with ethyl acetate. After removal of solvent, the residue was subjected to counter-current distribution (100 transfers) with the solvent system used for isolating quinazomycin. The contents of the individual tubes were evaporated to dryness and taken up in water (1.0 ml). The UV spectra, radio-activity (liquid scintillation counter) and antibiotic activity (*S. aureus*) were determined of aliquots. It was found that the antibiotic material was similarly located (tubes 16–35) as in the earlier experiments, had UV maxima at 244 and 320–330 nm. This echinomycin (2.2 mg) had a total radioactivity of 200 000 cpm. accounting for the synthesis of 29 per cent of the antibiotics peptide moiety. This would suggest that a substantial amount of preformed peptide sequence was already present in the cell-free extract. This fact is not, however, consistent with the finding that no antibiotic is synthesized in the absence of cysteine.

Studies on the complete cell-free system revealed that antibiotic synthesis was maximum at pH 7.0 and sharply reduced at pH below 6·0 or about 7.5. Replacement of Mg^{2+} with Ca^{2+} or Mn^{2+} reduced antibiotic synthesis by 25 and 50 per cent respectively and no antibiotic was synthesized in the presence of Cd^{2+}. Mercaptoethanol could be replaced with glutathione but no antibiotic was synthesized when neither was present.

Antibiotic synthesis was completely inhibited when the enzyme solution was treated with RNase or protamine but only slightly reduced when treated with DNase, alkaline conditions, chloramphenicol or cycloheximide.

After dialysis of the enzyme solution, neither dialysate nor diffusate had any antibiotic synthesizing activity. This activity was, however, reconstituted by mixing these two materials. Fractionation by ammonium sulphate precipitation yielded no active fractions. The three precipitates obtained with one-fourth, half and three-fourths saturation of ammonium sulphate all regained activity on mixing with the freeze-dried diffusate obtained from the dialysis experiment.

BIQUINAZOMYCIN[8]

To test whether analogues of echinomycin could be obtained by cell-free synthesis, quinazol-4-one-3-acetic acid was incorporated into the cell-free echinomycin synthesizing medium in place of quinoxaline-2-carboxylic acid. After incubation, the pH of the solution was adjusted to 6.0 and the antibiotic material extracted with *n*-butanol. After removal of solvent, the residue was subjected to counter-current separation using the same solvent system as for quinazomycin. Almost all the antibiotic activity was located in tubes 79 to 96. Removal of solvent yielded quinazol-4-one-3-acetic acid and an antibiotic, which was isolated by preparative TLC on silica plates with *s*-butanol–acetic acid–water (3 : 3 : 1) as the solvent. The antibiotic was obtained as a white powder that did not melt below 300° and had UV maxima at 225 and 290 nm. Parallel hydrolytic and chromatographic experiments with biquinazomycin, quinazomycin and echinomycin indicated that all three antibiotics had the same amino acids and 6N hydrochloric acid hydrolysis at 40° for 5 days of biquinazomycin, followed by removal of hydrochloric acid and silica gel column chromatography yielded only quinazol-4-one-3-acetic acid and no quinoxaline-2-carboxylic acid.

It follows that biquinazomycin (I, $R_1 = R_2 =$ III) is an analogue of echinomycin containing quinazol-4-one-3-acetyl residues in place of quinoxaline-2-carboxyl ones. The amino acid residues in both antibiotics are the same but it is still to be proven that the sequence is the same.

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