NOTES

PREPARATION OF RADIOACTIVE POLYOXINS A, B, C AND I

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(Received for publication November 6, 1974)

The polyoxins[1,2] are antifungal antibiotics produced by *Streptomyces cacaoi* var. *asoensis*.[3] We have investigated the mechanism of polyoxin-resistance of the phytopathogenic fungus *Alternaria kikuchiana*, with the use of labelled antibiotics.[4] In the present paper, a biosynthetic method for the preparation of radioactive polyoxins using the polyoxin-producing organism is described.

Orotic acid-6-^{14}C (62 mCi/mmole), uracil-2-^{14}C (62 mCi/mmole), thymine-2-^{14}C (60 mCi/mmole) and uracil-6-^{3}H (24.9 Ci/mmole) were purchased from the Radiochemical Centre, England. Polyoxins A, B, C, D, E, F, G, K, L and M were obtained from the Kaken Chemical Co., Ltd. Polyoxins H, I, J, carbamoylpolyoxamic acid,[1] polyoxamic acid[1] and 5-hydroxymethyluracil[1] were the generous gifts of Dr. K. ISONO, the Institute of Physical and Chemical Research. Polyoximic acid[1] was prepared by alkaline hydrolysis[1] of polyoxin A.

A strain of *S. cacaoi* var. *asoensis* was kindly supplied by Dr. S. SUZUKI of the institute referred to above. Spores of the organism were inoculated into a 300-ml Erlenmeyer's flask containing 50 ml of liquid medium.[3] After incubation at 27°C for 28 hours with reciprocal shaking, 5 ml of the culture broth was inoculated to 50 ml of the same medium and incubated again under the above conditions. Mycelial pads were collected by filtration and washed twice with sterilized 0.066 M phosphate buffer, pH 6.47. For experiments incorporating the labelled compounds into the polyoxins, 10 g of washed mycelium were suspended in 100 ml of the phosphate buffer.

A typical incubation mixture contained a labelled compound of an appropriate radioactivity and 70 ml of the mycelial suspension. The mixture was incubated at 27°C for 30 hours with reciprocal shaking. The filtrate of the incubated mixture was treated with 15 g of activated carbon. The carbon was washed with distilled water and then the absorbates were eluted with 1 liter of 60% aqueous acetone. The eluate was concentrated to a small volume under reduced pressure and introduced to a 50-ml column of Dowex 50W ×8 (H-form). The polyoxins were eluted with 1 liter of 0.3 N ammonium hydroxide. After concentration the eluate was subjected to chromatography on a 50-ml column of Amberlite IR-4B (Cl-form). The effluent and washings were combined and concentrated under reduced pressure to dryness. As noted by ISONO *et al*,[5] the residual fraction contained the polyoxin complex excluding polyoxins D, E and F. Radioactivity in an aqueous solution was determined as follows: An aliquot of the solution was suspended in 15 ml of liquid scintillation fluid, containing 10% naphthalene, 0.8% 2,5-diphenyloxazole, 0.02% 1,4-di-2-(4-methyl-5-phenyloxazole)-benzene and 2% ethylene glycol made up in diethylene dioxide. The radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

For Avicel thin-layer chromatography (TLC), the following solvents were used: mixture I, butanol-acetic acid-water (4:1:2 or 4:1.2:2.5); mixture II, 75% phenol; mixture III, methanol-pyridine-acetic acid-water (6:6:1:3); mixture IV, isobutyric acid-1 N ammonium hydroxide (5:3). Radioactivity on the TLC plate was located with an Aloka thin-layer chromatogram scanner (model TRM 1B) or with radioautography using Fuji X-ray film.

Incorporation of ^{14}C-Compounds into Polyoxin Complex

When orotic acid-6-^{14}C, uracil-2-^{14}C and thymine-2-^{14}C were incubated with the washed mycelia of *S. cacaoi* var. *asoensis*, incorporation of radioactivity into the polyoxin complex was observed. As Table 1 shows, orotic acid was incorporated most efficiently of the three into the polyoxin complex.

Table 1. Incorporation of ^{14}C-compounds into polyoxin complex by *Streptomyces cacaoi* var. *asoensis*

^{14}C-Compounds	Radioactivity incorporated into polyoxin complex (μCi)
Orotic acid-6-^{14}C	1.13
Uracil-2-^{14}C	0.36
Thymine-2-^{14}C	0.07

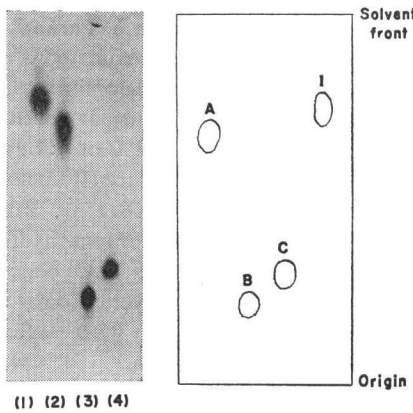
The experiment was carried out as described in the text except that the incubation mixture contained 10 μCi of each compound.

Preparation of ^{14}C-labelled Polyoxins A, B, C and I from Orotic Acid-6-^{14}C

From 250 μCi of orotic acid-^{14}C, 45 μCi of the polyoxin complex was obtained. It contained at least eight radioactive components. Four major components **1** (9.5 μCi), **2** (15.4 μCi), **3** (6.3 μCi) and **4** (2.1 μCi) were isolated from the complex by Avicel TLC using the solvent mixtures from I~IV (Fig. 1). The

Fig. 1. Avicel thin-layer chromatogram of ^{14}C-labelled compounds (**1**), (**2**), (**3**) and (**4**) prepared from orotic acid-6-^{14}C.

Chromatography was performed in mixture II. Left, a radioautogram; right, authentic samples of polyoxins detected by ultraviolet absorption.



four components gave positive ninhydrin reactions and exhibited ultraviolet absorption. Each of the components was submitted again to Avicel TLC in the above solvent mixtures with authentic samples of polyoxins A~M. The Rf values of **1**~**4** were identical with those of polyoxins I, A, B and C, respectively. And the ultraviolet absorption spectra of **1**~**4** in 0.05 N hydrochloric acid and in 0.05 N sodium hydroxide showed the same maximums as those[1] of polyoxins I, A, B and C, respectively. Alkaline hydrolysis with 0.5 N sodium hydroxide at 65°C for 4 hours yielded two radioactive products, that were identified by Avicel TLC as 5-hydroxymethyluracil and polyoxin C. In addition, nonradioactive product from **1** and **2** identified as polyoximic acid, and from **2** and **3** identified as polyoxamic acid were found.

From the results described above, it is concluded that **1**, **2**, **3** and **4** are ^{14}C-labelled polyoxins I, A, B and C, respectively. And also that orotic acid-6-^{14}C is incorporated into the pyrimidine moiety of the polyoxins.

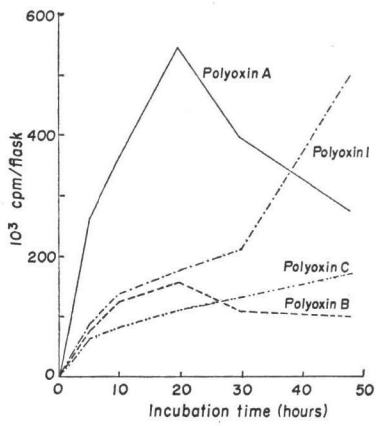
The total amounts of labelled polyoxins A (5.9 mg), B(2.1 mg), C(0.4 mg) and I (3.4 mg) were assayed by ultraviolet absorption at 262 nm, and the specific radioactivities were calculated as 1.61, 1.53, 1.76 and 1.41 μCi/μmole, respectively. The radiochemical purities of the labelled polyoxins were 98% as analysed by Avicel TLC.

Time Course of Orotic Acid-^{14}C Incorporation into Polyoxins

The incubation mixtures contained 10 μCi of orotic acid-6-^{14}C and 10 ml of the mycelial suspension in 50-ml flasks. The mixtures were incubated at 27°C for various time intervals with shaking. Polyoxins A-, B-, C- and I-^{14}C were isolated from each of the incubation mix-

Fig. 2. Time course of orotic acid-^{14}C incorporation into polyoxins

The experiment was carried out as described in the text.



tures and their radioactivities were determined. The results obtained are plotted in Fig. 2. The radioactivities incorporated into polyoxins A and B reached their maxima at 20 hours decreasing beyond that time. Polyoxins C- and I-^{14}C increased gradually throughout the incubation period with the latter compound rapidly increasing after 30 hours.

Preparation of ^{3}H-Labelled Polyoxins A, B, C and I from Uracil-6-^{3}H

Polyoxins A(33.6 μCi), B(4.1 μCi), C(3.2μCi) and I(26.5μCi) labelled with tritium were obtained from 1 mCi of uracil-6-^{3}H according to the method described. The specific activities were approximately 3.1 μCi/μmole, as calculated from ultraviolet absorptions at 262 nm.

Acknowledgements

We wish to thank Drs S. SUZUKI and K. ISONO for their gifts and advice.

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