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Preparation of [2a,8a,10a,14a-^{3}H]Geldanamycin by Biosynthesis

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SUMMARY

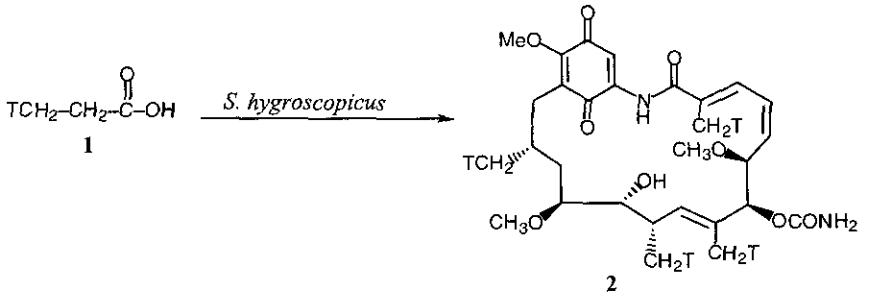
[3-^{3}H]Propionic acid (**1**) was prepared by catalytic hydrogenation of 3-bromopropionic acid with tritium gas. Fermentations of *S. hygroscopicus* were treated with **1** to produce [2a,8a,10a,14a-^{3}H]geldanamycin (**2**) with a specific activity of 76.2 mCi/mmol. The effect of the amount of **1** used and the dosing pattern on the specific activity of **2** was investigated.

Keywords: geldanamycin, tritium, fermentation, biosynthesis

INTRODUCTION

The technique of tracing the biosynthesis of natural products by treating organisms with labeled materials is a well established science with an extensive literature. This procedure, however, is usually not considered feasible for producing labeled materials for pharmacological work because of the low amount of labeled precursor incorporated into the desired product. We were interested to find whether sufficiently high amounts of simple, inexpensive radioactive precursors could be used to yield biologically labeled natural product drugs with a specific activity useful for drug development work.

We chose geldanamycin (**2**) for this investigation because of the considerable current interest in its potential as an anticancer drug (1) and its novel mechanism of action (2), and because its complex structure would be difficult to specifically label by



1 **2**

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conventional chemical methods. Geldanamycin is an ansamycin antibiotic produced by *Streptomyces hygroscopicus geldanus nova,* UC-5208. Previous biosynthesis studies (3) with carbon-14 suggested that [3-^{3}H]propionate would be incorporated into geldanamycin in metabolically and chemically stable positions.

RESULTS AND DISCUSSION

In order to determine the optimum time for dosing with the labeled propionate, a preliminary fermentation was run and monitored for geldanamycin by HPLC. The fermentation medium reported by DeBoer (4) was used. In our hands geldanamcin was first detected five days after inoculation of the production medium and appeared to maximize at about day ten. We used four label dosing regimens to determine the effect of the amount of radioactivity used and the dosing schedule on the specific activity of the labeled product.

[3-^{3}H]propionic acid **1** was prepared at a specific activity of 19.3 Ci/mmol by catalytic reduction of 3-bromopropionic acid with tritium gas. Three concentrations of radiolabel were tried in the fermentations, 0.333 mCi/mL, 0.666 mCi/mL, and 2.50 mCi/mL. Dosing was done on the day before the expected start of geldanamycin production. The 0.333 mCi/mL dose gave a specific activity of 48.5 mCi/mmol; the 0.666 mCi/mL dose gave 76.2 mCi/mmol. The 2.50 mCi/mL dose killed the bacteria, and no geldanamycin was produced. A fermentation was done with a final concentration of label at 0.666 mCi/mL, but with the dose divided into two parts, half given the day before geldamanycin production and half given two days later. The divided dose fermentation gave a specific activity of 53.0 mCi/mmol.

These results show that the specific activity of the product is, as expected, increased with increasing concentration of available label, limited by the tolerance of the organism to the radioactivity. The fact that the divided dose gave a lower specific activity indicates that the propionate is incorporated into geldanamycin precursors early in the biosynthetic scheme. ^{3}H NMR shows that the tritiated propionate was incorporated into the molecule as predicted, with the labeled carbon-3 forming the four methyl groups on the macrocyclic ring. No other positions were labeled. The suitability of [2a,8a,10a,14a-^{3}H]geldanamycin prepared in this way for pharmacological studies has been demonstrated by its use in the development of a novel drug delivery mechanism (5).

The radioactivity did not cause any observable morphological mutation of the bacteria, but a fresh seed culture was produced for each fermentation.

EXPERIMENTAL

Proton and tritium NMR spectra were recorded on a Bruker AM500 spectrometer. Analytical TLC were performed using E. Merck silica-gel 60F-254 plates. Preparative TLC was performed using Analtech silica-gel GF, 2000 microns. Solvents were removed from solutions on a rotary evaporator under water aspirator vacuum at ambient temperature. Radioactive samples were counted on a Packard Tri-carb

2100TR liquid scintillation counter. Developed TLC plates were scanned on a Bioscan Sys. 200A imaging scanner. HPLC was done using a Waters Associates model 510 dual pump system with a model U6K septumless injector and an IN/US Systems model 1B β-RAM flow radioactivity monitor.

A reference sample of geldanamycin was provided by The National Cancer Institute. A culture sample of *Streptomyces hygroscopicus geldanus nova*, UC-5208, was provided by Pharmacia & Upjohn, Inc. The active culture was maintained on petri dishes of Trypticase Soy Agar. Fermentations were incubated with shaking in a New Brunswick Scientific Series 25 Environmental Shaker with a 1 inch orbital rotation set at 100 RPM at room temperature. All media were autoclaved for 20 min at 121 °C. Fermentations were done protected from light, and purification and analysis of geldanamycin was done under red light.

[3-^{3}H]Propionic acid, 1

A solution of 12 mg of 3-bromopropionic acid in 0.5 mL of 1N NaOH with 5 mg of 10% Pd on carbon catalyst was exposed to carrier-free tritium gas at a starting pressure of 679 mm Hg for 4 h. The catalyst was filtered away, and the solvent was removed on a vacuum line. The residue was exchanged 3 times with water-methanol on the vacuum line. The yield was 1.518 Ci with a specific activity of 19.3 Ci/mmol (321 mCi/mg), determined by UV spectrum.

For dosing, 1 Ci was diluted to 45 mL of water and adjusted to pH 7 with HCl. The solution was sterilized by membrane filtration.

[2a,8a,10a,14a-^{3}H]Geldanamycin, 2

A seed culture for the production fermentation was prepared by incubation of *S. hydroscopicus* from the stock TSA plates in a medium composed of 1.0 g of glucose monohydrate, 1.0 g of peptone, 0.25 g of yeast extract, and 100 mL of water until a satisfactory growth was indicated by the development of white spherical colonies.

A production medium composed of 60 g of glucose monohydrate, 3.75 g of peptone, 3.75 g of tryptone, 3.75 g of yeast extract, 15 mL of molasses and 7.5 g of oatmeal in 1500 mL of water was prepared. The pH was adjusted to 7.0 with 1 N NaOH, and 100 mL was added to each of fifteen 500 mL baffled culture flasks. The culture flasks were inoculated with the seed culture and incubated for 3 days. Each culture flask was dosed with 3 mL of the [3-^{3}H] propionic acid solution (66.6 mCi), and incubation was continued for another 5 days.

Celite was added to each flask, and the medium was filtered through paper. The cell bodies were washed with ethanol and chloroform, which was added to the filtrate. The filtrate was extracted 3 times with 250 mL of chloroform, and the extract was dried ($NaSO_4$) and evaporated to give 210 mg of crude product. The impure material was chromatographed on 4 silica gel plates eluted with chloroform-methanol 95:5. The yellow geldanamycin bands were combined, and the product was washed from the

silica with chloroform-ethanol 1:1. The product still contained exchangeable tritium, which was removed by back exchange in ethanol. The yield was 6.25 mCi (46 mg) of **2** with a specific activity of 76.2 mCi/mmol (135 μCi/mg). The product was 95% radiochemically pure by TLC [silica, chloroform-methanol 95:5, Rf 0.48] and HPLC [Zorbax Rx C_8, methanol-acetonitrile-water (30:30:40), 1 mL/min, t_R 18 min] with retentions the same as a standard sample. The UV spectrum and ^1H NMR spectrum were the same as a standard sample. ^3H NMR ($CDCl_3$): δ 2.05, (t, 1, J = 15 Hz, 2aCH_3), 1.82, (t, 1, J = 15 Hz, 8aCH_3), 0.99 (m, 2, 14a and 10aCH_3).

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