# PREPARATION OF $^{14}C$-LABELED SHOWDOMYCIN

YOSHIHIDE KOMATSU

Shionogi Research Laboratory, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka, Japan

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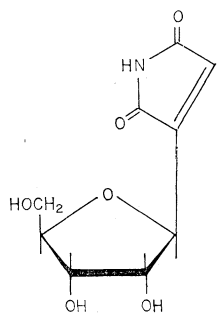
When *Streptomyces showdoensis* ($N_2$-209-56) mycelia were grown in the presence of sodium acetate-2-$^{14}C$, the radioactive carbon from acetate-2-$^{14}C$ was fairly well incorporated into the showdomycin molecule; with approximately 14 % of the total radioactivity of the added sodium acetate-2-$^{14}C$ being incorporated during a 10-hour incubation. Although three antibiotic substances were detected in the fermentation broth, the radioactive carbon from acetate-2-$^{14}C$ was incorporated only into showdomycin. The radioactive showdomycin was extracted with methanol from the lyophilized powder of the fermentation broth, re-extracted with *n*-butanol, and finally purified by chromatography on a silicic acid column. Over 98.1 % of the total radioactivity of this preparation was due to the $^{14}C$-showdomycin and approximately 0.33 $\mu$Ci of the preparation corresponded to 1 $\mu$mole of showdomycin. About 2.8 % of the radioactivity of the added sodium acetate-2-$^{14}C$ was recovered as that of the purified showdomycin-$^{14}C$.

Showdomycin (SHM), a maleimide-C-riboside antibiotic (Fig. 1)[1,2,3] inhibits growth of bacteria[4], EHRLICH ascites tumor cells in mice[4,5] and the syntheses of various macromolecules (DNA, RNA, protein and cell wall) in *Escherichia coli* cells[6,7]. These inhibitions were cancelled by the addition of nucleosides[4,6,7,8]. The inhibitory action of the structurally related analog, N-ethylmaleimide (NEM), was not reversed by the addition of nucleosides.

Fig. 1. Structure of showdomycin




Since in cell-free extracts of *E. coli* the antibiotic inhibits both the ribonucleotide reductase system and the dTMP synthetase system[9] and there was also a corresponding decrease in the intracellular pool of the corresponding deoxyribonucleotides[7], it seemed likely that inhibition of these enzyme systems would be a possible cause of the inhibition of the DNA synthesis in *E. coli* cells. However, in cell-free systems the inhibitory action of the antibiotic on both enzyme systems was not reversed by nucleosides[9]. Thus, the question arose why nucleosides cannot reverse the inhibitory action of SHM in a cell-free system.

SUHADOLNIK *et al.*[10] reported that acetate serves as the precursor for either two or four of the carbons of the maleimide ring of SHM.

In order to study the mechanism for the reversal of the SHM-inhibition by nuleosides, the effect of nucleosides on the transport of SHM into *E. coli* K-12 cells was examined, with $^{14}C$-labeled SHM (SHM-$^{14}C$)[11,12]. The present paper describes a simple

procedure for the preparation of SHM-$^{14}$C by incubating acetate-2-$^{14}$C with *Streptomyces showdoensis* $N_2$-209-56.

## Materials and Methods

Paper chromatographic analysis of SHM-$^{14}$C: The analytical sample, *e. g.* fermentation broth, supernatant fluid after removal of the mycelium, butanol extract or fractions, was mixed with reference non-labeled SHM and developed with solvent I, *n*-butanol - ethanol - water (50 : 15 : 35) or, where necessary, with solvent II, *n*-butanol - formic acid - water (77 : 10 : 13) or with solvent III, *n*-butanol - methanol - water (20 : 7 : 8) on Whatman 3MM paper for 15~18 hours by the ascending method. For identification of the radioactive SHM on the paper chromatograms, the spot of reference non-labeled SHM was located using UV lamp. The chromatograms were then cut into pieces (0.5~1.0 cm) and counted using a liquid scintillation spectrometer with a toluene phosphor solution.

*Streptomyces*: *Streptomyces showdoensis* $N_2$-209-56 was obtained from Dr. M. MAYAMA. This strain is a mutant of *Streptomyces showdoensis* C-224, which produced SHM in a higher yield than the original strain with synthetic medium[13].

Chemicals: Sodium acetate-2-$^{14}$C was purchased from Schwarz BioResearch Inc. Silicic acid [100 mesh (powder), $SiO_2 \cdot xH_2O$] was purchased from Mallinckrodt Chemical Works.

## Results

### SHM-$^{14}$C Production by Growing Mycelium of *Streptomyces showdoensis* $N_2$-209-56

Lyophilized spores of *Streptomyces showdoensis* $N_2$-209-56 were suspended in 5 ml of BENNETT's medium (1 % D-glucose, 0.2 % Bacto Yeast Extract, 0.1 % Bacto Beef Extract and 0.2 % Difco Casamino Acid) and inoculated onto BENNETT's agar slants. After incubation for one week at 28°C, one loop from an agar slant of the *Streptomyces* was inoculated into 15 ml of BENNETT's medium and incubated at 28°C on a reciprocal shaker. After 22.5 hours, 2 ml of this seed culture were added to a flask

Fig. 2-A. Paper chromatograms of the fermentation broth at 2-minute and 2-hour incubation.

The fermentation broth at 2-minute incubation or that at 2-hour incubation (each 20 μl) was mixed with reference non-labeled SHM and developed with solvent I for 18 hours by ascending method.

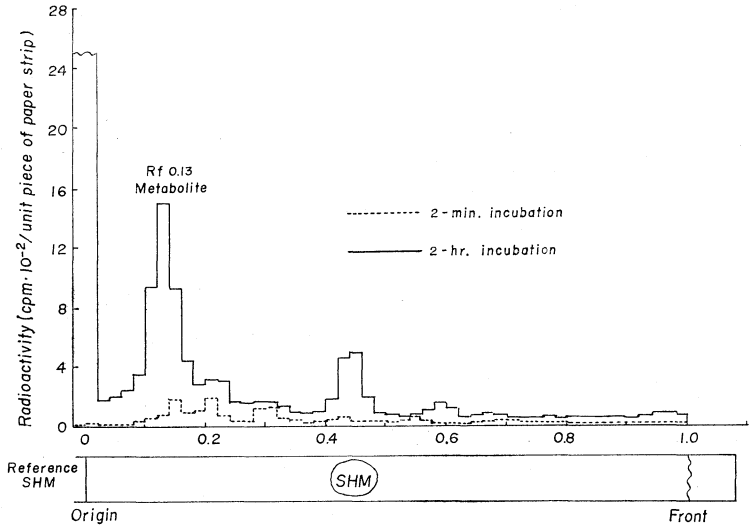
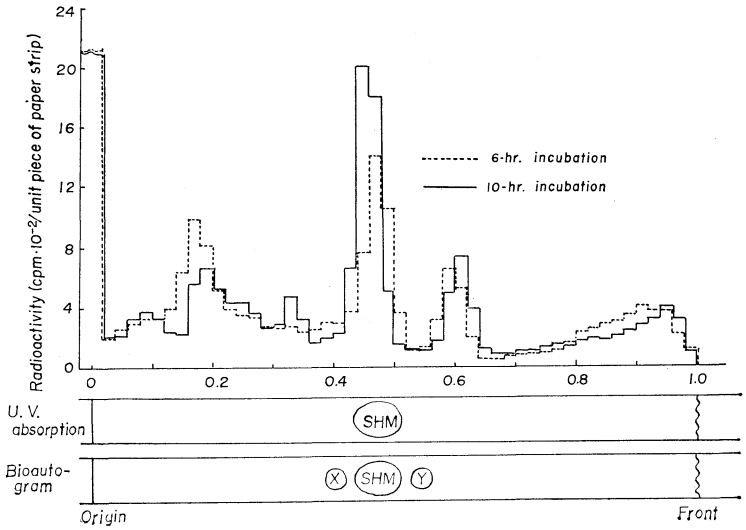


Fig. 2-B. Paper chromatograms of the fermentation broth at 6-hour and 10-hour incubation.

The fermentation broth at 6-hour incubation or that at 10-hour incubation (each 20 $\mu$l) was developed with solvent I for 18 hours by ascending method. The paper chromatogram of the fermentation broth at 10-hour incubation was bioautographed with *E. coli* K-12 as test organism.




containing 50 ml of the medium for production [2 % D-glucose, 0.4 % urea, 0.1 % $K_2HPO_4$, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % KCl, 0.001 % $FeSO_4 \cdot 7H_2O$ and tap water (pH 5.5)]. A sterile solution (0.4 ml) of sodium acetate-2-$^{14}$C (250 $\mu$Ci/4.9 $\mu$moles/ml) was added to the flask just after inoculation.

The nature of the antibiotics produced was examined by paper chromatography of the fermentation broth and bioautography of the developed chromatogram with *E. coli* K-12 as the test organism.

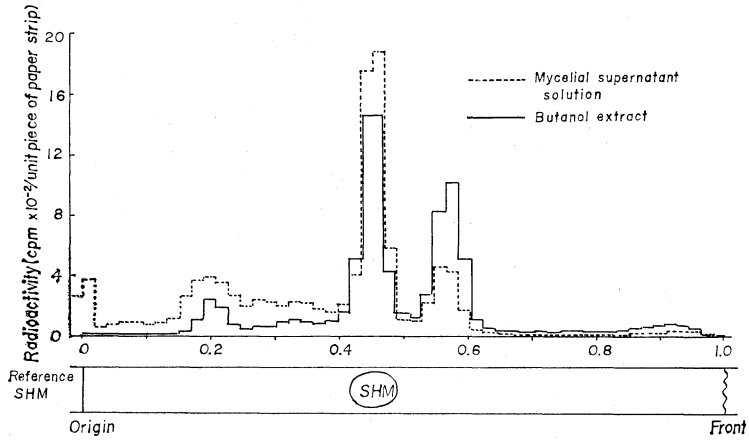
The incorporation of acetate-2-$^{14}$C into the mycelium and into various metabolites of the *Streptomyces* was followed at given time intervals. In the early stages of the incubation, the radioactive carbon from acetate-2-$^{14}$C was actively incorporated into an unknown metabolite in the culture fluid, located at 0.13 Rf value on the paper chromatogram developed with solvent I (Fig. 2-A). Incorporation of the radioactive carbon from acetate-2-$^{14}$C into SHM gradualy increased during further incubation, while that into the unknown metabolite described above actually declined (Fig. 2-B). Papaer-chromatographic analysis showed that approximately 3.5, 7.8, 10.8, 12.9 and 14.2 % of the total radioactivity of added acetate-2-$^{14}$C were detected at the same Rf value as the SHM reference sample after 2, 4, 6, 8 and 10-hour incubation, respctively.

In addition to SHM, two small spots (X and Y) were found on the bioautogram of the fermentation broth. It is of interest to note that the radioactive carbon from acetate-2-$^{14}$C was not significantly incorporated into these unknown antibiotics (Fig. 2-B).

Since most of the radioactivity detected at the same Rf value as the SHM reference sample remained in the supernatant solution after removal of the mycelium by centrifugation (Fig. 3), it seems that most of the SHM is released from the mycelium during fermentation.

Fig. 3. Paper chromatograms of the mycelial supernatant solution and the butanol extract.

The mycelial supernatant solution (20 $\mu$l) or the butanol extract (10 $\mu$l) was mixed with reference non-labeled SHM and developed with solvent I for 18 hours by ascending method.

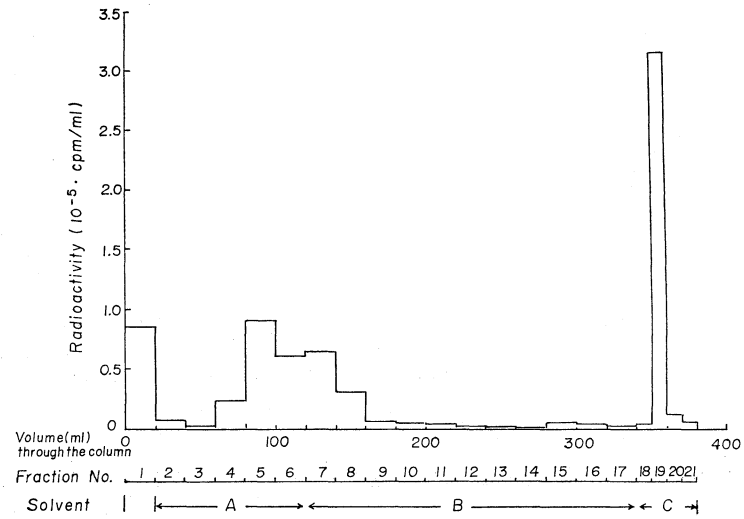



## Isolation of SHM-$^{14}$C

After 10-hour incubation the mycelium of the *Streptomyces showdoensis* was removed by centrifugation. The supernatant fluid (47 ml) was lyophilized, and then extracted three times with 15 ml of methanol. The methanol extracts were combined and evaporated to about one-tenth volume under reduced pressure. To the concentrated solution 15 ml of *n*-butanol was added and the methanol was exhaustively evaporated off under reduced pressure. The resulting precipitate was removed by centrifugation. The clear butanol extract containing SHM-$^{14}$C (Fig. 3) was charged onto a silicic acid column (1×10 cm, packed with benzene). The column was washed with 100 ml of benzene - acetone (9 : 1), and then with 200 ml of benzene - acetone (4 : 1). SHM was eluted with benzene - acetone (1 : 4) as shown in Fig. 4. The fractions

Fig. 4. Column chromatography of the butanol extract on silicic acid.

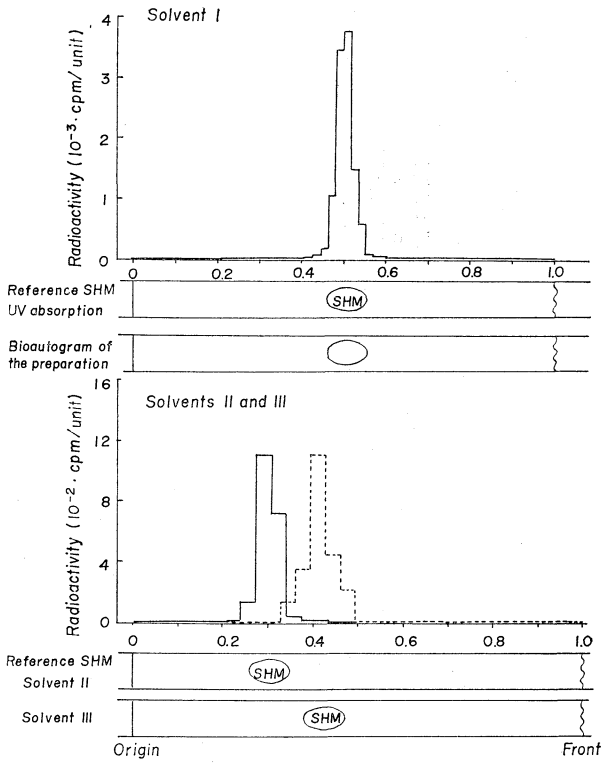
Solvent A : benzene - acetone (9 : 1) Solvent B : benzene - acetone (4 : 1)
Solvent C : benzene - acetone (1 : 4) For further explanation see text.



containing SHM-$^{14}$C (fraction 19 and 20) were combined and evaporated to dryness. Paper chromatographic analysis showed that over 98.1 % of the total radioactivity of this preparation was due to SHM-$^{14}$C (Fig. 5). This preparation showed an antibacterial activity identical to that of authentic SHM. Approximately 0.331 $\mu$Ci of the preparation corresponded to 1 $\mu$mole of SHM in an antimicrobial assay employing *E. coli* K-12 as a test organism. Total radioactivity of the SHM-$^{14}$C obtained was approximately 2.792 $\mu$Ci. This value corresponded to about 2.8 % of the sodium acetate-2-$^{14}$C employed.

Fig. 5. Paper chromatograms of the SHM-$^{14}$C preparation.

The SHM-$^{14}$C preparation was developed with solvent I, II or III for 15 hours by the ascending method. The paper chromatogram developed with solvent I was bioautographed with *E. coli* K-12 as test organism.
—— chromatogram developed with solvent I or that with solvent II.
----- chromatogram developed with solvent III.




## Discussion

The radioactive carbon from acetate-2-$^{14}$C was fairly well incorporated into the SHM molecule under the incubation conditions described above; approximately 14 % of the total radioactivity of added acetate-2-$^{14}$C (at 37.4 $\mu$M initial concentration) was incorporated into SHM during the 10-hour incubation.

It is of interest to note that the radioactive carbon from acetate-2-$^{14}$C was incorporated exclusively into SHM among the three antibiotic substances present in the fermentation broth of the *Streptomyces*.

The specific activity of the SHM-$^{14}$C obtained from the fermentation broth after 10-hour incubation was approximately 0.65 % that of the acetate-2-$^{14}$C. This value would probably be increased with increasing addition of acetate-2-$^{14}$C or with shortening of the incubation period, since the acetate-2-$^{14}$C given at a concentration of 37.4 $\mu$M was almost exhausted after 6-hour incubation. In fact, the specific activity of the preparation obtained from a fermentation broth incubated for 8 hours was 2.6 times that of the preparation obtained from a fermentation broth incubated for 10 hours.

We could easily isolate the SHM-$^{14}$C by extraction with methanol from the lyophilized powder of the mycelial supernatant solution, re-extraction from the methanol extract with *n*-butanol and use of silicic acid column chromatography. About 2.8 % of the radioactivity of the added acetate-2-$^{14}$C was recovered in the purified SHM-$^{14}$C.

Acknowledgement

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## References


1) DARNALL, K. R.; L. B. TOWNSEND & R. K. ROBINS : The structure of showdomycin, a novel carbon-linked nucleoside antibiotic related to uridine. Proc. Natl. Acad. Sci. 57 : 548～553, 1967
2) NAKAGAWA, Y.; H. KANO, Y. TSUKUDA & H. KOYAMA : Structure of a new class of C-nucleoside antibiotic showdomycin. Tetrahedron Letters No. 42 : 4105～4109, 1967
3) TSUKUDA, Y.; Y. NAKAGAWA, H. KANO, T. SATO, M. SHIRO & H. KOYAMA : The crystal structure of showdomycin and their derivatives. Chem. Commun. 9 : 975～976, 1967
4) NISHIMURA, H.; M. MAYAMA, Y. KOMATSU, H. KATO, N. SHIMAOKA & Y. TANAKA : Showdomycin, a new antibiotic from a *Streptomyces* sp. J. Antibiotics, Ser. A 17 : 148～155, 1964
5) MATSUURA, S.; O. SHIRATORI & K. KATAGIRI : Antitumor activity of showdomycin. J. Antibiotics, Ser. A 17 : 234～237, 1964
6) KOMATSU, Y. & K. TANAKA : Mechanism of action of showdomycin. I. Effect of showdomycin on the syntheses of nucleic acids and proteins in *E. coli* K-12. Agr. Biol. Chem. 32 : 1021～1027, 1968
7) KOMATSU, Y. & K. TANAKA : Mechanism of action of showdomycin. II. Effect of showdomycin on the synthesis of deoxyribonucleic acid in *Escherichia coli*. Agr. Biol. Chem. 34 : 891～899, 1970
8) NISHIMURA, H. & Y. KOMATSU : Reversal of inhibiting action of showdomycin on the proliferation of *Escherichia coli* by nucleosides and thiol compounds. J. Antibiotics 21 : 250～254, 1968
9) KOMATSU, Y. & K. TANAKA : Mechanism of action of showdomycin. III. Effect of showdomycin on the syntheses of deoxyribonucleotides in cell-free extracts of *Escherichia coli*. Agr. Biol. Chem. 35 : 526～536, 1971
10) SUHADOLNIK, R. J. : Nucleoside antibiotics. p. 393, John Wiley & Sons, Inc., New York, 1970
11) KOMATSU, Y. : Mechanism of action of showdomycin. IV. Interactions in the methanisms for transport of showdomycin and various nucleosides in *Escherichia coli*. Agr. Biol. Chem., in press.
12) KOMATSU, Y. : Mechanism of action of showdomycin. V. Reduced ability of showdomycin-resistant mutants of *Escherichia coli* for showdomycin and nucleoside uptake. in preparation
13) MAYAMA, M.; H. NAGATA & K. MOTOKAWA : Showdomycin, a new nucleoside antibiotic. III. Showdomycin production by a mutant of *Streptomyces showdoensis*. Annual Report of Shionogi Research Laboratory 18 : 13～22, 1968