SYNTHESIS OF CARBON-14 AND TRITIUM LABELED SAGAMICIN

TAKASHI DEGUCHI, SHUZO OKUMURA, AKIO ISHII and MASAO TANAKA

Pharmaceuticals Research Laboratory, Kyowa Hakko Co., Ltd.
1188 Shimotogari, Nagaizumi-machi, Suntogun, Shizuoka, Japan

(Received for publication June 4, 1977)

Sagamicin, an aminoglycoside antibiotic, was labeled with tritium by means of a platinum catalyzed hydrogen exchange reaction and by carbon-14 in two biosynthetic procedures. ^{14}C-Methyl-L-methionine was used as the radioactive precursor in these biological processes involving *Micromonospora sagamiensis*. The distribution of radioactivity in ^{14}C-sagamicin and gentamicin C_1 was studied by mild acid hydrolysis and HOFMANN degradation. The results showed that both C- and N-methyl groups were derived from methionine. One of the biosynthetic processes involved the conversion of gentamicin into sagamicin with resting cells and labeled methionine.

Sagamicin is a new antibiotic produced by *Micromonospora sagamiensis*[1] characterized as 6′-N-methyl-gentamicin C_{1a}[2]. For studying the metabolic behaviors of this antibiotic in animals[3], it was desirable to prepare radioactive sagamicin. ^{3}H-Gentamicin was reported to be obtained by catalytic hydrogen exchange reaction[4]. Labeling with tritium has intrinsically restricted applications for radiography or radioimmunoassay, unless high specific activity is developed.

Biosynthetic labeling of gentamicin has been studied in detail and ^{14}C-methyl-L-methionine (^{14}C-methyl-Met) was found to be the best precursor.[5,6] Only the N-methyl carbon atoms of the complex were attributed to methionine[7]. However the data seemed to show both C- and N-methyl carbon atoms were equally derived from methionine. Labeling experiments using ^{13}C-methyl-Met and ^{2}H-methyl-Met have shown that all of methyl groups in gentamicins are derived from methionine[8].

In the present paper we wish to report the preparation of radioactive sagamicin by means of catalytic hydrogen exchange reaction with tritiated water, biosyntheses from ^{14}C-methyl-Met, and analytical results on the distribution of radioactivity in the ^{14}C-labeled antibiotics.

Materials and Methods

Radioactive Materials

Tritiated water (5 Ci/ml) was purchased from the Radiochemical Center. ^{14}C-Methyl-L-methionine (53.7 mCi/mmol) and S-adenosyl-L-methionine-methyl-^{14}C (58.0 mCi/mmol) were purchased from New England Nuclear Co. (U.S.A.).

Exchange Reaction

Sagamicin (400 mg as sulfate), 0.4 ml of tritiated water (2 Ci) and prereduced platinum oxide (200 mg) were combined and heated at 120°C for 18 hours in a sealed tube. Ten ml of water was added to the contents in the tube and after removal of the catalyst, the reaction mixture was lyophilized. The lyophilizate was dissolved in 10 ml of water and lyophilized again. This procedure was repeated 4 times to remove the tritiated water completely. The resultant product was chromatographed on silicagel. The column was developed with the lower phase of a chloroform - isopropyl alcohol - ammonium hydroxide mixture. The fraction containing sagamicin was concentrated *in vacuo*, adjusted to pH 4.5 with 4 N sulfuric acid and precipitated by addition of 10 volumes of methyl alcohol.

De-Novo Biosynthesis of Sagamicin

A loop of *Micromonospora sagamiensis* spore suspension was used to inoculate 10 ml of seed medium in a test tube and this was incubated at 30°C for 3 days on a shaker. The seed medium consisted of 2% dextrin, 0.5% glucose, 0.5% Polypeptone, 0.5% yeast extract, 0.1% corn steep liquor, 0.3% meat extract and 0.2% calcium carbonate (pH was adjusted to 7.5 before autoclaving). Three ml of the first seed culture was inoculated into 30 ml of the same type of medium in a 250-ml Erlenmeyer flask and incubated similarly. Radioactive methionine was added 48 hours after inoculation. Finally, the grown organisms were transferred into a 2-liter Erlenmeyer flask containing 300 ml of the medium (calcium pantothenate was used instead of carbonate) and incubated as above for 8 days.

Bioconversion of Gentamicin to Sagamicin

A 3-day fermented broth was centrifuged at 4,500 *g* for 10 minutes at 0°C and the mycelium was washed once with an equal volume of cold saline water. The mycelium was then suspended in 0.15 M phosphate buffer (pH 7.0) to a final optical density of 1.0 at 660 nm after addition of 5 μCi of a radioactive precursor, 5 mg of gentamicin C_{1a}, 10 mg of adenosine 5′-triphosphate (Kyowa Hakko Kogyo Co.), and 0.5 ml of metal sulfate solution containing 10 mM of magnesium, 0.1 mM of zinc and 2.7 mM of iron. Then final volume in the tube was 5 ml.

Isolation of Sagamicin and the Gentamicins

The pH's of the de-novo synthesis fermentation broth and bioconversion resting-cell suspensions were adjusted to 2 with a saturated oxalic acid aqueous solution. After the acidified solutions were shaken for half an hour, the mycelia were separated by centrifugation, washed once with water, and the pH's of the supernatants were adjusted to 6.5 with 2 N ammonium hydroxide. The neutralized supernatants were percolated through columns of Amberlite IRC-50 (NH_4^+). After washing the columns with water, sagamicin and gentamicin complexes were eluted with 1 N ammonium hydroxide. The eluates were concentrated *in vacuo* to small volumes, adjusted to pH 4.5 with 2 N sulfuric acid and lyophilized. The resultant mixtures of antibiotics were further purified by silica gel chromatography, as mentioned above. The fractions containing sagamicin or gentamicin C_1 were concentrated *in vacuo*, adjusted to pH 4.5 with 4 N sulfuric acid and lyophilized.

Degradation of Sagamicin

(1) Acid Hydrolysis: Several mg of sagamicin were dissolved in 1 ml of 6 N hydrochloric acid, sealed under reduced pressure and incubated at 40°C for 4 days[9]. The resultant hydrolysate was evaporated *in vacuo* and separated by tlc. Radioactivity on the tlc plate was detected by autoradiography with X-ray film (Sakura type N). Active zones were removed and radioactivity was quantitated by a liquid scintilation counter.

(2) HOFMANN Degradation: The free base of sagamicin was obtained by the ion-exchange treatment mentioned above. A mixture of 320 mg of sagamicin base, 0.3 ml of methyl iodide, 370 mg of sodium bicarbonate and 5 ml of methyl alcohol was heated under gentle reflux for 72 hours.[10] In the course of the reaction, 0.3 ml of methyl iodide was added twice. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. To the residue, 464 mg of silver oxide and 10 ml of water were added and the mixture was agitated at room temperature overnight. Silver iodide and excess silver oxide were removed by filtration, and the filtrate was lyophilized. Several mg of the lyophilized derivative of ^{14}C-sagamicin were placed in a test tube equipped with an inlet tube for nitrogen and an outlet tube that led to a trap containing hydrochloric acid. The test tube was heated to 160°C in an oil bath undergentle stream of nitrogen. Every 15 minutes the acid trap was exchanged with a new one. The radioactivity of the acid-trap fractions was determined directly. The pyrolysis residue was submitted to a sample oxidizer and the radioactivity was determined. The same procedure was carried out for gentamicin C_1.

Analytical Experiments

Tlc was conducted under an ascending procedure with the lower phase of a chloroform - methyl alcohol - 29% ammonium hydroxide (1 : 1 : 1, by volume) mixture on silica gel plates (Kieselgel 60, Merck; Wakogel B-5 FM, Wako). Aminoglycosides on tlc plates were detected by iodine exposure,

fluorescence or radioactivity. For determination of fluorescence obtained by the fluorescamine assay (F. Hoffman-La Roche)[11], a fluorophotometer (Hitachi MPF-2A) was used. For measurements of radioactivity, a sample oxidizer (Aloka LSC-112), a liquid scintilation counter (Tricarb 3320, Packard), and a chromato scanner (Aloka TLC-101) were used. Thermal analyses were conducted under nitrogen streams by micro TG-DTA (thermogravimetry-differential thermal analyzer, Shimadzu DT 20B). NMR spectra were obtained with JEOL JNM-PFT-100. Glc were performed on glass columns (3 mm × 2 m) packed with Chromosorb 103 (80~100 mesh) in a Shimadzu GC-5A. Operating conditions were as follows: the column temperature 170°C, the flow rate of nitrogen 40 ml/min. Antimicrobial assays were conducted by an agar diffusion assay with *Bacillus subtilis* ATCC 6633.

Results and Discussion

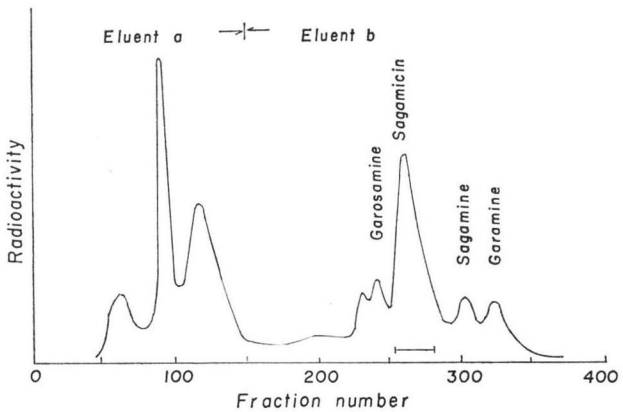
Hydrogen Exchange Reaction of Sagamicin

^{3}H-Sagamicin obtained by the method described above had a 4.18 μCi/mg specific activity and 97.0% radiochemical purity. The yield was 133 mg (from 400 mg of sagamicin). Degradation products formed during the reaction included garosamine, sagamine and garamine (Fig. 1). These components, identified by tlc, were radiochemically minor and observed to be formed in a control run with unlabeled H_2O. The less polar components (peaks eluted earlier in Fig. 1) were more densely labeled.

Incorporation of ^{14}C-Methyl-L-methionine into Sagamicin

The mode of addition of ^{14}C-methyl-Met was investigated with gentamicin[5], and it was determined that the addition at the initial stage of fermentation was not recommended. In the present experiment, the radioactive precursor was added at 48 hours after inoculation. A time course study of the fermentation was conducted, and incorporation of radioactivity into gentamicin C_1 was observed to increase at the final stage of the fermentation (day 9 and 10) was reported by LEE *et al.*[5] However, with our *Micromonospora sagamiensis*, that produces both sagamicin and the gentamicins, radioactivity was readily incorporated into sagamicin in the earlier phase of the fermentation (from days 6 to 8). The broth obtained by the fermentation for 8 days was subjected to the purification process described previously. The radioactivity balance in the fermentation broth is indicated in Table 1. One half of the radioactivity was recovered as ^{14}C-methyl-Met in the effluent of IRC-50, and one third was in the mycelia. The aminoglycosides complex contained 5.2% of the radioactivity, primarily in the sagamicin and gentamicin C_1. The specific activity of ^{14}C-sagamicin was 2.53 μCi/mg and the radiochemical purity was 94.5%. Gentamicin C_1 was used for further investigation without iso-

Fig. 1. Chromatographic separation of ^{3}H-sagamicin
Column: Wakogel C-200, 4.5×47 cm
Fraction volumes: 10 ml
Eluent a: Lower phase of a chloroform - isopropyl alcohol - 17% ammonium hydroxide (2: 1: 1) mixture
Eluent b: Lower phase of a chloroform - isopropyl alcohol - 29% ammonium hydroxide (1: 1: 1) mixture



lation of a solid fraction. The specific activity and radiochemical purity were 1.85 μCi/mg and 95.0% respectively (by fluorometrical analysis).

In order to achieve higher efficiency of utilization of ^{14}C-methionine, biotransformation with intact cells was studied further. S-Adenosylmethionine was used as a methyl donor except methionine, since gentamicin C_{1a} conversion to sagamicin (gentamicin C_{2b}) involved only one reaction with *Micromonospora purpurea*[12]. The radioactivity incorporated into sagamicin was 16.7% and 4.1% with methionine and S-adenosylmethionine respectively. The low efficiency of incorporation observed with the latter compound should be attributed to the instability of the compound in the medium and/or poor transportation through the cell membrane. Since this system is useful for obtaining labeled sagamicin of desired specific activity, further studies are in progress.

Distribution of Methyl-^{14}C in Sagamicin

The distribution of radioactivity among the hydrolysis products is indicated in Table 2. Under the mild condition described previously, the antibiotics were hydrolyzed into two subunits (Chart 1) and the tlc autoradiogram in the case of sagamicin was shown in Fig. 2. The ratios of radioactivity between sagamine and

Table 1. Balance of radioactivity in the fermentation broth[a]

Precipitate[b]	Supernatant[b]			
	Eluate[c]			Effluent[c]
	Sagamicin	Gentamicin C_1	Others	
33.5	2.3	1.8	1.1	56.0

a) 7.2 mCi of L-methionine-methyl-^{14}C was used for 2.1 liters of the medium (balance was expressed as per cent).
b) fractions obtained by centrifugation
c) fractions obtained by IRC-50 treatment

Table 2. Distribution of methyl-^{14}C in sagamicin and gentamicin C_1 (acid hydrolysis)

Compound	Radioactivity of subunit (μCi$\times 10^{-2}$)		
	Sagamine	C_1-Gentamine	Garosamine
Sagamicin	1.99 (1.0)		4.02 (2.0)
Gentamicin C_1		15.3 (1.9)	16.1 (2.0)

The values in parentheses were ratios between corresponding subunits.

Fig. 2. Autoradiogram of sagamicin hydrolyzate (tlc: Kieselgel 60)

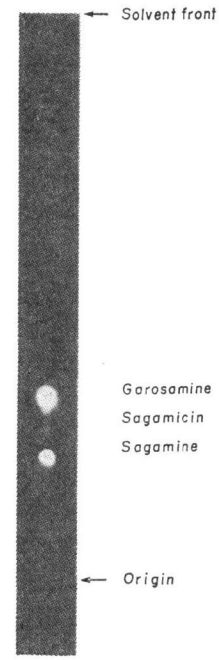


Chart 1. Subunits of sagamicin, gentamicin C_{1a}, C_1 and C_2.

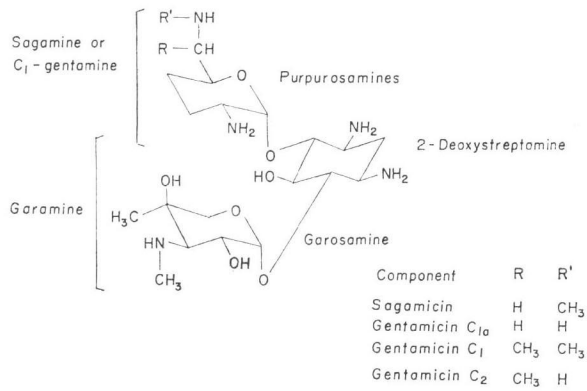


Fig. 3. Proton NMR spectrum of the quaternary ammonium hydroxide of sagamicin in D_2O solution.

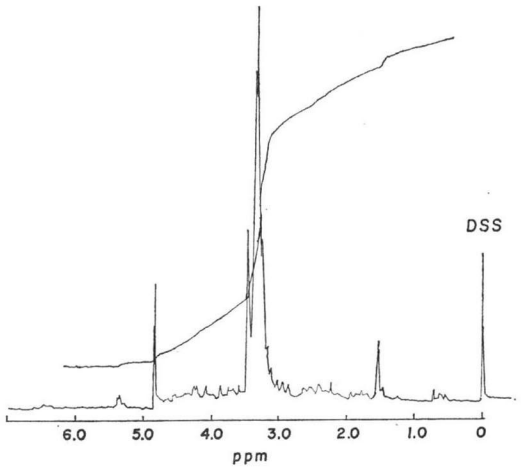
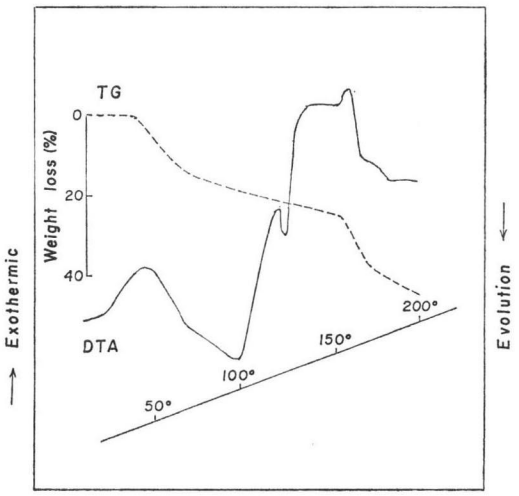


Fig. 4. TG-DTA curves of the quaternary ammonium hydroxide of sagamicin.
Conditions: heating rate 10°C/min, standard sample Al_2O_3



garosamine were 1:2 in sagamicin, and the ratios between C_1-gentamine and garosamine were 1:1 in gentamicin C_1. These ratios correspond to those of methyl groups in the subunits. Under the extreme conditions gentamicin C_1 gave 1:1 radioactivity ratios between C_1-purpurosamine and garosamine, whereas gentamicin C_2 gave 1:1.13 ratios between C_2-purpurosamine and garosamine[7]. These results would be explained if C-methyl groups are derived from methionine[18] and methionine labels to some extent N-methyl groups. If all methyl groups are attributed to methionine, 1:2 ratios would be expected between C_2-purpurosamine and garosamine. The discrepancy could be caused by the extreme hydrolysis to the unit carbohydrates.

Table 3. Distribution of methyl-^{14}C in sagamicin and gentamicin C_1 (HOFMANN degradation)

Compound	Radioactivity in fraction ($\mu Ci \times 10^{-3}$)	
	Trimethylamine	Pyrolysis residue
Sagamicin	11.9 (1.8)	6.6 (1.0)
Gentamicin C_1	15.5 (1.0)	15.4 (1.0)

The values in parentheses were ratios between corresponding fractions.

In order to further elucidate the origin of the methyl groups, N-methyl groups were eliminated by exhaustive methylation followed by HOFMANN degradation. Sagamicin was converted to the quaternary ammonium derivative and the NMR spectrum was taken in FT-mode (Fig. 3). C-Methyl peak appeared at 1.55 ppm and multiple N-methyl peaks at around 3.4 ppm. The result strongly supported that all the amino or imino groups in the molecule were permethylated (the presence of O-methyl groups in the quaternary hydroxide was excluded in the present method). From the thermal analysis stepwise release of trimethylamine was observed at about 50°C and 160°C (Fig. 4). According to the TG curve, 1.78 mg (30 μmoles) of trimethylamine liberated from 4.5 mg (6.1 μmoles) of the quaternary hydroxide. Identification of trimethylamine was carried out by glc (retention time 3.33 minutes). Although the first weight loss at 50°C could involve some contribution of adsorbed water, both changes at 50°C and 160°C were exothermic. For the radioactive derivative, the degradation products were divided into two fractions, trimethylamine (trapped in hydrochloric acid) and the pyrolysis residue. The degradation was completed in 15 minutes at 160°C. As shown in Table 3, the ratios of radioactivity

between trimethylamine and pyrolysis residue fractions corresponded to those of N- and C-methyl groups in either of sagamicin or gentamicin C_1. Therefore it was concluded that all the C- and N-methyl groups in sagamicin or gentamicin C_1 were derived from methionine and they were labeled to an equal degree.

References

1) NARA, T.; I. KAWAMOTO, R. OKACHI, S. TAKASAWA, M. YAMAMOTO, S. SATO, T. SATO & A. MORIKAWA: New antibiotic XK-62-2 (Sagamicin). II. Taxonomy of the producing organism, fermentative production and characterization of sagamicin. J. Antibiotics 28: 21~28, 1975
2) EGAN, R. S.; R. L. DEVAULT, S. L. MUELLER, M. I. LEVENBERG, A. C. SINCLAIR & R. S. STANASZEK: A new antibiotic XK-62-2. III. The structure of XK-62-2, a new gentamicin C complex antibiotic. J. Antibiotics 28: 29~34, 1975
3) ISHII, A.; H. MINAGAWA, S. OKUMURA & T. DEGUCHI: Absorption, distribution and excretion of labeled KW-1062 in rat and mouse. Chemotherapy 25: 1880~1886, 1977
4) WILSON, T. W.; D. KADAR, T. INABA & G. E. JOHNSON: Purification of tritiated gentamicin. J. Lab. Compds. 9: 559~562, 1973
5) LEE, B. K.; R. T. TESTA, G. H. WAGMAN, C. M. LIU, L. MACDANIEL & C. SCHAFFNER: Incorporation of L-methionine-methyl-^{14}C into gentamicins. J. Antibiotics 26: 728~731, 1973
6) LEE, B. K.; R. G. CONDON & G. H. WAGMAN: Incorporation of L-methionine-methyl-^{14}C into gentamicins. II. Large scale preparation of methyl-^{14}C-gentamicins. J. Antibiotics 27: 822~825, 1974
7) LEE, B. K.; R. G. CONDON, A. MURAWSKI & G. H. WAGMAN: Incorporation of L-methionine-methyl-^{14}C into gentamicins. III. Chromatographic separation and degradation of components of methyl-^{14}C-gentamicin complex. J. Antibiotics 28: 163~166, 1975
8) DANIELS, P. J. L.; A. YEHASKEL & J. B. MORTON: The biosynthetic origin of the methyl groups of the gentamicin antibiotics. 16th Intersci. Conf. on Antimicr. Agents & Chemoth., Chicago, Oct. 1976
9) KOBAYASHI, S.: Paper in preparation.
10) COPE, A. C. & A. S. MEHTA: Mechanism of the HOFMANN elimination reaction: An ylide intermediate in the pyrolysis of a highly branched quaternary hydroxide. J. Am. Chem. Soc. 85: 1949~1952, 1963
11) UDENFRIEND, S.; S. STEIN, P. BOHLEN, W. DAIRMAN, W. LEIMGRUBER & M. WEIGELE: Fluorescamine: A reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science 871~872, 1972
12) TESTA, R. T. & B. C. TILLEY: Biotransformation, a new approach to aminoglycoside biosynthesis. II. Gentamicin. J. Antibiotics 29: 140~146, 1976
13) RINEHART, K. L., Jr. & R. M. STROSHANE: Biosynthesis of aminocyclitol antibiotics. J. Antibiotics 29: 319~353, 1976