STUDIES ON BIOSYNTHESIS OF KASUGAMYCIN. V

BIOSYNTHESIS OF THE AMIDINE GROUP

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During production of kasugamycin, ^{15}N-glycine was added, yielding ^{15}N-kasugamycin. ^{15}N-Kasugamycin was treated with baryta, yielding ammonia from the carboxyformidoyl group (the side chain). The two amino groups of kasuganobiosamine which was obtained by baryta treatment were degraded to ammonia. Mass spectroscopic analysis of ammonia from these two sources showed that the nitrogen atom of glycine is incorporated into the imino nitrogen of the carboxyformidoyl group.

As reported in a previous paper[1], the two carbons of the carboxyformidoyl group (the side chain) of kasugamycin are derived from the two carbons of glycine. The present study shows that the nitrogen atom of the carboxyformidoyl group is derived from the nitrogen of glycine.

Materials and Methods

Preparation of ^{15}N-kasugamycin: A strain of *Streptomyces kasugaensis* was shake-cultured in a medium consisting of maltose 1.5 %, soybean meal 1.5 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.1 %, and NaCl 0.3 % (without adjustment of pH). During the period from 72 to 105 hours of fermentation, ^{15}N-glycine was added and the fermentation continued. The ^{15}N-glycine employed (30 atom percent ^{15}N excess) was purchased from Daiichi Pure Chemicals Co., Ltd.

As a control, U-^{14}C-glycine was added with the same procedure as ^{15}N-glycine and the results compared to those with ^{15}N-glycine.

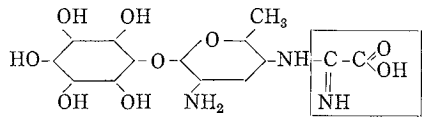
The cultured broth (250~500 ml) was combined and centrifuged at 3,000 r.p.m. to remove the mycelium. The supernatant solution was passed through a column of 100 ml of Amberlite XE-100 resin in NH_4^+ form and the adsorbed kasugamycin was eluted with 150 ml of 0.1 N NH_4OH. The eluate was neutralized with 1 N HCl and concentrated *in vacuo* to 20 ml. The concentrated solution was passed through a column of 30 g of carbon and the adsorbed kasugamycin was eluted with 200 ml of 0.05 N HCl. The eluate was neutralized with Dowex-3 resin in OH^- form and concentrated. After standing overnight in an ice box, crystals of kasugamycin hydrochloride monohydrate were collected.

Determination of ^{15}N-atom percent excess in ^{15}N-kasugamycin: To 50 ml of ^{15}N-kasugamycin·HCl·H_2O, 100 mg of $CuSO_4 \cdot 5H_2O$ and 900 mg of K_2SO_4 were added and, under stirring, 5.0 ml of conc. H_2SO_4 was added. The mixture was subjected to KJELDAHL digestion. The digested mixture was made to 20 ml with distilled water and, using 15 ml of the solution, ammonia in the solution was steam distilled into 10 ml of 0.1 N H_2SO_4. The ammonia concentration in the solution was estimated by back-titration using a part of the solution. The rest of the trapped ammonia solution was concentrated to about 3.0 ml and subjected to mass spectroscopic analysis.

Determination of ^{15}N-atom percent excess in the nitrogen atom of the carboxyformidoyl group and in the two nitrogen atoms of kasuganobiosamine: One hundred mg of ^{15}N-kasugamycin·HCl·H_2O was dissolved in 2.0 ml of distilled water and 5.0 ml of saturated barium hydroxide solution was added. Under bubbling with N_2 gas at a rate of 2~3 bubbles/second, it was heated at 100°C for 10 hours. The effluent gas was passed into 20 ml of 0.1 N H_2SO_4. Using a part of the solution, the ammonia concentration in the solution was measured by back-titration. The rest of the trapped ammonia solution derived from the carboxyformidoyl group was concentrated to 3.0 ml and subjected to mass spectroscopic analysis. The result of this analysis indicated ^{15}N-atom percent excess of the carboxyformidoyl group. The residual solution containing kasuganobiosamine was neutralized with dry ice to remove barium and passed through 10 ml of Amberlite IRC-50 resin (H^+). The adsorbed kasuganobiosamine was eluted with 0.1 N HCl. The fractions giving positive ninhydrin reaction were collected, neutralized with Dowex-3 resin in OH^- form and lyophilized. The total amount of the lyophilized powder thus obtained was subjected to ^{15}N analysis by the same procedure as that for ^{15}N-kasugamycin as described above. This result indicated ^{15}N-atom percent excess of the two nitrogens in kasuganobiosamine. Mass spectrometry of ^{15}N was carried out at Kandachi Laboratory, Department of Agricultural Chemistry, The University of Tokyo.

Results and Discussion

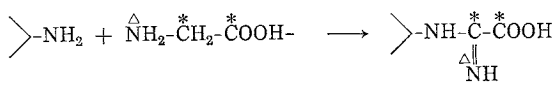
In the first experiment, 125 ml of the medium was placed in each flask and 11.5 mg/flask of ^{15}N-glycine was added ten times during the period 72~105 hours of the fermenture. The cultured broth from 4 flasks was harvested at 120 hours. In this case, at 72 hours of the culture, 688 mcg/ml of kasugamycin was produced and the cultured broth at 120 hours contained 904 mcg/ml of kasugamycin. From 4 flasks, 370.8 mg of crystalline kasugamycin hydrochloride monohydrate was isolated. The degradation of 50 mg of this ^{15}N-kasugamycin gave 4.55 mg of nitrogen (theoretical yield 4.84 mg) as ammonia and ^{15}N-atom percent excess of this nitrogen was determined by mass spectroscopic analysis to be 1.387 %. The incorporation of ^{15}N-glycine was calculated to be 13.87 % (1.387×3/30). As control, the same amount of U-^{14}C-glycine (11.5 μc, 11.5 mg) was added to another flask and the incorporation of U-^{14}C-glycine into kasugamycin was 12.77 %. Baryta treatment of 100 mg of ^{15}N-kasugamycin hydrochloride monohydrate gave 1.66 mg nitrogen (theoretical yield 3.23 mg) as ammonia from the nitrogen atom of the carboxyformidoyl group, and the degradation of kasuganobiosamine obtained by baryta treatment gave 3.06 mg nitrogen (theoretical yield 6.46 mg) as ammonia from the two amino groups. The ^{15}N-atom percent excess in ammonia derived from the carboxyformidoyl group was 1.939 and that derived from the two amino groups of kasuganobiosamine was 0.659. Thus, the amino group of glycine is preferentially incorporated into the imino nitrogen of the carboxyformidoyl group.



In order to confirm this, in another experiment 125 ml of the medium was placed in each of 4 flasks. After 72 hours of the shaking culture of *S. kasugaensis*, 5.0 mg of ^{15}N-glycine (30 atom percent ^{15}N excess) was added to each flask, and after 24 hours

^{15}N-kasugamycin was harvested from two flasks. To the other two flasks, 5.5 mg/flask of ^{15}N-glycine was added and ^{15}N-kasugamycin was harvested 24 hours later. Kasugamycin production in the broth was as follows: 285 mcg/ml at 72 hours, 620 mcg/ml at 96 hours and 850 mcg/ml at 120 hours. From the harvest at 96 hours, 59.5 mg of ^{15}N-kasugamycin hydrochloride monohydrate was obtained. Baryta degradation of this ^{15}N-kasugamycin gave 0.972 mg nitrogen (theoretical yield 1.919 mg) as ammonia from the carboxyformidoyl group and 1.864 mg nitrogen (theoretical yield 3.839 mg) as ammonia from the two amino groups of kasuganobiosamine. The ^{15}N-atom percent excess of the carboxyformidoyl group was 5.648 % and that of two amino groups of kasuganobiosamine was 0.953 %. From the cultured broth harvested at 120 hours, 91.0 mg of ^{15}N-kasugamycin was obtained, and baryta degradation gave 1.564 mg nitrogen (theoretical yield 2.935 mg) as ammonia and the further degradation gave 3.799 mg nitrogen (theoretical yield 5.871 mg) as ammonia. The ^{15}N-atom percent excess of the carboxyformidoyl nitrogen was determined to be 4.916 % and that from the two amino groups of kasuganobiosamine was 1.518 %. The ratio of ^{15}N-atom percent excess in the carboxyformidoyl nitrogen to that in kasuganobiosamine is higher in ^{15}N-kasugamycin harvested at 96 hours of culture than that harvested at 120 hours as follows: $5.648/0.953\times2>4.916/1.518\times2$. This indicates that the amino nitrogen of glycine is first incorporated into the carboxyformidoyl group and as the fermentation continues, the incorporation of glycine nitrogen into kasuganobiosamine increases. As reported previously[1], the two carbons of glycine are almost exclusively incorporated into the two carbons of the side chain. Thus, it appears that glycine is deaminated during fermentation and the ammonia is utilized for synthesis of kasuganobiosamine.

The results indicate that glycine is incorporated in the carboxyformidoyl group without fragmentation as shown below:



Reference

1) Fukagawa, Y.; T. Sawa, T. Takeuchi & H. Umezawa: Biosynthesis of kasugamycin. II. Biosynthesis of the two-carbon side chain of kasugamycin. J. Antibiotics 21: 182~184, 1968.