ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1972, p. 242-246

Vol. 1, No. 3
Printed in U.S.A.

Origin of Glycine from Acid Hydrolysis of the β-Lactam Antibiotic A16886B

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Received for publication 26 November 1971

Structural analysis of two new β-lactam antibiotics, A16884A and A16886B, indicated that they, like cephalosporin C, were composed of modified valine and cysteine residues, and α-aminoadipic acid. However, acid hydrolysis of A16886B and A16884A produced three times as much glycine as did hydrolysis of cephalosporin C under the same conditions. Samples of A16886B-^{14}*C-6* and A16886B-^{14}*C-8* were prepared by the addition of cysteine-^{14}*C-3* and cystine-^{14}*C-1* to fermentations of *Streptomyces clavuligerus*. The specific activity of glycine obtained from hydrolysis of A16886B-^{14}*C-6* was considerably higher than that from hydrolysis of A16886B-^{14}*C-8*. An explanation for the difference in amounts of glycine obtained from hydrolysis of these antibiotics is discussed.

Structure elucidation of two new β-lactam antibiotics from *Streptomyces lipmanii* and *S. clavuligerus* established them as 7-(5-amino-5-carboxyvaleramido) - 7 - methoxycephalosporanic acid (A16884A) and 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - carbamoyloxymethyl-3-cephem-4-carboxylic acid (A16886B), respectively (5; M. Gorman, C. E. Higgens, and R. Nagarajan, Belgium Patent 754,693, 1971; M. Gorman et al., Abstr. 11th Intersci. Conf. Antimicrob. Ag. Chemother., p. 7, 1971; R. L. Hamill et al., Abstr. 11th Intersci. Conf. Antimicrob. Ag. Chemother., p. 7, 1971). During the initial isolation and structure studies on the antibiotics from *S. clavuligerus*, it was found that, upon acid degradation, antibiotic A16886B yielded, in addition to α-aminoadipic acid, approximately three times more glycine than did the β-lactam antibiotics A16886A or cephalosporin C (5; Gorman et al., Belgium Patent 754,693, 1971; Abstr. 11th Intersci. Conf. Antimicrob. Ag. Chemother., p. 7, 1971). A second novel 7-methoxy containing β-lactam antibiotic, A16884A, isolated from *S. lipmanii*, also produced a relatively large amount of glycine upon acid hydrolysis (Hamill et al., Abstr. 11th Intersci. Conf. Antimicrob. Ag. Chemother., p. 7, 1971). Structural analysis indicated that glycine was not a formal component of the new β-lactams. The structures of the compounds are shown in Fig. 1.

We have determined the origin of glycine obtained upon acid hydrolysis of A16886B and now propose a mechanism to explain its facile formation.

MATERIALS AND METHODS

Preparation of A16886B-^{14}C-6 and A16886B-^{14}C-8. *S. clavuligerus* NRRL 3585 was fermented under conditions previously described (6). To 1.0 liter of a 72-hr culture was added 1.1×10^8 disintegrations/min, 50.0 μCi/1.2 mg of DL-cysteine-^{14}*C-3*. After 24 hr, the culture was filtered, and the labeled A16886B-^{14}*C-6* was isolated according to the published procedure (Gorman et al., Belgium Patent 754,693, 1971; Abstr. 11th Intersci. Conf. Antimicrob. Ag. Chemother., p. 7, 1971). Paper chromatograms developed in propanol-pyridine-acetic acid-acetonitrile-water (45:30:9:40:36) were used to obtain radioautograms and also bioautograms, with *Pseudomonas solanacearum* used as an assay organism.

Because of the difficulty of obtaining crystalline samples of these antibiotics, a unit assay was established for A16884A, A16886A, and A16886B. Purity of a preparation of these antibiotics was calculated on the basis of 3,000 units/mg for the pure compounds (6).

A procedure identical to that described above was used for the preparation of A16886B-^{14}*C-8*, except that 1.1×10^8 disintegrations/min, 56.7 μCi/mmole of DL-cystine-^{14}*C-1,1'* was added to 1.0 liter of the NRRL 3585 culture.

Acid hydrolysis of labeled A16886B antibiotics. Samples of the labeled C-6 and C-8 A16886B antibiotics were dissolved in 6 N HCl, ca. 1 mg/ml. The solutions were refluxed for 21 hr in an apparatus with a gas delivery tube immersed in a solution of Hyamine hydroxide (1 M in methanol, New England Nuclear Corp.). The hydrolysates were taken to dryness under vacuum, approximately 1 ml of water was added, and the solution was again taken to dryness. The dried sample was dissolved in buffer (2% thiodiglycol, 0.2 N Na^+, *p*H 2.2). The hydrolysates were then analyzed on a Beckman model 120C amino acid analyzer equipped with a BioCal model BC501 automatic sample injector.

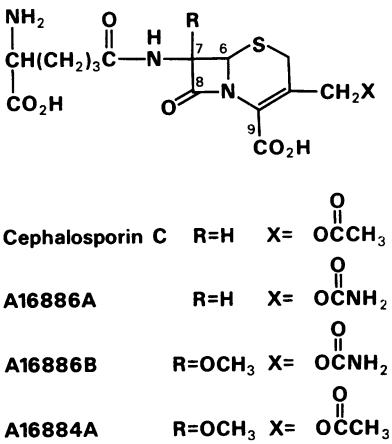


FIG. 1. *Chemical structures of cephalosporin C and three new β-lactam cephalosporin antibiotics.*

Amino acid separation was achieved on a column of Durrum DC-1A resin, 0.9 by 52 cm, 56.5 C, with a 70 ml/hr flow of buffer (0.2 N Na^+, *p*H 3.23, 0.5% thiodiglycol). A ninhydrin flow of 35 ml/hr was used when a ninhydrin response curve was desired. When the amino acids were eluted for radioactive scintillation counting, 1-min fractions were collected directly from the column.

Decarboxylation of glycine. The aqueous solution of labeled glycine obtained from hydrolysis of A16886B-^{14}C-*6* was decarboxylated by use of a procedure described by Chappelle and Luck (3). A 40% solution of potassium iodide, 0.5 ml, was added to a Warburg flask side arm, and to a second side arm was added 0.5 ml of an *N*-bromosuccinimide (NBS) suspension prepared by the addition of 2.5 g each of NBS and succinimide to 25 ml of a 1 M sodium acetate-acetic acid buffer, *p*H 4.7. To the main compartment of the vessel was added a 0.1-ml solution of glycine, 44 μg of PdCl, and 2.8 ml of a 10% solution of succinimide in 1 M sodium acetate, *p*H 4.7. In the center well was placed 0.2 ml of Hyamine hydroxide (1 M in methanol, New England Nuclear Corp.). After tipping the NBS-reagent into the main compartment, the reaction vessel was gently shaken at 30 C for 1 hr. The resulting Hyamine hydroxide-CO_2 solution was removed, placed in a scintillation vial with 20 ml of diatol, and counted for radioactivity.

RESULTS

When samples of A16884A and A16886B were subjected to hydrochloric acid hydrolysis and the resulting hydrolysates were analyzed, considerably more glycine was obtained than was expected on the basis of similar degradation of cephalosporin C and A16886A. The results of these hydrolyses are shown in Table 1.

Addition of DL-cysteine-^{14}C-*3* and DL-cystine-^{14}C-*1,1'* to fermentations of *S. clavuligerus* resulted in the isolation of labeled preparations of A16886B of the specific activity shown in Table 2. Radioactivity scanning of paper chromatograms showed only a single labeled component in each of the two A16886B samples. The radioactivity peak was superimposable with bioautograms of the paper chromatograms.

Duplicate samples of A16886B-^{14}C-*6* and A16886B-^{14}C-*8* were subjected to hydrochloric acid hydrolysis. One of the two hydrolysates of each labeled compound was chromatographed, and the resulting ninhydrin-positive products were quantitatively measured and identified by comparison of their elution times with those of standard amino acids. The second hydrolysate of each labeled compound was chromatographed by the same procedure. However, the components from the column were not reacted with ninhydrin, but were collected in 1-min fractions. A 0.1-ml sample of each fraction was assayed for radioactivity. The carbon dioxide emitted from hydrolysis of A16886B-^{14}C-*8* was collected in a methanolic solution of Hyamine hydroxide.

The precision of the amino acid analyzer was repeatedly shown to be sufficient to duplicate the elution time of a standard mixture of amino acids for two sequential analyses. Figure 2 demonstrates the ninhydrin response curves and the corresponding radioactivity counts obtained from hydrolysates of A16886B-^{14}C-*6* and A16886B-^{14}C-*8*. The fractions collected for radioactivity counting that corresponded to the elution time of glycine were

TABLE 1. *Glycine from acid hydrolysis of cephalosporins*

Substrate	Glycine (μmoles per mg of substrate)[a]
Cephalosporin C	0.13
A16884A	0.76
A16886A	0.13
A16886B	0.62

[a] Substrates were of comparable high purity.

TABLE 2. *Preparation of labeled A16886B by addition of labeled precursors to S. clavuligerus*

Precursor	Labeled A16886 obtained		Per cent incorporation
	A16886B / mg of isolated prepn[a]	Specific activity[b]	
DL-Cysteine-^{14}C-*3*	0.31	2.7×10^4	8.8
DL-Cystine-^{14}C-*1,1'*	0.21	4.2×10^4	10.9

[a] Based upon a unit assay.
[b] Disintegrations per minute per milligram.

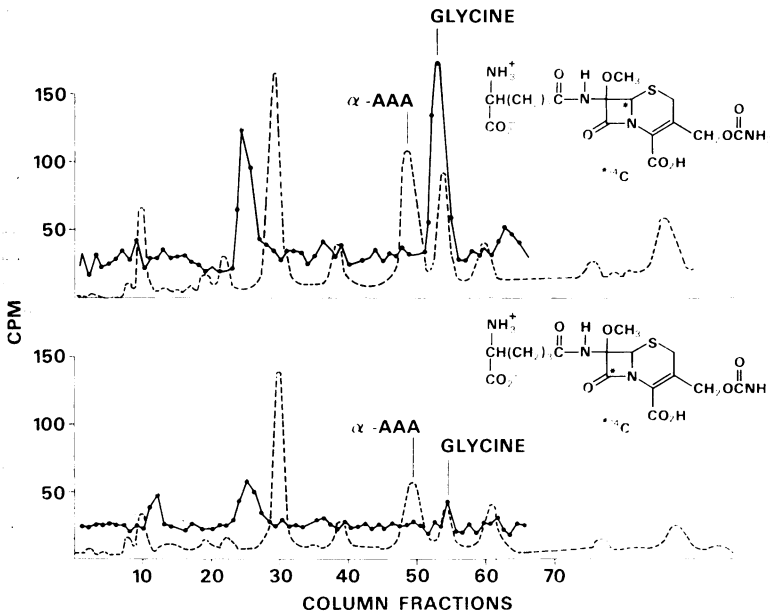


FIG. 2. *Ninhydrin response and radioactivity profiles of column elution fractions of the acid hydrolysates of A16886B-^{14}C-6 and A16886B-^{14}C-8. Dashed lines: ninhydrin response. Solid lines: counts per minute.*

TABLE 3. *Glycine obtained from hydrolysis of labeled A16886B*

Labeled cephalosporin hydrolyzed			Glycine recovered		CO_2 recovered[a]
Substrate	Amt (mg)	Specific activity[a]	Amt (μmoles/mg of A16886B)	Specific activity[a]	
A16886B-^{14}C-6	1.48	2.7×10^4	0.83	4.5×10^4	2.57×10^2
A16886B-^{14}C-8	0.74	4.2×10^4	0.69	2.2×10^3	1.5×10^4

[a] Disintegrations per minute per milligram of A16886B.

TABLE 4. *Decarboxylation of labeled glycine samples*

Glycine samples from	Amt (μg)	Disintegrations/min	Radioactivity in CO_2[a]	Per cent recovery of label
A16886B-^{14}C-6	31.4	1,413	947	67
Authentic glycine-^{14}C-2	55.7	178	22	12
Authentic glycine-^{14}C-1	55.0	342	250	73

[a] Disintegrations per minute.

concentrated and subjected to amino acid analysis. Glycine was the only amino acid detected in these fractions.

As shown in Table 3, approximately the same molar amounts of glycine were obtained from hydrolysis of A16886B-^{14}C-8 and A16886B-^{14}C-6. However, the specific activity of the glycine obtained from A16886B-^{14}C-6 was about 20 times greater than that obtained from A16886B-^{14}C-8. Collection of the carbon dioxide from the hydrolysis of A16886B-^{14}C-8 gave 40 times the amount of radioactivity obtained from A16886B-^{14}C-6 hydrolysis.

The composition of the non-ninhydrin-positive radioactive components shown in Fig. 2 was not determined. It is interesting that, in total, less radioactivity was detected in the chromatography fractions of the hydrolysate of A16886B-^{14}C-8 than from A16886B-^{14}C-6, as shown in Fig. 2. This is in agreement with the collection of a significant amount of labeled carbon dioxide from the hydrolysis of labeled A16886B-^{14}C-8.

The chromatography fractions containing the glycine from the hydrolysis of A16886B-^{14}C-6 were combined and concentrated. Decarboxylation of the glycine in the resulting aqueous solution with *N*-bromosuccinimide yielded labeled

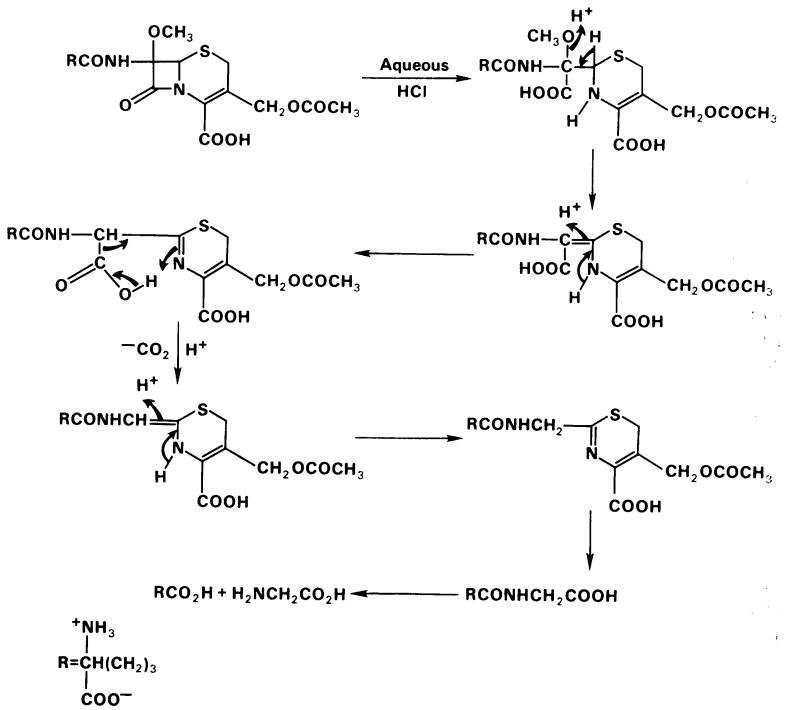
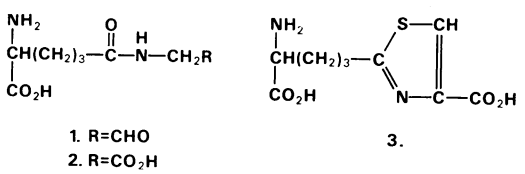


FIG. 3. *Proposed mechanism of acid hydrolysis of A16886B.*



1. R=CHO
2. R=CO_2H

3.

FIG. 4. *Compounds obtained upon acid hydrolysis of cephalosporin C.*

carbon dioxide, as shown in Table 4. Under similar conditions, glycine-^{14}C-*2* gave only a trace of radioactive carbon dioxide.

DISCUSSION

Early in the work on the structure of A16886B and A16884A, it was thought that perhaps the peptides contained a glycine residue. However, as their structures became apparent, an explanation of the relatively large amounts of glycine obtained upon their hydrolysis had to be sought elsewhere. Since the yield of glycine from A16886A was similar to that from cephalosporin C, the novel carbamate at C-3′ could not be the influencing factor. Thus, only the presence of the methoxy group at C-7 was left to account for the significant increase in glycine.

Addition of cysteine-^{14}C-*3* and cystine-^{14}C-*1,1′* to fermentations of *S. clavuligerus* allowed the isolation of A16886B-^{14}C-*6* and A16886B-^{14}C-*8*, respectively.

The location of the label in the labeled samples of A16886B is based upon the following arguments. Previous labeling experiments in penicillin biosynthesis have shown that cystine, upon reduction to cysteine, is a direct precursor to the β-lactam ring (2). The ratio of label in penicillin biosynthesized from DL-cystine-^{14}C-*3*, ^{15}N, ^{35}S has been found to be similar to that in the precursor. Secondly, as shown in Fig. 2, acid hydrolysis of the labeled A16886B samples did not produce any labeled α-aminoadipic acid. If a significant amount of label had been present in cellular pools due to breakdown of the labeled precursors, its occurrence in α-aminoadipic acid would have been expected.

The specific activity of the glycine derived from acid hydrolysis of A16886B-^{14}C-*6* was 20 times greater than of that derived from A16886B-^{14}C-*8*. This would imply that the glycine produced upon hydrolysis of A16886B is derived predominantly from C-6 and C-7, and not from C-7 and C-8. The relatively fewer total counts obtained in the A16886B-^{14}C-*8* hydrolysate and the relatively greater number of counts obtained in the carbon dioxide would suggest facile decarboxylation of the potential C-8 carboxyl of A16886B.

Subsequent decarboxylation of the labeled

glycine obtained from hydrolysis of A16886B-^{14}C-6 resulted in the collection of a significant amount of labeled carbon dioxide. This suggests that, during acid hydrolysis of A16886B, the C-6 carbon is oxidized to the carboxyl of glycine, and that the C-7 amino group, and not N-5, of the antibiotic becomes the amino group of glycine. The mechanism shown in Fig. 3 can be envisioned to explain the above findings.

During studies that eventually led to the structure of cephalosporin C, Jeffery, Abraham, and Newton (4) found that, when cephalosporin C was heated in 0.1 N hydrochloric acid at 100 C, carbon dioxide was evolved and a mixture of ninhydrin-positive degradation products was formed. Two of the products obtained were α-aminoadipic acid and a trace of glycine. Oxidation of a third component (compound 1, Fig. 4) gave a product, δ-amino-δ-carboxylvalerylglycine (compound 2, Fig. 4), identical to that obtained previously by Abraham and Newton (1) by similar treatment of penicillin N. These workers found that compound 1 readily gave α-aminoadipic acid and glycine upon hydrochloric acid hydrolysis. A fourth product obtained from hydrolysis of cephalosporin C was the acid-stable thiazole shown as compound 3 in Fig. 4.

It was proposed that the thiazole was derived, in part, from C-6, C-7, and C-8 of cephalosporin C. Therefore, the formation of glycine from cephalosporin C, via compound 2, is competitive with the formation of thiazole (compound 3).

Thus, glycine is apparently derived from the C-6 and C-7 β-lactam carbons of both cephalosporin C and the 7-methoxy cephalosporins A16884A and A16886B. At this time, the only explanation for relatively larger amounts of glycine from the latter compounds must be based on the acid-catalyzed loss of the C-7 methoxy by a mechanism that subsequently leads to decarboxylation of a C-8 carboxyl group containing intermediate. The 7-methoxy group, therefore, directs the acid degradation of A16886B and A16884A to give an intermediate which can further degrade to glycine. In contrast, the driving force of the acid degradation of cephalosporin C results in the formation of stable thiazole (compound 3, Fig. 4), a minor amount of dipeptide (compound 2, Fig. 4), and, subsequently, glycine.

ACKNOWLEDGMENTS

We thank E. H. Flynn for valuable advice and N. L. Holbrook for technical assistance.

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