Communications to the editors

CHEMISTRY OF SARAMYCETIN. II SEQUENCE STUDIES

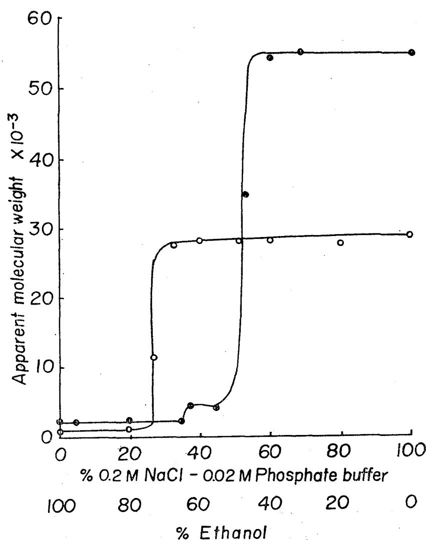
Sir :

General structural features of the antibiotic saramycetin have been discussed previously[1]. We wish to communicate here the results of structural studies carried out on a large fragment of the saramycetin molecule, for which we propose the sequence :

Glycyl–threonyl–saramycetic acid I–yl–cysteinyl–saramycetic acid II–yl–aspartyl–proline.

Upon mild acid hydrolysis of saramycetin with 0.03 N HCl at 100°C for 4 hours, a fragment of the saramycetin molecule split off and formed an insoluble precipitate. This precipitate was filtered and then purified by acid–base reprecipitation and chromatography on cellulose using methanol as solvent. The product's homogeneity was ascertained by paper electrophoresis (dimethylamino ethanol - acetic acid pH 9.3, 10 volts/cm, 2.5 hours, producing migration of 2.3 cm towards the cathode ; then formic acid–pyridin pH 3.3, 10 volts/cm, 2.5 hours, producing migration of 1.6 cm towards the cathode) and ultracentrifugation. Molecular weight of this product of hydrolysis was 1,050 daltons ("1,050 fragment") compared with 2,100 daltons for the parent saramycetin, both determined by ultracentrifugation[2].

Fig. 1. Apparent molecular weight of saramycetin (•) and of the "1,050 fragment" (○) as function of buffer concentration (0.2 M NaCl～0.02 M phosphate) in ethanol.



The "1,050 fragment" showed in the analytical ultracentrifuge many of the properties expected for half of the saramycetin molecule. For example, saramycetin formed aggregates of 55,000 daltons between pH 3.2 and 12 in aqueous buffers ; the "1,050 fragment" formed aggregates of 2,800 daltons under the same conditions. The dependence of formation of aggregates of saramycetin or of the "1,050 fragment" on the concentration of phosphate buffer in ethanol is shown in Fig. 1. Aggregation of the "1,050 fragment" occurred at 26 % buffer concentration and of the saramycetin at 50 % buffer concentration.

Potentiometric titration of the "1,050 fragment" in dimethylformamide - H_2O indicated the existence of two types of titratable acidic functions. The found equivalent weight for the first type was 585 and for the second, 1,100, suggesting the presence of three acidic functions in the "1,050 fragment". One acidic function is attributed to the end carboxyl group, one to the free carboxyl group of the aspartic acid and one, yielding the equivalent weight of 1,100, to the aromatic carboxyl group in saramycetic acid II.

Sulfhydryl group titration with aqueous iodine of the "1,050 fragment" yielded an equivalent weight of 1,020. Elemental analysis : C 46.87, H 4.85, N 13.38, S 12.28. Calculated for $C_{39}H_{44}N_{11}O_{13}S_4$: C 46.70, H 4.39, N 15.36, S 12.77. Acetylation of the "1,050 fragment" with acetic anhydride in pyridine yielded material containing 4.7 % O–acetyl and 4.9 % N–acetyl groups, equivalent to one O–acetyl and one N–acetyl group per molecule. (The threonine hydroxyl and terminal amino group, respectively.)

Paper chromatography of the product of hydrolysis of the "1,050 fragment" with 6 N HCl revealed the presence of cysteine,

aspartic acid, glycine, threonine, proline, saramycetic acid I[1] (2-[2-acetyl-thiazole-4-yl]-4-thiazole-carboxylic acid), and saramycetic acid II[3] (2-[3-amino-1-carboxy-2-aza-1-butenyl]-benzthiazole-5-carboxylic acid). Modified STEIN-MOORE analysis showed the presence of one mole of each of these amino acids in the molecule. Also, nearly one mole of saramycetic acid I could be extracted from the acid hydrolysate with chloroform.

Oxidation of the "1,050 fragment" with performic acid (0°C, 4 hours) yielded a product that has a fourth acidic function, in addition to the titratable acidic functions already mentioned. After oxidation of the "1,050 fragment", sulfhydryl groups could no longer be detected.

Amino acid analysis, sulfhydryl group titration, elemental analysis, and other results indicated the presence of one cysteine molecule in the "1,050 fragment" and confirmed that the other three sulfur atoms were present in ring structures and, thus, were parts of saramycetic acid I and II.

The "1,050 fragment" could not be degraded enzymatically by aminopeptidase-M, carboxypeptidase-B, papain, subtilisin, or chymotrypsin.

EDMAN degradation[4] showed that glycine is at the amino end of the "1,050 fragment"; hydrazinolysis showed proline at the carboxyl end.

Hydrolysis of the "1,050 fragment" with 0.03 N HCl failed to yield any fractions of value in sequence analysis. In contrast, oxidation of the "1,050 fragment" with performic acid, followed by this mild acid hydrolysis, yielded three fractions useful for such analysis.

When the oxidized "1,050 fragment" was hydrolyzed with 0.03 N HCl for 3.5 hours at 100°C, subsequent electrophoresis (formic acid-acidic acid pH 1.9, 10 volts/cm, 3 hours) revealed a ninhydrin-positive spot at 17.5 cm towards the cathode (Fraction I). Elution of the spot, followed by hydrolysis and STEIN-MOORE analysis, showed the presence of glycine and threonine, in equal amounts. The homogeneity of Fraction I was confirmed by papar chromatography and EDMAN[4] degradation indicated the sequence of glycyl-threonine. The hydroxyl group in the threonine molecule, which could be acetylated, corresponds to the O-acetyl group mentioned above.

Partial acid hydrolysis of the oxidized "1,050 fragment" with 0.03 N HCl also yielded two fractions that could be isolated by cellulose TLC (Macherey Nagel MN 300). In the solvent system butanol - acetic acid - water (4 : 1 : 5), two yellow spots were located at Rf 0.6 (Fraction II) and Rf 0.4 (Fraction III). These fractions were eluted with methanol and subsequently purified by rechromatographing. Homogeneity of these fractions was demonstrated by means of several paper chromatographic systems, electrophoresis, and ultracentrifugation.

Fraction II, dissolved in ethanol, has a molecular weight of 550 daltons by ultracentrifugation. Electrophoresis (formic acid-acidic acid pH 2.0, 10 volts/cm, 3 hours) showed a single spot at 2.0 cm towards the cathode visible under ultraviolet light. Hydrolysis of this fraction with 6 N HCl yielded saramycetic acid I, saramycetic acid II, and cysteic acid, as detected by paper chromatography. Hydrazinolysis indicated that saramycetic acid I and cysteic acid were present as hydrazides, but saramycetic acid II was not. This experiment established that saramycetic acid II was at the carboxyl end of fraction II.

Fraction II did not react with dinitrofluorobenzene and therefore has no free amine group, thus excluding the possibility that cysteic acid was at the amino end. Saramycetic acid I has a ketone group, which is presumably formed from an amide group during acid hydrolysis. If saramycetic acid I were at the "amino end" of Fraction II, a dinitrophenylhydrazone could be formed. Fraction II was allowed to react with dinitrophenylhydrazine in acetic acid and ethanol. After the evaporation of all volatile materials, the elimination of unreacted dinitrophenylhydrazine, and reprecipitation, the precipitate was chromatographed on paper. In the solvent system butanol - acetic acid - water (4 : 1 : 5), a major yellow spot at Rf 0.4 and a minor yellow spot at Rf 0.6 were visible. In the same solvent system, dinitrophenylhydrazine has an Rf

of 0.9 and Fraction II an Rf of 0.6. The ultraviolet spectrum of the dinitrophenylhydrazine derivative of Fraction II, eluted from the spot at Rf 0.4, indicated that a hydrazone had been formed. Fraction II showed absorption at 222 and 278 mμ, whereas the dinitrophenylhydrazine derivative absorbed at 292 and 370 mμ. Absorption at 370 mμ is typical for a conjugated dinitrophenylhydrazone[5] (dinitrophenylhydrazine absorbs at 350 mμ). We concluded that saramycetic acid I is the first moiety in Fraction II.

The sequence of Fraction II is, then, saramycetic acid I-yl-cysteic acid-yl-saramycetic acid II.

Fraction III, dissolved in ethanol, has a molecular weight of 670 daltons by ultracentrifugation. Electrophoresis (pH 2.0, 10 volts/cm, 3 hours) showed that this fraction remained at the origin. Hydrolysis with 6 N HCl yielded saramycetic acid I, saramycetic acid II, cysteic acid, and threonine, as detected by paper chromatography. Hydrazinolysis showed that saramycetic acid II was at the carboxyl end of Fraction III. EDMAN degradation indicated that threonine was at the amino end of this molecule. The sequence of Fraction III is, therefore, threonyl-saramycetic acid I-yl-cysteic acid-yl-saramycetic acid II.

Sequential studies on Fraction I, II, and III and end group analysis of the "1,050 fragment" indicated the position of all components of this fragment except aspartic acid. The only possible position for aspartic acid is between saramycetic acid II and proline.

We propose for the "1,050 fragment", which is half the molecule of the antibiotic saramycetin, the sequence:

Glycyl-threonyl-saramycetic acid I-yl-cysteinyl-saramycetic acid II-yl-aspartyl-proline.

The composition of the second half of the antibiotic saramycetin, as well as the relation of the "1,050 fragment" and the relation of the sulfhydryl group present in this fragment to the intact antibiotic, remains to be established.

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A. ASZALOS
J. KIRSCHBAUM
O. KOCY
F. RUSSO-ALESI
J. ALICINO

The Squibb Institute for Medical Research
Georges Road
New Brunswick, N. J.
08903, U.S.A.

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