A NEW PEPTIDE ANTIBIOTIC COMPLEX S-520. II
FURTHER CHARACTERIZATION AND DEGRADATIVE STUDIES

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(Received for publication July 18, 1970)

For further resolution of the peptide antibiotic complex S-520, a dinitrophenyl derivative and an acetyl derivative were prepared. Two forms (I and II) were isolated on thin-layer chromatograghy of the derivatives, but they were found to be interconvertible, and were suggested to be δ-lactone and acid forms respectively. Studies on the acid hydrolysate of the antibiotic complex indicated the presence of glycine (1 mole), D-valine (*ca.* 0.74 moles), D-isoleucine (*ca.* 0.18 moles), ornithine (*ca.* 0.75 moles), lysine (*ca.* 0.27 moles) and four previously unknown amino acids (named a-I, *n*-I, *n*-II, *n*-III). The construction of the antibiotic complex was discussed.

The isolation of a new peptide antibiotic complex S-520 from *Streptomyces diastaticus* has been reported[1]. The hydrochloride of the antibiotic exhibited a single spot (Rf 0.58 ± 0.05) on thin-layer chromatography (T.L.C.) using Silica gel GF with *n*-butanol - acetic acid - water (3 : 1 : 1). For further resolution of the antibiotic complex, it was necessary to prepare derivatives possessing increased solubility, because of the extremely limited solubility of the native antibiotic, as reported in the previous paper. A dinitrophenyl derivative (DNP-S-520) was prepared for this purpose. The elemental analysis and molecular weight determination corresponded to $C_{40}H_{58\sim99}O_{10}$-$N_{8\sim9}Cl(C_6H_3O_4N_2)$, which indicated that a single dinitrophenyl group was introduced for each mole of peptide in the complex. An acetyl derivative (Acetyl-S-520) was also prepared, and gave an analytical result corresponding to $C_{40}H_{58\sim59}O_{10}N_{8\sim9}Cl\cdot CH_3CO$. DNP-S-520 gave two spots on T.L.C. using Silica gel GF with chloroform - methanol (4 : 1), which were tentatively designated DNP-S-520-I (Rf 0.80) and DNP-S-520-II (Rf 0.20). Acetyl-S-520 also gave two spots on the same T.L.C. system; acetyl-S-520-I (Rf 0.70) and acetyl-S-520-II (Rf 0.10).

These spots were isolated by preparative T.L.C., and analyzed by an automatic amino acid analyzer after acid hydrolysis. DNP-S-520-I and II gave identical amino acid analyses, differing from that of the native antibiotic in that the basic amino acids ornithine and lysine were lacking. Acetyl-S-520-I and II gave identical amino acid analyses to each other and to the native antibiotic. The only difference between the I and II forms of DNP-S-520 was seen in their IR spectra; an absorption at 1725 cm^{-1} was observed with DNP-S-520-I, but not with DNP-S-520-II.

The relative contents of I and II in the antibiotic complex S-520 was determined by the following procedure: dinitrophenylation, separation of DNP-S-520-I and II by

T.L.C., then measurement of the optical density at 350 mμ due to dinitrophenyl group. In the usual preparations of S-520, contents of I and II were estimated as 50~60 % and 40~50 %, respectively. When a preparation of S-520 was treated with dilute hydrochloric acid in aqueous mothanol, increased content of I to approximately 80 % was observed. Reversely, treatment with dilute sodium hydroxide or ammonium hydroxide increased the content of II to approximately 80 %. When alternative treatments with acid and alkali were carried out successively, alternate increases in I and II occurred.

From the above results, it seems that between S-520-I and S-520-II, there is no essential difference as a component of the complex, they have the same constituents as found so far, and they are readily convertible. The only difference, found in their IR absorptions, suggests that I contains a δ-lactone-like moiety in its structure and that II is its acid form. The antimicrobial activity, tested on an assay plate with *Bacillus subtilis*, was similar for both I-rich and II-rich preparations. So far then, isolation of components which make up the antibiotic complex S-520 has not been successful.

The acid hydrolysate of S-520 was examined by two-dimensional paper chromatography; development in one direction was by *n*-butanol - acetic acid - water (4 : 1 : 2) migration in the other direction was effected by electrophoresis at 300 volt for 3 hours in 1 N acetic acid. As shown in Fig. 1, two acidic substances (a-I and a-II), six neutral substances (glycine, valine, isoleucine, *n*-I, *n*-II and *n*-III), and two basic substances (ornithine and lysine) were detected by ninhydrin reaction. By automatic amino acid analysis (Fig. 2), glycine, valine, isoleucine, ornithine, lysine and ammonia were identified by direct comparison with respective authentic specimens. It is of interest to note that the peaks of *n*-II and *n*-III were observed under the analytical condition for basic amino acids, though they were neutral amino acids.

These amino acids were isolated from the hydrolysate. Glycine, D-valine, D-

Fig. 1. Two dimensional paper chromatogram of S-520 hydrolysate.

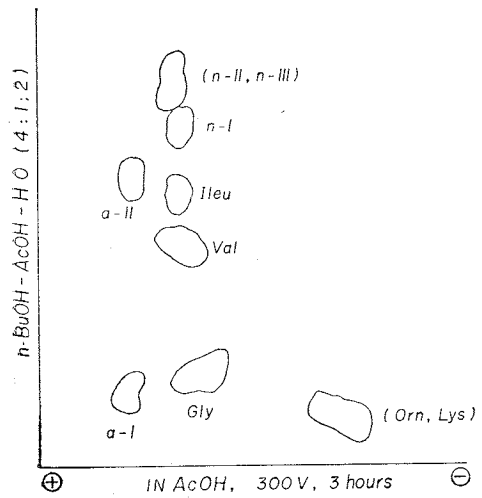

Fig. 2. Amino acid analysis of S-520 hydrolysate.

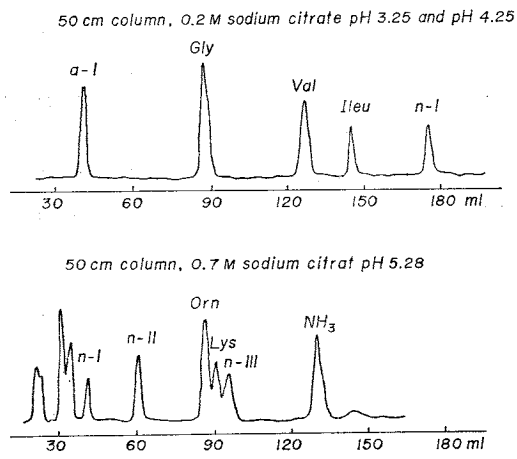

Table 1. Amino acids found (moles per mole)

Sample tested	a-I	Gly	Val	Ile	*n*-I	*n*-II	*n*-III	Orn	Lys	Amm
S-520 hydrochloride (mixture of Lot. 3 and 4)	0.461	1.041	0.761	0.265	0.297	0.430	0.218	0.816	0.179	—
S-520 hydrochloride (Lot. 5)	0.449	0.995	0.771	0.165	0.198	0.361	0.383	0.761	0.275	0.678
DNP-S-520 (Lot. 5)	0.509	0.944	0.657	0.170	0.179	0.301	0.363	0	0	0.650
Acetyl-S-520 (Lot. 5)	0.560	1.078	0.769	0.180	0.178	0.349	0.380	0.673	0.311	0.768
S-520 hydrochloride (Lot. 6)	0.563	1.070	0.763	0.118	0.370	0.396	0.138	0.766	0.306	0.662

isoleucine were confirmed from their analytical data and optical activities. Preparative isolation of ornithine and lysine was not yet carried out, but they are thought to be in the D-series from a measurement of the optical rotatory dispersion (O.R.D.) of their mixture. The a-II found by paper chromatography could not be isolated. It seemed to be degraded by ion-exchange resin procedures, and was not observed on automatic amino acid analysis. Isolation and structure elucidation of the unknown amino acids, a-I, *n*-I, *n*-II and *n*-III, will be reported in following papers[2,3].

Quantitative analyses of these amino acids were carried out on several batches of S-520 hydrochloride as well on as DNP-S-520 and acetyl-S-520. The molar contents were calculated on the basis of a molecular weight of 898, which was tentatively assumed for the antibiotic complex hydrochloride as an average molecular weight. As seen in Table 1, glycine was found to be one mole per mole. The amino acid a-I was found to be approximately 0.50 mole per mole. However, the content of this amino acid was deduced to be one mole, because its recovery rate by the same condition of acid hydrolysis was estimated as approximately 50 %. The yields for the other amino acids varied somewhat with the production batches used. However, sum totals of valine and isoleucine; *n*-I, *n*-II and *n*-III; and ornithine and lysine, were consistently shown to be approximately one mole.

These data allow us to make the following postulation: the antibiotic complex S-520 is composed of several closely related peptides, which have three replaceable units in their structures; the first unit is valine or isoleucine in each component of the complex, the second is *n*-I, *n*-II or *n*-III, and the third is ornithine or lysine. Examples such as this are commonly found in peptide antibiotics produced by actinomycetes. Usually these occur as a complex consisting of several peptides, as in the actinomycin group antibiotics[4], quinoxaline antibiotics[5], ostreogricin-vernamycin B group antibiotics[6] and so on.

In the case of the antibiotic complex S-520, isolation of each of the components which make up the complex, seems to be difficult because of its limited solubility.

Experimental

Dinitrophenyl derivative of S-520 (DNP-S-520): Some 400 mg of S-520 hydrochloride was dissolved in 20 ml of methanol and to it a solution of sodium bicarbonate (400 mg) in water (20 ml) was added. The solution, in which a gel-like precipitate formed, was then mixed with 20 ml of 10 % 2,4-dinitrofluorobenzene in methanol. The mixture was stirred for 2 hours at room temperature. A yellow gel-like precipitate which formed was filtered and washed with 50 % aqueous methanol. The precipitate was then dissolved in

15 ml of hot methanol and again precipitated by addition of 5 ml of water to yield a yellow powder (438 mg). The re-precipitation procedure was repeated twice more to give a yellow powder (180 mg) free from dinitrophenol, m.p. 254~258°C (dec.).

Anal. Found: C 53.55, 54.14; H 6.16, 6.30; N 14.20, 14.20; Cl 3.05, 3.52; MW 917 (osmometry in pyridine).

Calcd. for $C_{40}H_{59}O_{10}N_9Cl(C_6H_3O_4N_2)$: C 53.72, H 6.03, N 14.99, Cl 3.45, MW 1027.5

$C_{40}H_{58}O_{10}N_8Cl(C_6H_3O_4N_2)$: C 54.52, H 6.02, N 13.83, Cl 3.51, MW 1012.5

λ_{max}^{MeOH}: 227 mμ ($E_{1cm}^{1\%}$ 472), 265 mμ ($E_{1cm}^{1\%}$ 122.5), 290 mμ ($E_{1cm}^{1\%}$ 81.3), 305.5 mμ ($E_{1cm}^{1\%}$ 74.2), 350 mμ ($E_{1cm}^{1\%}$ 178).

Acetyl derivative of S-520 (Acetyl-S-520): S-520 hydrochloride (200 mg) was dissolved in pyridine (10 ml), and acetic anhydride (10 ml) was added. The solution was kept in an ice box for 1 hour. Ice water (50 ml) was added to the solution, which was then extracted with ethyl acetate (50 ml). The extract was dehydrated with sodium sulfate, and concentrated to dryness under reduced pressure. The residue was dissolved in a small amount of methanol-ethyl acetate, and ether was added to give a colorless powder (120 mg), m.p. 245~255°C (dec.).

Anal. Found: C 56.49, H 6.95, N 12.41, Cl 3.83

Calcd. for $C_{40}H_{59}O_{10}N_9Cl\cdot CH_3CO$: C 55.78, H 6.86, N 13.95, Cl 3.93, MW 903.5

$C_{40}H_{58}O_{10}N_8Cl\cdot CH_3CO$: C 56.72, H 6.87, N 12.61, Cl 4.00, MW 888.5

λ_{max}^{MeOH}: 227 mμ ($E_{1cm}^{1\%}$ 408), 283 mμ ($E_{1cm}^{1\%}$ 57.5), 290 mμ ($E_{1cm}^{1\%}$ 58.6), 299.5 mμ ($E_{1cm}^{1\%}$ 43.1).

Conversion of I and II forms of S-520 by acid and alkali: Treatment with acid: A preparation of S-520 hydrochloride (10 mg) was dissolved in methanol (1 ml), and 1 N HCl (1 ml) was added. The solution was kept at 37°C for respective periods. Sample solutions to be tested were concentrated and then lyophilized.

Treatment with alkali: A preparation of S-520 hydrochloride (10 mg) was dissolved in methanol (1 ml), and mixed with 0.2 N NaOH (1 ml). The solution was kept at 37°C for respective periods. The sample solution was then neutralized by adding dry ice (CO_2) to pH 6~7, concentrated and lyophilized.

Measurement of I and II: The residues prepared as above were dissolved in methanol, and admixed with water (1 ml) and sodium bicarbonate (10~20 mg). To that, 0.5 ml of 10% 2,4-dinitrofluorobenzene in methanol was added. The mixtures were shaken for 30 min. at room temperature and then a small amount of water was added to ensure complete precipitation. The yellow precipitate (DNP-S-520) was collected by centrifuge, washed with water and dried. The residue was applied to a Silica gel GF plate and developed with chloroform–methanol (9 : 1). The separated spots of DNP-S-520-I and II were extracted with 4 ml of methanol, and optical densities at 350 mμ were measured. The relative contents of I and II were calculated from their optical densities. The results are shown in Table 2.

Table 2. Contents of I and II of S-520 after treatments with acid and alkali

Time of treatment	Treatment with HCl		Treatment with NaOH	
	I	II	I	II
0 hour	48%, 62%	52%, 38%	18%	82%
20 hours	83%, 77%	17%, 23%	21%, 22%	79%, 78%
44 hours	82%	18%	16%	84%

When S-520 hydrochloride (10 mg) was treated with methanol (1 ml) and 1.5 N NH_4OH (1 ml) for 24 hours at 37°C, the content of II was measured as approximately 80% by the same manner as above.

Amino acid analyses: Analysis was carried out by a Hitachi autuomatic amino acid analyzer with a column, 50×0.9 cm, packed with Hitachi spherical resin No. 3105, under a flow rate of 30 ml/min. at 50°C. For analytical condition of acidic and neutral amino acids, 0.2 M sodium citrate buffer pH 3.25 was run, followed after 170 min. by 0.2 M sodium citrate buffer pH 4.25. For analytical condition of basic amino acids, 0.7 M

sodium citrate buffer pH 5.28 was used.

The results are shown in Fig. 2 and Table 1.

Isolation and identification of known amino acids: S-520 hydrochloride (1.447 g) was hydrolyzed with 6 N HCl for 24 hours at 105°C. The hydrolysate was evaporated to dryness, and dissolved into 50 ml of water. The aqueous solution was extracted with *n*-butanol (50 ml). The aqueous fraction, which contained ornithine, lysine, a-I, glycine, valine, isoleucine and small amounts of *n*-I, *n*-II and *n*-III, was subjected to preparative paper chromatography carried out on Toyo Roshi No. 525 with *n*-butanol - acetic acid - water (4 : 1 : 2). Two mixtures (ornithine and lysine; and a-I and glycine), valine and isoleucine were isolated. These were extracted with acidic aq. methanol (HCl) from the paper and concentrated to dryness to give respective crude preparations: a mixture of ornithine and lysine (181 mg), a mixture of a-I and glycine (176 mg), valine (78 mg) and isoleucine (30.4 mg). The mixture of a-I and glycine was passed through an IR-4B (OH) column (7×0.9 cm). The effluent was dried to give glycine, which was then crystallized from water-ethanol as colorless crystals.

Anal. Found: C 31.61, H 6.66, N 18.10.

Calcd. for $C_2H_5O_2N$: C 32.00, H 6.71, N 18.66 %.

Valine hydrochloride was crystallized from water-ethanol as colorless plates.

Anal. Found: C 38.75, H 7.82, N 9.58.

Calcd. for $C_5H_{11}O_2N \cdot HCl$: C 39.08, H 7.81, N 9.12 %.

O.R.D.: $[\phi]_{250}$ −1644, $[\phi]_{227}$ −3644 (trough), $[\phi]_{215}$ −2480 (*c*, 0.0171, 0.5 N HCl).

$[\alpha]_D^{23}$ −16.2°±0.5° (*c*, 0.554, 1 N HCl).

The crude preparation of isoleucine was adsorbed on a small column of Dowex 50×8 (NH_4 type) and eluted with 1 N NH_4OH. Concentration of the eluate and crystallization from water-ethanol gave colorless plates of isoleucine.

Anal. Found: C 55.49, H 9.60, N 10.83, O 24.18.

Calcd. for $C_6H_{13}NO_2$: C 54.94, H 9.99, N 10.68, O 24.40 %.

O.R.D.: $[\phi]_{250}$ −1121, $[\phi]_{225}$ −4180 (trough), $[\phi]_{212}$0 (*c*, 0.01638, 0.5 N HCl).

$[\alpha]_D^{23}$ −33.3°±1.5° (*c*, 0.489, 1 N HCl).

The n.m.r. spectrum measured on D_2O solution was identical with that of D-isoleucine.

Ornithine and lysine: The crude mixture of ornithine and lysine was examined by paper chromatography on Toyo Roshi No. 51 with isopropanol - acetic acid - water (3 : 1 : 1). The spots of ornithine (Rf 0.24) and lysine (Rf 0.28) were confirmed by direct comparison with respective authentic specimens and by color reaction with vanillin reagent.

O.R.D. was measured with this mixture; ornithine/lysine (approximately 4 : 1).

O.R.D.: $[\phi]_{300}$ −220, $[\phi]_{250}$ −534, $[\phi]_{225}$ −1666 (trough), $[\phi]_{215}$ −796 (*c*, 0.1328, 0.5 N HCl).

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