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Azaserine, Synthetic Studies. I

BY JAMES A. MOORE, JOHN R. DICE, ERNEST D. NICOLAIDES, ROGER D. WESTLAND AND EUGENE L. WITTLE

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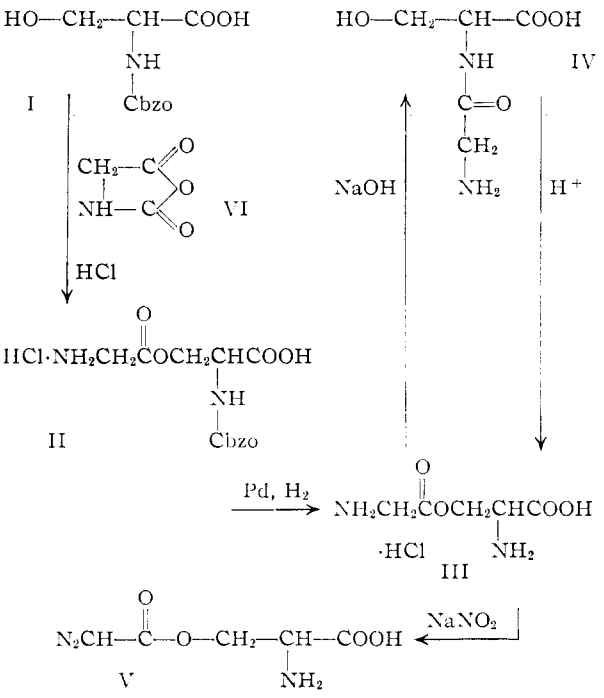
The synthesis of the antibiotic azaserine, O-diazoacetyl-L-serine, has been accomplished by selective diazotization of O-glycyl-L-serine. This intermediate was prepared by the reaction of N-carboxyglycine anhydride with N-carbobenzoxy-L-serine followed by debenzylation or, less satisfactorily, by sulfuric acid isomerization of N-glycyl-L-serine.

In a previous communication from these laboratories,[1] the isolation from fermentation cultures of a Streptomyces of azaserine, an antibiotic which inhibits the growth of various neoplasms, was reported. Degradation studies[2] have shown that this antibiotic is O-diazoacetyl-L-serine (V). We now wish to describe the work leading to the synthesis of this unique compound.

The synthetic route which was chosen for the preparation of this substance comprises the preparation and diazotization of O-glycyl-L-serine monohydrochloride (III). This compound, which has not been described previously, has been obtained by several methods which are described in this and the following paper. Throughout the work, most of the reactions were performed with both DL- and L-serine and hereafter the term serine refers to both series.



Although preferential acylation of the hydroxyl group of serine has been effected in strongly acidic media,[3] the esterification of a serine derivative in which the amino function is protected by an easily removed protective group was considered to be the most versatile approach to the introduction of the glycyl group or a suitable precursor. This was accomplished by the use of N-carboxyglycine anhydride (VI) as indicated in the diagram. When a solution of N-carbobenzoxyserine in dioxane or ethyl acetate was warmed with the Leuchs anhydride (VI) in the presence of one equivalent of hydrogen chloride, a reaction took place with the separation of crude O-glycyl-N-carbobenzoxyserine hydrochloride (II) as an oil. It was possible to obtain a small amount of the O-glycyl-N-carbobenzoxy-DL-serine hydrochloride in crystalline form although it was preferable to hydrogenate the total crude product and to isolate the resulting O-glycylserine monohydrochloride. This condensation was difficult to control in large scale preparations. Side reactions could not be avoided, and although an excess of N-carbobenzoxyserine was used, some glycylglycine and probably higher polymers were formed. Over-all yields of III in the two steps were 20–40%.

As an alternative to the use of a protective group in the serine molecule, the possibility of effecting the transfer of a glycyl group from the nitrogen to oxygen atom was explored. Although the formation of the oxazoline and its conversion to the ester is a well known procedure, and has been used for the preparation of O-benzoylserine, this method is not readily applicable to the amino acyl compound.[4] A more suitable means of carrying out the acyl shift has been described by Elliott, in which N-peptidyl residues were transferred to the adjacent hydroxyl group in serine-containing proteins.[5] In applying this procedure to N-glycylserine the peptide was dissolved in cold concentrated sulfuric acid and after prolonged standing the reaction mixture was processed to furnish crude O-glycylserine sulfate which was used directly in the subsequent diazotization step. This procedure, like the foregoing, is not well adapted to large scale preparations.

The O-glycylserine monohydrochloride (III) in both the DL- and L-series has been obtained analytically pure by crystallization from an aqueous solution by the addition of alcohol. The infrared spectra (Fig. 1, a and b) show well-defined ester bands and no amide absorption. While these compounds are stable in acid solution, they rearrange rapidly and completely to N-glycylserine in neutral or alkaline solution. Thus O-glycyl-DL-serine mono-

(1) J. Ehrlich, L. Anderson, G. L. Coffey, A. B. Hillegas, M. A. Knudsen, H. J. Koepsell, D. L. Kohlberger and J. E. Oyaas, *Nature*, **173**, 72 (1954).

(2) S. A. Fusari, T. H. Haskell, R. P. Frohardt and Q. R. Bartz, THIS JOURNAL, **76**, 2881 (1954); S. A. Fusari, R. P. Frohardt, A. Ryder, T. H. Haskell, D. W. Johannessen, C. C. Elder and Q. R. Bartz, *ibid.*, **76**, 2878 (1954).

(3) *Cf.* W. Sakami and G. Toennies, *J. Biol. Chem.*, **144**, 203 (1942).

(4) Attempts in this direction have been described by M. Bergmann and A. Miekeley, *et al.*, in *Z. Physiol. Chem.*, **140**, 128 (1924); **143**, 108 (1925); **146**, 247 (1925).

(5) D. F. Elliott, *Biochem. J.*, **50**, 542 (1952).

hydrochloride at *p*H 7.5 for 24 hours gave a 94% yield of N-glycyl-DL-serine.

Diazotization of the O-glycyl ester (III) must be performed within a very narrow *p*H range. Unlike other diazoesters which have been prepared by direct diazotization, the product in this case is an amino acid which cannot readily be removed from the reaction mixture as it is formed. This reaction was carried out by allowing an aqueous solution of the O-glycylserine monohydrochloride to react with nitrite ion at a *p*H between 4.5 and 5.0.

The reaction was allowed to proceed, usually at room temperature, until no further increase in the ultraviolet absorption maximum at 250 mμ was observed. The reaction rate was much greater at lower *p*H values, but concomitant destruction of the product resulted in lower yields. A surprising feature of this reaction was the finding that the use of a several-fold excess of the nitrosating agent significantly improved the yield, despite the fact that selective diazotization of one amino group was required. The product was isolated by adsorption on activated charcoal and elution with aqueous acetone, a procedure which was developed for the isolation of the antibiotic from culture filtrates.[2]

The yield of O-diazoacetylserine produced in the reaction as measured by inhibition of the test organism *Kloeckera brevis* was considerably lower than that calculated on the basis of ultraviolet absorption at λ 250 mμ. This indicated that other diazo compounds were formed in the diazotization, and evidence of the presence of another, highly unstable diazo compound was obtained in the chromatography. This material, however, could not be isolated or identified, and no other products were characterized.

The O-diazoacetyl-DL-serine (V) obtained in this process was recrystallized from water by the addition of alcohol, m.p. 153–155° dec., $\lambda_{max}^{H_2O}$ 250 mμ, log 4.30; the infrared spectrum is shown in Fig. 1c. This material has one-half the activity of azaserine in the *K. brevis* growth inhibition assay.

The synthetic O-diazoacetyl-L-serine was identical in all physical and biological properties with the antibiotic azaserine; the infrared spectra (Fig. 1d) are superimposable. The position of the diazo group in azaserine has been firmly established by the isolation of O-acetyl-L-serine and O-glycolyl-L-serine after reduction and treatment with dilute aqueous acid, respectively.[2] This structure proof, together with the identity of the synthetic and fermentation products, serves to demonstrate unequivocally the constitution of the compound obtained in this synthesis, eliminating the possibility that diazotization had occurred at the α-amino group of the serine.

Acknowledgment.—The authors wish to thank Dr. T. H. Haskell and Carole C. Elder for the initial purifications of azaserine and O-diazoacetyl-DL-serine on carbon columns and for their advice. They are indebted to Dr. J. M. Vandenbelt, R. Bruce Scott, E. J. Schoeb and Carola Henrich for the infrared and ultraviolet absorption spectra and to C. E. Childs, E. E. Meyers, Claire Johnston and Virginia Pawlik for the microanalyses. They also wish to express their appreciation to Dr. Harry M. Crooks, Jr., for his advice and encouragement.

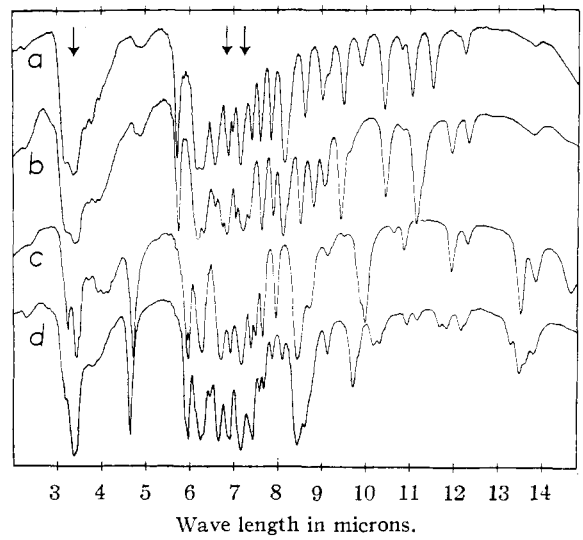


Fig. 1.—Infrared absorption spectra of: (a) O-glycyl-DL-serine monohydrochloride; (b) O-glycyl-L-serine monohydrochloride; (c) O-diazoacetyl-DL-serine; (d) O-diazoacetyl-L-serine, azaserine (in Nujol mulls, arrows indicate major Nujol bands.)

Experimental

N-Carbobenzoxy-L-serine (I).—Large quantities of this compound as well as the corresponding derivative of DL- and D-serine were required in this work; since published procedures[6] gave only moderate yields, it seems desirable to describe the procedure used.

In a 1-l. beaker, cooled in an ice-bath and containing a stirrer and *p*H electrodes, was placed 44.3 g. (0.42 mole) of L-serine ($[\alpha]^{27}D$ +14.4°, *c* 10 in 1 *N* HCl) and 212 ml. of 2 *N* sodium hydroxide. To the well-stirred solution, which had a *p*H of 9.8, at 5–8°, was added a small amount of benzyl chloroformate from which the toluene solvent had been removed. At 10–15 min. intervals, portions (4–5 g.) of benzyl chloroformate were added; as the *p*H of the solution fell to 9.0, sodium hydroxide (1 *N*) was added dropwise to shift the *p*H to 9.8–10.0. The alternate addition was continued until 80 g. (0.46 mole) of benzyl chloroformate had been added. The *p*H was then maintained at 10 for 0.5 hour at 10° in order to hydrolyze any O,N-biscarbobenzoxy-L-serine. A total of about 450 ml. of 1 *N* sodium hydroxide was usually required. The basic solution was extracted with 150 ml. and then 100 ml. of ether.

The alkaline aqueous solution was placed in a 2-l. beaker, 1 l. of ethyl acetate was added and, with vigorous stirring, the solution was acidified to *p*H 3 by the careful addition of 30–35 ml. of concd. hydrochloric acid. The layers were separated and the aqueous layer was extracted with 250 ml. and 150 ml. of ethyl acetate. The combined ethyl acetate solution was dried over magnesium sulfate and concentrated *in vacuo* at 50° until a considerable amount of crystalline N-carbobenzoxy-L-serine had precipitated.

The solution was gently warmed to redissolve the product and was then allowed to cool. The product was filtered, washed with a little ethyl acetate and air dried at 50°, weight 47 g., m.p. 114–118°. The mother liquor was concentrated further *in vacuo* and a second crop was obtained, weight 32 g., m.p. 113–116°, total yield 79 g. (78%). Recrystallization from ethyl acetate gave a pure product, m.p. 117–119°, $[\alpha]^{26}D$ +5.8° (*c* 6 in acetic acid).

O-Glycyl-N-carbobenzoxy-DL-serine Hydrochloride (II).—To a suspension of 12 g. of N-carbobenzoxy-DL-serine in 100 ml. of dry ethyl acetate containing 4 g. of anhydrous hydrogen chloride was added 5 g. of N-carboxyglycine an-

(6) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942); M. Bergmann and L. Zervas, *Ber.*, **65**, 1196 (1932).

hydride.[7] The mixture was heated for 0.3 hour at 60° and was then allowed to stand at 25° overnight. The ethyl acetate was decanted from the gummy precipitate and the precipitate was washed with hot ethyl acetate. The gum was dissolved in 25 ml. of absolute alcohol and the solution was evaporated to dryness *in vacuo*, leaving 13 g. of viscous oil. (In most cases this oil was used directly in subsequent reductions.) The oil was dissolved in ethanol and fractionally precipitated by the addition of ethyl acetate and ether. Some of these fractions could be crystallized from methanol and then recrystallized from methanol–ethanol to yield 140 mg. of O-glycyl-N-carbobenzoxy-DL-serine hydrochloride, m.p. 182–184° dec. The infrared spectrum had sharp bands at 5.67 μ (COOH); 5.78 μ (ester); 5.92 μ and 6.5 μ (amide); 13.2 and 14.4 μ (associated with carbobenzoxy).

Anal. Calcd. for $C_{13}H_{17}O_6N_2Cl$: C, 46.92; H, 5.15; N, 8.42. Found: C, 47.22; H, 5.22; N, 8.57.

O-Glycyl-DL-serine Monohydrochloride (III).—A solution of 25 g. of O-glycyl-N-carbobenzoxy-DL-serine monohydrochloride in 100 ml. of water was shaken with hydrogen and 1 g. of palladium black catalyst for 2 hours at 3 atm. pressure. The catalyst was removed by filtration and the aqueous filtrate was concentrated *in vacuo* to a small volume. Ethanol (100 ml.) was added, and the clear solution was warmed and allowed to stand; 8 g. (54% yield) of O-glycyl-DL-serine monohydrochloride separated as fine white needles, m.p. 159–161° dec. The infrared spectrum is shown in Fig. 1a.

Anal. Calcd. for $C_5H_{11}O_4N_2Cl$: C, 30.23; H, 5.58; N, 14.11. Found: C, 29.97; H, 5.77; N, 14.04.

The decomposition points of this compound and of the L-isomer varied considerably with the rate of heating. On paper chromatography in a *t*-butyl alcohol–acetic acid–water mixture (50:25:25), this compound had an R_f value of 0.27 to 0.32. The chromatograms usually showed traces of other ninhydrin reacting substances which are probably due in part to the migration of different ionic species and to the rearrangement of O-glycyl-DL-serine to N-glycyl-DL-serine.

Rearrangement of O-Glycyl-DL-serine Monohydrochloride.—To a solution of 2 g. (0.01 mole) of O-glycyl-DL-serine monohydrochloride in 50 ml. of water was added sufficient 1 *N* sodium hydroxide solution to give a *p*H of 7.5. The solution was allowed to stand overnight, acidified to *p*H 3.5 with dilute hydrochloric acid and then neutralized to *p*H 7 with ammonium hydroxide. The solution was evaporated *in vacuo* to a small volume and absolute ethanol was added. An oil was obtained which slowly solidified. Two crystallizations from water–ethanol gave small white needles, m.p. 196–196.5° dec., yield 1.5 g. (94%). The infrared spectrum confirmed the identity of this product as N-glycyl-DL-serine (IV).

O-Diazoacetyl-DL-serine (V).—To a solution of 990 mg. (5 meq.) of O-glycyl-DL-serine monohydrochloride in 30 ml. of water was added a solution of 863 mg. (12.5 meq.) of sodium nitrite in 20 ml. of water. After standing for 0.5 hour the solution was shell-frozen and lyophilized. The residue had an absorption maximum of $E^{1\%}_{1\text{ cm}}$ 152 at $\lambda^{H_2O}_{max}$ 250 mμ. It was dissolved in 10 ml. of water and chromatographed on a column (22 mm. dia.) containing a mixture of 15 g. of Darco G-60 charcoal and 15 g. of Celite 545. The column was washed with 130 ml. of water and was then eluted with 2% aqueous acetone. After 90 ml. of clear eluate had been obtained, 50 ml. of pale yellow eluate with a positive ninhydrin reaction was collected. This second fraction was shell-frozen and evaporated *in vacuo* to yield 161 mg. of yellow powder, $E^{1\%}_{1\text{ cm}}$ 842 at $\lambda^{H_2O}_{max}$ 250 mμ. This material was recrystallized from a mixture of 1 ml. of pyridine, 1.5 ml. of alcohol and 0.3 ml. of water to give 70 mg. (8%) of yellow crystalline product, $E^{1\%}_{1\text{ cm}}$ 1140 at $\lambda^{H_2O}_{max}$ 250 mμ (ϵ 19,700; log ϵ 4.3), m.p. 153–155° dec. The infrared spectrum is shown in Fig. 1c. This substance showed one half the activity of azaserine (175 times the standard) in the *K. brevis* assay.

Anal. Calcd. for $C_5H_7O_4N_3$: C, 34.69; H, 4.08; N, 24.27. Found: C, 34.75; H, 4.28; N, 24.02.

(7) Prepared by the method of V. Y. Go and H. Tani, *Bull. Chem. Soc. Japan*, **14**, 510 (1939); *C. A.*, **34**, 1971 (1940).

O-Glycyl-N-carbobenzoxy-L-serine Hydrochloride (II).—To a suspension of 25 g. (0.105 mole) of N-carbobenzoxy-L-serine in 200 ml. of dry ethyl acetate containing 8 g. (0.21 mole) of hydrogen chloride gas was added 10 g. (0.1 mole) of N-carboxyglycine anhydride. The mixture was warmed to 50–60° for 0.5 hour during which time all of the solid dissolved and a large amount of gas was evolved. After one hour a colorless oil began to precipitate. The mixture was allowed to stand overnight, the solvent was decanted from the oil, and the oil was washed with hot ethyl acetate. The product was dissolved in 50 ml. of ethanol and the solution was evaporated to dryness yielding 22 g. (67%) of pale yellow oil.

O-Glycyl-L-serine Monohydrochloride (III).—The 22 g. (0.067 mole) of product from the above reaction was dissolved in 100 ml. of water and was shaken with 200 mg. of palladium black catalyst for 2 hours at 2 atm. of hydrogen. The catalyst was removed by filtration and the filtrate was concentrated *in vacuo* to small volume. Absolute ethanol (100 ml.) was added and a colorless oil precipitated. The mixture was warmed to 40° and the oil solidified. The solution was cooled and the solid was filtered, washed with alcohol and ether and air-dried to yield 5 g. (37%) of white crystals, m.p. 152.5° dec. The product was recrystallized from water–ethanol to give small white plates, m.p. 161.5° dec., $[\alpha]^{28}D$ +10.4° (*c* 5 in water). The infrared spectrum is shown in Fig. 1b.

Anal. Calcd. for $C_5H_{11}O_4N_2Cl$: C, 30.23; H, 5.58; N, 14.11. Found: C, 29.99; H, 5.70; N, 14.12.

O-Diazoacetyl-L-serine; Azaserine (V).—A solution of 990 mg. (5 meq.) of O-glycyl-L-serine monohydrochloride in 75 ml. of water was treated with 1.54 g. (10 meq.) of silver nitrite at 25°. The mixture was shaken for 15 minutes and the dark silver chloride was removed by filtration through Celite. Sodium chloride (150 mg.) was added and the silver chloride was again removed. The clear yellow solution, free of silver ion, was shell-frozen and lyophilized to give a light tan powder, weight 900 mg., $E^{1\%}_{1\text{ cm}}$ 449 at $\lambda^{H_2O}_{max}$ 250 mμ. This solid was dissolved in 10 ml. of water and put on a column (22 mm. dia.) containing a mixture of 11 g. of Darco G-60 and 11 g. of Celite 545. The column was washed with 2 holdup volumes (120 ml.) of water and then with water containing 2% acetone. The eluate giving a positive ninhydrin test (50 ml.) was collected and evaporated to dryness from the frozen state to yield 266 mg. of yellow powder $E^{1\%}_{1\text{ cm}}$ 907 at $\lambda^{H_2O}_{max}$ 250 mμ. This was recrystallized from water and alcohol to give 170 mg. (20% yield) of yellow green crystalline solid, m.p. 153–155° dec., $E^{1\%}_{1\text{ cm}}$ 1125 at $\lambda^{H_2O}_{max}$ 250 mμ (ϵ19,500; log ϵ 4.29), $[\alpha]^{27}D$ −0.6° ± 0.6 (*c* 5.0 in H_2O). This material had the infrared spectrum shown in Fig. 1d, identical with that of azaserine.[2] It showed complete activity (330 times the standard) in the *K. brevis* assay.[1]

Anal. Calcd. for $C_5H_7O_4N_3$: C, 34.69; H, 4.08; N, 24.27. Found: C, 34.74; H, 4.39; N, 23.93.

Rearrangement of N-Glycyl-DL-serine (IV) to O-Glycyl-DL-serine (III) and Diazotization.—N-Glycyl-DL-serine (2.4 g.) was added slowly with constant agitation to 38 ml. of 100% sulfuric acid at 0°. The clear solution was allowed to stand at 25° for 3–5 days. The mixture was cooled to −30 to −40° in a Dry Ice–isopropyl alcohol bath and was added cautiously to 500 ml. of anhydrous ether at −30°. The white hygroscopic precipitate was filtered rapidly, washed with dry ether, and dissolved in 50 ml. of water. Barium hydroxide solution (1 *N*) was immediately added dropwise until a *p*H of 4.5 was reached and the barium sulfate was removed by means of a small basket centrifuge. A cold solution of 1.2 g. of sodium nitrite in 130 ml. of water was added in portions with stirring over five minutes. After standing at 0° for 0.5 hour and at 25° for 4.5 hours, the solution was chromatographed on activated carbon as described previously. The O-diazoacetyl-DL-serine (V) thus obtained was recrystallized from water–alcohol to give yellow crystals, yield 150 mg., $E^{1\%}_{1\text{ cm}}$ 1000 at $\lambda^{H_2O}_{max}$ 250 mμ. The material had an infrared spectrum identical with that in Fig. 1c; the *K. brevis* assay (175 times the standard) was one half that of azaserine.

Rearrangement of N-Glycyl-L-serine to O-Glycyl-L-serine and Diazotization.—Two and four-tenths grams of N-glycyl-L-serine (m.p. 201–202°, $[\alpha]^{27}D$ −9.2°) was treated exactly

as in the above rearrangement. Diazotization of the L-compound gave 106 mg. of crude O-diazoacetyl-L-serine (V), $E^{1\%}_{1\,cm}$ 1065 at $\lambda^{H_2O}_{max}$ 250 mμ. After recrystallization from aqueous alcohol, a value of $E^{1\%}_{1\,cm}$ 1140 at $\lambda^{H_2O}_{max}$ 250 mμ was obtained. The infrared spectrum and microbiological assay (330 times standard) were identical with those of azaserine.

DETROIT 32, MICHIGAN

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF PARKE, DAVIS & COMPANY]

Azaserine, Synthetic Studies. II

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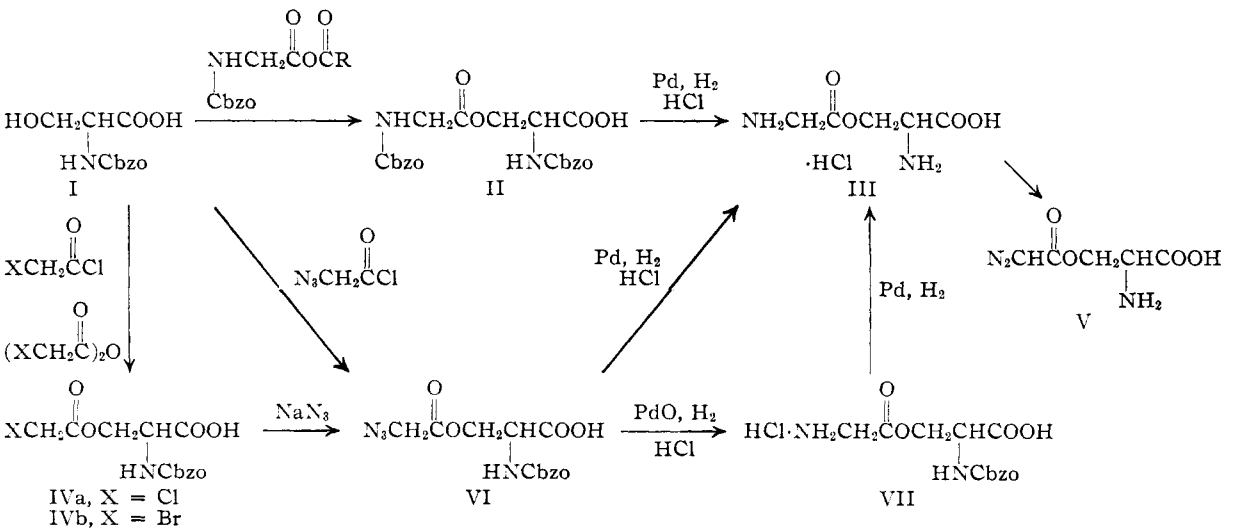
Two methods for the preparation of O-glycyl-L-serine monohydrochloride, the intermediate in the synthesis of the antibiotic azaserine, are presented. The esterification of N-carbobenzoxy-L-serine with a haloacetyl halide or anhydride and an azide displacement of the halide group followed by hydrogenation and debenzylation has given a moderate yield of the desired intermediate. Alternately, the esterification of N-carbobenzoxy-L-serine with mixed anhydrides of carbobenzoxyglycine and various acids followed by debenzylation, gave satisfactory yields of O-glycyl-L-serine monohydrochloride. Selective diazotization of O-glycyl-L-serine has produced azaserine. These reactions have also been carried out with DL- and D-serine.

In the preceding paper,[1] the preparation of O-glycyl-L-serine monohydrochloride (III) by two methods and its diazotization to the antibiotic azaserine (V) has been described. Since a more suitable method for the preparation of this intermediate was desired, other potential syntheses were investigated. This paper describes two additional methods for the preparation of the intermediate. As with the previous work, all the reactions were first carried out with DL-serine. For comparative purposes O-glycyl-D-serine monohydrochloride also was prepared and converted to O-diazoacetyl-D-serine.

While the direct introduction of the amino acyl group into a serine derivative has been successful, as described in the previous paper, in the present work it has been found more advantageous first to introduce an amine precursor or protected amino group and subsequently generate the amine.



One of the methods developed for the synthesis of O-glycylserine monohydrochloride (III) involved the preparation of O-haloacetyl-N-carbobenzoxyserine (IV), a nucleophilic displacement of the halide group by the azide ion and a subsequent hydrogenation of the azide and carbobenzoxy groups in the presence of hydrogen chloride.

The reported instances of serine derivatives in which the hydroxyl group of serine is esterified are limited,[2-5] and the O-halo- or O-azidoacetylserine derivatives have not been previously reported.

In the present work the reactions of chloroacetyl chloride, chloroacetic anhydride and bromoacetyl bromide with N-carbobenzoxy-L- or DL-serine (I) have been studied. A diversity of anhydrous solvents have been used in the presence or absence of a tertiary base. In the DL-series, crystalline products could be obtained in every case, while in the L-series, due to increased solubility and lower melting points, nearly all of the reaction products were oils. It was possible in the L-series, however, to obtain small amounts of crystalline product from three of the reactions which enabled characterization of every intermediate. The reaction of chloroacetic anhydride with N-carbobenzoxy-DL-serine in refluxing benzene or ethyl acetate without a base

(1) J. A. Moore, J. R. Dice, E. D. Nicolaides, R. D. Westland and E. L. Wittle, THIS JOURNAL, **76**, 2884 (1954).

(2) W. Sakami and G. Toennies, *J. Biol. Chem.*, **144**, 203 (1942).

(3) M. Frankel and M. Halmann, *J. Chem. Soc.*, 2735 (1952).

(4) R. L. M. Synge, *Biochem. J.*, **33**, 1924 (1939).

(5) M. Bergmann and A. Miekeley, *Z. physiol. Chem.*, **140**, 128 (1924).