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O-Carbazyl-DL-serine, an Inhibitory Analog of Glutamine

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O-Carbazyl-DL-serine has been prepared by hydrogenolysis of the intermediate formed by the interaction of O-chloroformyl-N-carbobenzoxy-DL-serine benzyl ester (formed from phosgene and N-carbobenzoxy-DL-serine benzyl ester) and carbobenzoxyhydrazine. The interaction of the O-chloroformyl derivative directly with hydrazine and subsequent hydrogenolysis produced the disubstitution product, O,O′-bicarbamylserine, but interaction with phenylhydrazine by the same procedure gave O-(3-phenylcarbazyl)-serine. O-Carbazylserine inhibits the growth of *Streptococcus lactis* and the inhibition is reversed competitively by glutamine.

The introduction of an oxygen atom in place of a methylene group of a natural metabolite has resulted in several instances in a structural analog which is a competitive antagonist of the metabolite.[1-5] O-Carbamylserine, for example, is a competitive antagonist of glutamine.[4] Also, inhibitory analogs with a hydrazide in place of an amide group have been reported frequently; for example, γ-glutamylhydrazine is a competitive antagonist of glutamine in several biological systems.[6] It was of interest to determine whether an analog with dual modifications of an oxygen in place of a methylene and a hydrazide group in place of an amide group would be an antagonist with unusual activities. Accordingly, O-carbazylserine was prepared and found to be a competitive inhibitor of glutamine in several microörganisms.

An initial attempt was made to synthesize O-carbazylserine *via* a similar route used in the preparation of O-carbamylserine[4] using hydrazine in place of ammonia as a reactant with the intermediate condensation product produced from the interaction of N-carbobenzoxy-DL-serine benzyl ester and phosgene. However, the material isolated after hydrogenolysis of the reaction mixture proved to be the dimer, O,O′-bicarbamylserine, with only a trace of the desired compound. The chemical reactivity of hydrazine in this type reaction is demonstrated by adding carbobenzoyl chloride to a large molar excess of hydrazine at room temperature; the disubstituted hydrazine is formed in about 80% yield. Both of the above disubstituted derivatives failed to react with active carbonyl reagents which suggests the absence of an unsubstituted H_2N–grouping and that the symmetrical derivative is the more probable structure. To obtain the desired compound, one of the amino groups of hydrazine was protected prior to interaction with the intermediate phosgene condensation product of N-carbobenzoxyserine benzyl ester. This was accomplished by preparing carbobenzoxy hydrazine through hydrazinolysis of dibenzyl carbonate and allowing this derivative to react with the chloroformyl intermediate, as indicated in the accompanying equations. The product on hydrogenolysis yielded the desired amino acid derivative which was found to inhibit the growth of several microörganisms.

The chemical reactivity of the hydrazine molecule presented the possibility of various side reactions which might produce biologically active compounds; *i.e.*, that of hydrazinolysis of the benzyl ester and/or carbobenzoxy group to yield the corresponding hydrazine derivatives. In order to demonstrate that a small contaminant of these derivatives did not produce the observed biological activity, N-carbobenzoxyserine benzyl ester was allowed to react with one equivalent of hydrazine in ethanol under reflux during a four-hour period to produce N-carbobenzoxyserine hydrazide; and upon heating with an excess of hydrazine the N-carbazylserine hydrazide was produced. The comparative stability of the carbobenzoxy group under the normal synthesis conditions is demonstrated by allowing N-carbobenzoxyserine to stand in the presence of hydrazine for 12 hours; under these conditions the compound produced is the N-carbobenzoxyserine hydrazonium salt. Of these latter derivatives, only the hydrazonium salt possessed biological activity, and in this case the inhibitory properties result from the hydrazine of the salt.

O-Carbazylserine is very soluble in water, and yields a typical purple ninhydrin reaction on a paper chromatogram with R_f values, when determined by the ascending technique in 1-butanol–acetic acid–water (3:1:1) and 65% pyridine, of 0.27 and 0.46, respectively. A solution of the compound after standing for several days decreases 85% in inhibitory activity upon the growth of *S. lactis*. Thus, the compound is somewhat unstable in aqueous solution. O-Carbazylserine rapidly interacts with carbonyl reagents such as formaldehyde and benzaldehyde to form the corresponding hydrazone derivatives.

O-(3-Phenylcarbazyl)-DL-serine was prepared by the interaction of the intermediate phosgene condensation product of N-carbobenzoxy-DL-serine benzyl ester with phenylhydrazine. The product eventually isolated after hydrogenolysis did not interact with carbonyl reagents indicating that the condensation occurred with the terminal amino nitrogen. This derivative was about one-tenth as active as the unsubstituted O-carbazylserine in the *S. lactis* assay subsequently described.

O-Carbazyl-DL-serine inhibits the growth of *Streptococcus lactis* 8039 at a concentration of 3 γ per ml. as indicated in Table I. This toxicity is

(1) N. H. Horowitz and A. M. Srb, *J. Biol. Chem.*, **174**, 371 (1948).

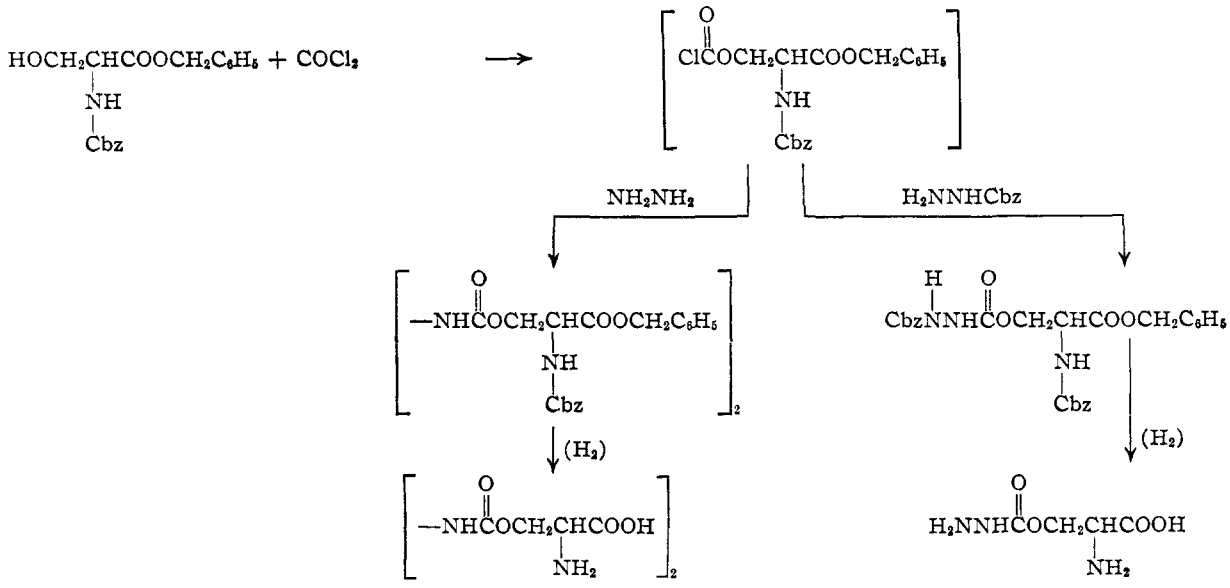
(2) B. E. Volcani and E. E. Snell, *ibid.*, **174**, 893 (1948).

(3) M. Rabinovitz, M. E. Olson and D. M. Greenberg, This Journal, **77**, 3109 (1955).

(4) C. G. Skinner, T. J. McCord, J. M. Ravel and W. Shive, *ibid.*, **78**, 2412 (1956).

(5) T. J. McCord, J. M. Ravel, C. G. Skinner and W. Shive, *ibid.*, **79**, 5693 (1957).

(6) H. McIlwain, J. A. Roper and D. E. Hughes, *Biochem. J.*, **42**, 492 (1948).



reversed competitively by glutamine, and the inhibition index (ratio of concentrations of analog to metabolite) for half-maximal growth is approximately 2. O-Carbamylserine, azaserine and γ-glutamylhydrazine also exert their initial inhibitory effect in the same concentration range of 2 to 4 γ per ml. The toxicity of either O-carbamylserine or γ-glutamylhydrazine (but not azaserine) is reversed competitively by glutamine. The results obtained with γ-glutamylhydrazine are indicated in Table I. The presence of an oxygen in place of a methylene of γ-glutamylhydrazine does not appreciably alter the toxicity of the analog for *S. lactis*.

TABLE I

REVERSAL OF O-CARBAZYLSERINE AND γ-GLUTAMYLHYDRAZINE TOXICITY BY L-GLUTAMINE IN *Streptococcus lactis*[a]

Analog, γ/ml.	L-Glutamine, γ/ml. 0	1	3	10	30
	Galvanometer readings				
O-Carbazyl-DL-serine					
0	48	48	48	48	44
1	48	47	41		
3	11	27	41	41	
10	0	2	10	33	44
30			3	14	26
100				4	10
γ-Glutamylhydrazine					
0	48	55	51	48	50
1	17	45	50		
3	2	6	24	36	
10			5	25	47
30				8	32
100					2

[a] Incubated at 30° for 27 hours.

Evidence that both O-carbazylserine and γ-glutamylhydrazine inhibit biosyntheses of certain metabolites in which essential glutamyl roles are involved is presented in Table II. Citrulline, hypoxanthine and uracil, each exerting an independent effect, increase the amount of the analogs necessary for inhibition. A combination of all three increases 5-to 10-fold the amount of analog necessary for half-maximal growth inhibition, and glutamine in an amount which alone gives a comparable reversing effect augments the reversal to approximately the same degree indicating that these products increase the inhibition index obtained with glutamine.

TABLE II

EFFECT OF PRODUCTS OF GLUTAMYL FUNCTIONS ON GROWTH INHIBITION BY O-CARBAZYLSERINE AND γ-GLUTAMYLHYDRAZINE IN *Streptococcus lactis*

Supplement, γ/ml.	Analog necessary for half-maximal inhibition of growth, γ/ml. O-Carbazyl-DL-serine	γ-Glutamyl hydrazine
None	1.2	0.6
Citrulline, 20; hypoxanthine, 10; and uracil, 5	6.2	6.7
Glutamine, 10	12	6
Glutamine, 10; citrulline, 20; hypoxanthine, 10; and uracil, 5	45	34

In separate experiments with *Lactobacillus arabinosus* 17-5, O-carbazylserine was found to be considerably more inhibitory than azaserine, about equivalent to O-carbamylserine, but less inhibitory than γ-glutamylhydrazine. Preliminary results indicate that O-carbazylserine given daily has a potent carcinostatic effect in mice with RC mammary carcinoma implants, but some damage to the liver was observed at the higher levels.[7]

Experimental[8]

Biological Testing.—The method of obtaining *Streptococcus lactis* 8039 cells for inoculation of growth assays has been described.[9] The assay procedure and medium were the same as previously presented[10] with the following exceptions: arginine, adenine, guanine and uracil were omitted

(7) These data were obtained by Mr. G. F. McKenna, the Biochemical Institute, The University of Texas, and are to be reported elsewhere.

(8) All melting points were determined with a Fisher–Johns melting point apparatus, and are uncorrected. The authors are indebted to Mr. D. R. Ross for the chemical analyses.

(9) J. M. Ravel, J. Estes, B. F. Mollenhauer and W. Shive, *J. Biol. Chem.*, **229**, 93 (1957).

(10) J. M. Ravel, L. Woods, B. Felsing and W. Shive, *ibid.*, **206**, 391 (1954).

from the basal medium; 0.2 γ/ml. of calcium pantothenate was added; and the concentrations of aspartic acid and glutamic acid were increased to 100 γ/ml. O-Carbazyl-DL-serine, γ-glutamylhydrazine, citrulline and glutamine were dissolved in sterile water, neutralized and added aseptically to the previously autoclaved assay tubes as indicated in the tables. The assay tubes were incubated at 30° for the indicated periods of time and the amount of growth was determined turbidimetrically in terms of galvanometer readings so adjusted in the particular instrument that distilled water reads 0 and an opaque object reads 100.

Dibenzyl Carbonate.—An ether solution of benzyl alcohol and triethylamine was treated with carbobenzoxy chloride at 0–5°. After the reaction was complete, triethylamine hydrochloride was removed by filtration, and the ether phase was washed with 0.5 *N* hydrochloric acid, 5% potassium bicarbonate and water. After drying, the solvent was removed, and the residue was fractionally distilled over porous boiling chips, b.p. 180–190° (2 mm.), $n^{30}D$ 1.5446. Upon cooling, the dibenzyl carbonate solidified, m.p. 27–28°.[11]

Carbobenzoxyhydrazine.—Dibenzyl carbonate was treated with 85% hydrazine, and after the reaction was complete the excess hydrazine was removed by heating the reaction mixture at 100–105° (3–4 mm.) for several hours. The crude product was recrystallized from ether, m.p. 67–68°.[12]

O-Carbazyl-DL-serine.—A mixture of 6.6 g. of N-carbobenzoxy-DL-serine benzyl ester[4] partially dissolved in 75 ml. of toluene was saturated with phosgene at 0–5°. The reaction mixture was kept for 16 hours at room temperature in a rubber-stoppered flask to effect complete reaction. The solvent was removed under reduced pressure, with warming, to yield a pale yellow oil which was freed of residual phosgene and hydrogen chloride gas by repeated addition and evaporation of benzene. A solution of this oil dissolved in 30 ml. of dry dioxane was added over a period of 1.5 hours to a stirred mixture of 5.0 g. of carbobenzoxy hydrazine in 75 ml. of ethanol plus 1.1 g. of sodium carbonate in 20 ml. of water at 5–10°. After completing the addition, the reaction mixture was stirred for one hour with continued cooling and then at room temperature for 4 hours. After reduction in volume *in vacuo*, with warming, the residue was dried by repeated addition and evaporation of benzene. Then the residue was slurried with 75 ml. of warm ethanol, and the sodium chloride was removed by filtration. Finally, an additional 150 ml. of ethanol was added followed by water until a slight turbidity persisted. The resulting mixture was then stirred under hydrogen gas at atmospheric pressure and room temperature in the presence of 0.7 g. of palladium black for 6 hours. The catalyst was filtered, washed with warm water and the combined filtrates were taken to dryness under reduced pressure with warming. The solid residue was recrystallized from alcohol–water to yield 1.7 g. of product, m.p. 193–195° dec.

Anal. Calcd. for $C_4H_9N_3O_4$: C, 29.44; H, 5.56; N, 25.76. Found: C, 29.86; H, 5.76; N, 25.96.

O,O'-Bicarbamyl-DL-serine.—The reaction of 6.6 g. of N-carbobenzoxy-DL-serine benzyl ester, in the same general procedure as described for the O-carbazylserine derivative, with phosgene followed by condensation with 1.0 g. of 95% hydrazine in 25 ml. of ethanol in the presence of 1.1 g. of sodium carbonate in 15 ml. of water gave 7.1 g. of a clear colorless oil. This oil, in 100 ml. of ethanol, was treated with hydrogen gas for 8 hours by the same procedure described above to yield 1.9 g. of solid residue. The solid material was suspended in 25 ml. of boiling methanol, and water was added with heating until solution was complete. After standing in the refrigerator for several hours, a gelatinous solid precipitated out, which was collected, washed with cold methanol followed by ether, and dried over phosphorus pentoxide *in vacuo* to give 0.7 g. of product, m.p. 192–195° dec.

Anal. Calcd. for $C_8H_{14}N_4O_8 \cdot {}^1/_2H_2O$: C, 31.68; H, 4.98; N, 18.48. Found: C, 31.95; H, 4.95; N, 18.68.

O-(3-Phenylcarbazyl)-N-carbobenzoxy-DL-serine Benzyl Ester.—Using the same procedure as that for the O-carbazylserine derivative, 6.6 g. of N-carbobenzoxy-DL-serine benzyl ester after treatment with phosgene followed by condensation with 4.32 g. of phenylhydrazine hydrochloride in 50 ml. of ethanol in the presence of 2.75 g. of sodium carbonate in 25 ml. of water, yielded 7.5 g. of a straw-colored solid, m.p. 100–103°. A sample of the crude product when recrystallized from ethanol–water gave small white needles, m.p. 110–112°.

Anal. Calcd. for $C_{25}H_{25}N_3O_6$: N, 9.07. Found: N, 8.98.

O-(3-Phenylcarbazyl)-DL-serine.—A solution of 4.6 g. of O-(3-phenylcarbazyl)-N-carbobenzoxy-DL-serine benzyl ester dissolved in 150 ml. of dioxane–ethanol (1:1) was hydrogenolyzed, in the same manner as that described for the O-carbazylserine derivative, for 6 hours to yield 2.1 g. of residual solid. The residue was recrystallized from methanol–water in several fractions to yield 1.8 g. of product, m.p. 220–222°.

Anal. Calcd. for $C_{10}H_{13}N_3O_4$: C, 50.20; H, 5.48. Found: C, 50.46; H, 5.68.

Interaction of Hydrazine and Serine Derivatives.—A mixture of 3.29 g. of N-carbobenzoxy-DL-serine benzyl ester in 30 ml. of ethanol containing 3 ml. of hydrazine was heated to reflux for 4 hours. The solvent and excess hydrazine was removed under reduced pressure, and the residue was recrystallized from aqueous ethanol to yield 1.3 g. of N-carbazyl-DL-serinehydrazide, m.p. 155–157°.[13] Using 1.6 g. of the serine derivative and heating in the presence of 0.4 ml. of hydrazine for 6 hours, followed by reduction to dryness *in vacuo* and crystallization of the residue from aqueous ethanol, produced 1.0 g. of N-carbobenzoxy-DL-serinehydrazide, m.p. 150–151°.[14]

A mixture of 2.39 g. of N-carbobenzoxy-DL-serine in 30 ml. of ethanol containing 1.0 ml. of hydrazine was allowed to stand at room temperature for 12 hours. After removal of the solvent and excess hydrazine *in vacuo*, the residue was crystallized from aqueous ethanol to yield 2.2 g. of the corresponding hydrazonium salt, m.p. 80–83°.

Anal. Calcd. for $C_{11}H_{18}NO_5 \cdot N_2H_4$: N, 15.49. Found: N, 15.53.

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(11) C. A. Bischoff, *Ber.*, **36**, 159 (1903), reports a boiling point of 195–203° (12 mm.) and a melting point of 29° for this product prepared by a different procedure.

(12) The reported m.p. by a more elaborate procedure is 67–69°; K. Hofmann, A. Lindenmann, M. Z. Magee and N. H. Khan, THIS JOURNAL, **74**, 470 (1952).

(13) K. Schlögl, J. Derkosch and E. Wawersich, *Monatsh.*, **85**, 607 (1954), report a m.p. of 157–159° for this compound prepared from the methyl ester.

(14) E. A. Popenoe, D. G. Doherty and K. P. Link, THIS JOURNAL, **75**, 3469 (1953), report a m.p. of 153–154° for this compound made through the methyl ester.