[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH]

O-Carbamyl-L-serine, an Inhibitory Analog of L-Glutamine

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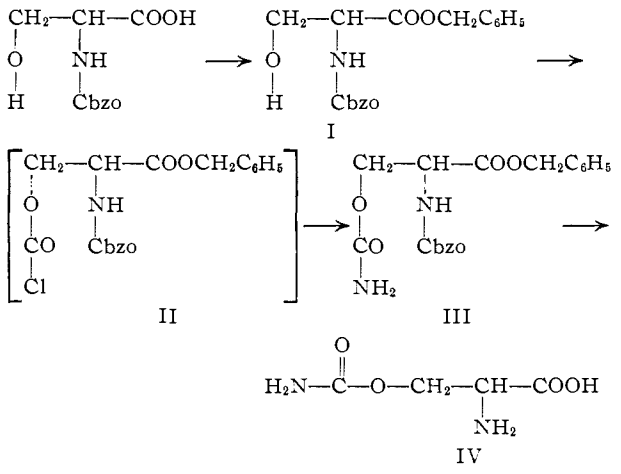
O-Carbamyl-L, D- and DL-serine have been prepared through interaction of L, D- and DL-N-carbobenzoxyserine benzyl ester with phosgene followed by treatment with ammonium hydroxide and hydrogenolysis. The L-derivative inhibits growth of *Streptococcus lactis* 8039, *Lactobacillus arabinosus* 17-5 and *Escherichia coli* 9723, whereas the D-isomer is not inhibitory even at high levels. The inhibition of growth of *S. lactis* by O-carbamylserine is reversed in a competitive manner by glutamine and less effectively by glutamic acid.

L-Azaserine (O-diazoacetyl-L-serine) has been reported to inhibit the synthesis of purines in soluble enzyme fractions of pigeon liver with the accumulation of glycinamide ribotide and its formyl derivative.[2] The ability of high levels of glutamine to overcome this inhibition suggests that some of the inhibitory effects of azaserine may result from its ability to act as a metabolite antagonist of glutamine.

In the present investigation, the synthesis of the O-carbamyl instead of the O-diazoacetyl derivative of L-serine was undertaken to produce a compound which, being more structurally similar to glutamine than azaserine, might be more effective in inhibiting the utilization of glutamine. Accordingly, O-carbamyl-DL-serine was prepared and found to be an effective inhibitory analog of L-glutamine for several microörganisms.

During the course of this investigation, a compound identified as O-carbamyl-D-serine by structural studies but not synthesis was reported to have been isolated from culture media of a new *Streptomyces strain*.[3] This derivative of D-serine was reported not to have antibiotic properties. Thus, the preparation of the O-carbamyl derivative of both D- and L-serine was desirable to establish that the biological activity of the racemic preparations resided in the derivative of L-serine.

Synthesis of the various O-carbamylserines was effected through the series of reactions indicated by the following equations. The N-carbobenzoxy derivative of D-, L- and DL-serine (I) was prepared through usual procedures in good yield; however, the benzyl ester of the optical isomers crystallized with difficulty in contrast to the racemic compound. Attempts to isolate the intermediate condensation product II with phosgene yielded only a yellow oil; therefore, the conversion to the carbamyl derivative III was made with the crude product since O-carbamyl-N-carbobenzoxy-serine benzyl ester precipitated nicely.



Hydrogenolysis was effected in a 20 × 40 cm. glass tube fitted with a porous plate through which hydrogen was bubbled. The catalyst was agitated with a magnetic stirring bar which was vigorously rotated on the porous plate. Hydrogenolysis was found to be more effective in aqueous mixtures than in anhydrous solvents.

The O-carbamylserines are soluble in water, give the typical purple ninhydrin color and decompose with vigorous evolution of gas at their melting points. Several attempts to condense directly N-carbobenzoxyserine and N-carbobenzoxyserine benzyl ester with potassium cyanate to yield the carbamyl derivative resulted only in the recovery of starting material.

O-Carbamyl-L-serine inhibits the growth of a number of microörganisms. Under the testing conditions subsequently described, this enantiomorph inhibits growth of *Streptococcus lactis*, *Lactobacillus arabinosus* 17-5 and *Escherichia coli* 9723 at concentrations of 4, 2 and 20 γ per ml., respectively, while the derivative of D-serine does not inhibit growth even at a concentration of 200 γ per ml. Azaserine inhibits growth of the same organisms at concentrations of 2, 40 and 0.06 γ per ml. under the same conditions. Thus, carbamylserine as a growth inhibitor is more effective for *L. arabinosus*, less effective for *E. coli* and slightly less effective for *S. lactis* than azaserine.

O-Carbamyl-DL-serine is half as effective as the L-form of the compound. The inhibition of growth of *S. lactis* by O-carbamyl-DL-serine is reversed in a competitive manner by both glutamic acid and glutamine as indicated in Table I. Glutamine is approximately 150 times as effective as glutamic acid in preventing the toxicity of carbamylserine. The toxicity of carbamylserine for *L. arabinosus* and *E. coli* is also prevented by glutamine and glutamic acid.

Although glutamine at a concentration of 20 γ per ml. prevents the toxicity of azaserine at concentrations below 60 γ per ml. for *S. lactis*, glutamine in concentrations of even 200 γ per ml. does not prevent the toxicity of 60 γ per ml. of azaserine.

(1) Rosalie B. Hite Post-doctoral Fellow, 1954–1956.

(2) S. C. Hartman, B. Levenberg and J. M. Buchanan, THIS JOURNAL, **77**, 501 (1955).

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TABLE I

REVERSAL OF O-CARBAMYLSERINE TOXICITY BY GLUTAMIC ACID AND GLUTAMINE

Test organism, *Streptococcus lactis* 8039, incubated 21 hr. at 30°.

DL-Glutamic acid, mg./5 ml.	O-Carbamyl-DL-serine, mg./5 ml.				
	0	0.1	0.3	1	3
0	54	3	0		
0.3		5			
1		47	1.0		
3		56	28	1.0	
10			54	25	3
30				53	23
100					53
L-Glutamine, γ/5 ml.					
0.3		3			
1		23	4		
3		53	8		
10			22	11	6
30			48	26	14
100			51	50	49
300					51

Thus, in contrast to the results obtained with O-carbamylserine, glutamine reverses the toxicity of only low concentrations of azaserine.

Experimental[4]

Biological Assays.—For *Streptococcus lactis* 8039 and *Lactobacillus arabinosus* 17-5, a previously described[5] amino acid medium was modified by increasing the concentrations of aspartic acid and glutamic acid to 500 and 300 γ, respectively, per 5 ml. assay tube. Calcium pantothenate which was inadvertently omitted from the list of constituents of the above indicated basal medium[5] was added at concentrations of 3 mg. per 30 ml. of vitamin supplement. For *Escherichia coli* 9723, a previously described[6] inorganic salts–glucose medium was employed. The assay methods were analogous to those indicated above.[5] Carbamylserine, azaserine and glutamine were dissolved in sterile water and added aseptically to the previously autoclaved assay tubes.

N-Carbobenzoxy-DL-serine.—The N-carbobenzoxy derivatives of serine were prepared by a procedure similar to that reported by Moore, *et al.*[7] DL-Serine (25 g.) was dissolved in 50 ml. of 4 *N* sodium hydroxide, cooled in an ice-bath and stirred mechanically while 45 g. of carbobenzoxy chloride was added slowly with intermittent additions of 2 *N* sodium hydroxide to maintain a *p*H of 9–10. The alkaline reaction mixture was extracted with ether after which 500 ml. of ethyl acetate was added to the aqueous solution and the *p*H adjusted to 3 with hydrochloric acid. After vigorous stirring the ethyl acetate was decanted and the water extracted again with fresh solvent. The organic layer was dried overnight over $CaSO_4$; the major portion of the solvent was then removed and crystallization allowed to occur in the refrigerator. There was recovered a total of 41.5 g. (73% of theory) of material, m.p. 120–122°.[8]

N-Carbobenzoxy-DL-serine Benzyl Ester.—The synthesis used was similar to the procedure of Ben-Ishai and Berger[9] for the preparation of N-carbobenzoxyamino acid esters. A mixture of 20.0 g. of N-carbobenzoxy-DL-serine partially dissolved in 200 ml. of benzene was heated to reflux in the presence of 20.0 g. of benzyl alcohol and 1.0 g. of *p*-toluenesulfonic acid. The water produced was removed azeotropically using a modified Stark and Dean distilling head. The resulting benzene solution was washed twice with 35-ml. portions of 5% potassium bicarbonate solution and finally dried over anhydrous sodium sulfate. The drying agent was removed, and the filtrate was concentrated to about one-fourth volume under reduced pressure. An equal volume of Skellysolve-C[10] was then added and the resulting solution placed in the refrigerator overnight. The needle-like crystals were recovered by filtration, washed with Skellysolve-C and placed in a vacuum desiccator over paraffin. There was recovered 23.5 g. (85% of theory) of product, m.p. 69–70°. After recrystallizing from benzene–Skellysolve-C mixture and drying at 56° over phosphorus pentoxide, the crystals melted 73–74°. Baer and Maurukas[11] report a melting point of 72.5–73.5° for this product prepared by a different procedure.

Anal. Calcd. for $C_{18}H_{19}O_5N$: C, 65.64; H, 5.81; N, 4.25. Found: C, 65.67; H, 5.94; N, 4.45.

O-Carbamyl-N-Carbobenzoxy-DL-serine Benzyl Ester.—To a cold solution of 30 g. of phosgene in 300 ml. of toluene was added, over a period of 30 minutes, 15.0 g. of N-carbobenzoxy-DL-serine benzyl ester. After warming to room temperature complete solution was effected, and the reaction mixture was stirred an additional 1.5 hr. The solvent was removed under reduced pressure to yield a pale yellow oil. This oil was cooled in an ice-bath and 50 ml. of ice-cold concentrated ammonium hydroxide added with efficient stirring. An immediate reaction occurred to yield 14.7 g. (87% of theory) of material, melting 126–127°, which was dried under vacuum over concentrated sulfuric acid. On recrystallization from ethanol the product melted from 135–136°.

Anal. Calcd. for $C_{19}H_{20}N_2O_6$: C, 61.28; H, 5.41; N, 7.52. Found: C, 61.69; H, 5.66; N, 7.46.

O-Carbamyl-DL-serine.—A solution of 3.8 g. of O-carbamyl-N-carbobenzoxy-DL-serine ester in 100 ml. of 50% dioxane was treated with hydrogen gas at atmospheric pressure and room temperature in the presence of 0.5 g. of palladium black for several hours. The reaction mixture was filtered, the catalyst was washed with hot water and the combined filtrates were evaporated to dryness under reduced pressure. The solid was recrystallized from alcohol–water to yield 1.42 g. (94% of theory) of product, m.p. 212–215° dec.

Anal. Calcd. for $C_4H_8N_2O_4$: C, 32.44; H, 5.45; N, 18.93. Found: C, 32.51; H, 5.64; N, 18.80.

O-Carbamyl-N-carbobenzoxy-D-serine Benzyl Ester.—The benzyl ester of N-carbobenzoxy-D-serine was prepared by the procedures described above for the DL-isomer. Baer and Maurukas[11] have reported the preparation of this intermediate by a different procedure.

Using the same procedure as that for the DL-isomer, 1.74 g. of N-carbobenzoxy-D-serine benzyl ester upon treatment with phosgene followed by ammonium hydroxide, yielded 1.50 g. (76% of theory) of the benzyl ester of O-carbamyl-N-carbobenzoxy-D-serine, m.p. 105–107°.

Anal. Calcd. for $C_{19}H_{20}N_2O_6$: N, 7.52. Found: N, 7.41.

O-Carbamyl-D-serine.—A solution of 1.5 g. of O-carbamyl-N-carbobenzoxy-D-serine benzyl ester dissolved in 60 ml. of 60% dioxane was treated with hydrogen gas at room temperature and atmospheric pressure in the presence of 0.25 g. of palladium black for 4 hr. The catalyst was removed and the resulting solution reduced in volume to yield 0.260 g. of product (44% of theory), m.p. 198° dec. After recrystallization from water–alcohol there was obtained crystalline material, m.p. 212–215° dec.; $[\alpha]_D$ −19.1° (*c* 2 in 1 *N* HCl). Hagemann, *et al.*,[3] reported for their isolated material a m.p. of 226–234° dec. and $[a]_D$ −19.6° (*c* 2 in 1 *N* HCl). The reported R_f value in pyridine (80 parts), isoamyl alcohol (40 parts) and water (70 parts) is 0.33; and in butyl alcohol (75 parts), formic acid (15 parts), water (10 parts) is 0.08. The synthetic product gave R_f values of 0.38 and 0.06, respectively, in the same solvents.

Anal. Calcd. for $C_4H_8N_2O_4$: N, 18.93; C, 32.44; H, 5.45. Found: N, 18.81; C, 31.59; H, 5.55.

(4) The authors are indebted to Mr. J. R. Claybrook for the chemical analyses and Mrs. Barbara Mollenhauer for the microbiological assays.

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(8) All melting points were determined with a Fisher–Johns melting point apparatus. E. Baer and J. Maurakas, *J. Biol. Chem.*, **212**, 32 (1955), report the melting point as 124–125°.

(9) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(10) Petroleum ether, b.p. 90–100°.

(11) E. Baer and J. Maurukas, *J. Biol. Chem.*, **212**, 25 (1955).

O-Carbamyl-N-carbobenzoxy-L-serine Benzyl Ester.—The intermediate, N-carbobenzoxy-L-serine benzyl ester, was prepared by the procedure described above for the DL-isomer. Baer and Maurukas[11] also prepared this intermediate by a different procedure.

Reaction of 1.0 g. of N-carbobenzoxy-L-serine benzyl ester, in the same general procedure as described for the DL-isomer, with phosgene followed by treatment with ammonium hydroxide gave 0.69 g. (61% of theory) of the benzyl ester of O-carbamyl-N-carbobenzoxy-L-serine, m.p. 104–106°.

Anal. Calcd. for $C_{19}H_{20}N_2O_6$: N, 7.52. Found: N, 7.68.

O-Carbamyl-L-serine.—A solution of 0.60 g. of O-carbamyl-N-carbobenzoxy-L-serine benzyl ester dissolved in 100 ml. of 50% dioxane, in the presence of 200 mg. of palladium black catalyst, was treated with hydrogen at room temperature and atmospheric pressure for 4 hr. The catalyst was filtered, washed with hot water and the combined filtrates were taken to dryness under reduced pressure. The residue was then recrystallized from alcohol–water to yield 85 mg. (36% of theory) of white needles, m.p. 206–209° dec.; $[\alpha]_D$ + 19.9° (*c* 2 in 1 *N* HCl).

Anal. Calcd. for $C_4H_8N_2O_4$: C, 32.44; H, 5.45; N, 18.93. Found: C, 32.70; H, 5.59; N, 18.73.

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[CONTRIBUTION FROM THE WESTERN UTILIZATION RESEARCH BRANCH][1]

Cross-linking of Bovine Plasma Albumin and Wool Keratin

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N-Ethylmaleimide is recognized as a specific additive reagent for thiol groups[2] with the advantages of mild reaction conditions and absence of by-products. N-Substituted bis-maleimides therefore have been studied as possible primary valence cross-linking reagents for proteins having reactive thiol groups. An instance of cross-linking by such a reagent has been shown in the reaction of bovine plasma albumin with N,N′-(1,3-phenylene)-bis-maleimide. Effects of treating reduced wool with N-phenylmaleimide and N,N′-bis-maleimides are described and evidence for cross-linking evaluated.

Introduction

Blood plasma albumin commonly includes a component, mercaptalbumin, having a single reactive thiol group per molecule.[3] The remainder of the albumin is closely similar in molecular weight, composition and other properties but contains no reactive thiol groups.

Mercaptalbumin forms about two-thirds of the albumins as usually isolated, but the reactive thiol content is affected by exposure of the sample to reducing media or prolonged storage. Because of this single reactive thiol group, bovine plasma albumin was selected as an appropriate protein for testing the cross-linking properties of bis-maleimides. The ultracentrifuge was used as a convenient instrument for demonstrating cross-linking and estimating the amount of "dimer" formed, since the centrifugal behavior of "dimerized" plasma albumin cross-linked by means of mercury through the thiol groups is known.[4]

Bis-maleimides have also been tested as cross-linking agents for reduced wool because of the desirability, in some applications, of replacing the disulfide cross-links by others more resistant to alkali, oxidation and reduction. Because evidence of cross-linking in wool is mainly indirect and ambiguous, comparative measurements of mechanical properties and resistance to chemical degradation have been made with fibers treated with difunctional and monofunctional reagents. These measurements have been supplemented by cystine analyses, stoichiometry of reagent uptake, and chromatographic demonstration of the expected cysteine derivative to provide as conclusive evidence as possible for the covalent cross-linking of wool by bis-maleimides.

Materials and Methods

Preparation of Cross-linking Agents.—N,N′-(1,2-Phenylene)-bis-maleamic acid was prepared by the directions given by Schönberg and Mustafa.[5] This method was also found satisfactory for the unreported meta isomer which decomposed near 216° after crystallization from ethanol–dimethylformamide solution.

Anal. Calcd. for $C_{14}H_{12}N_2O_6$: C, 55.5; H, 4.0; N, 9.2. Found: C, 55.5; H, 4.4; N, 9.2.

N-Phenylmaleimide was prepared by the method of Searle.[6] The bis-maleamic acids were converted to bis-maleimides by the same procedure. The unreported N,N′-(1,2-phenylene)-bis-maleimide (OPBM) melted at 243–244° after crystallization from dimethylformamide as fine white crystals.

Anal. Calcd. for $C_{14}H_8N_2O_4$: C, 62.7; H, 3.0; N, 10.5. Found: C, 62.6; H, 3.0; N, 10.5.

Plasma Albumin.—Armour's Bovine Plasma Albumin (Control N 128-175)[7] was used. Air-dry samples of 1.1 g. corresponding approximately to 1.0 g. dry weight were dissolved in 3.0 ml. of distilled water, each, in 12-ml. centrifuge tubes. The *p*H was adjusted to 8.0 in most experiments with a small amount of 50% NaOH or 28% NH_3. The quantities of $HgCl_2$ (mol. wt. 271.5) and N,N′-(1,3-phenylene)-bis-maleimide (MPBM) (mol. wt. 268.2) calculated to be equivalent to $^2/_3$ g. of mercaptalbumin (mol. wt. = 65,000) are 1.39 and 1.38 mg. Approximately this amount or $^1/_2$ of this amount was added as shown in Table I. The result of a preliminary experiment with excess N,N′-(1,2-phenylene)-bis-maleimide (OPBM) is also shown. Mercuric chloride was added as a solution in 1.0 ml. of distilled water, the organic reagents as dry crystalline powders. The tubes were stoppered, placed in the cold room at 7° and mechanically inverted at intervals of a few seconds. The bis-maleimides disappeared during reaction but remained undissolved when in excess. Samples without the cross-linking reagents were treated in the same way at the same time for use as controls.

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(7) Mention of a specific product does not imply endorsement by the U. S. Government.