[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF AGRICULTURE, UNIVERSITY OF WISCONSIN]

Interaction of 1,3,4,6-Tetraacetyl-β-D-glucosamine with Acyl Amino Acid Azides[1]

BY EDWIN A. POPENOE, DAVID G. DOHERTY[2] AND KARL PAUL LINK

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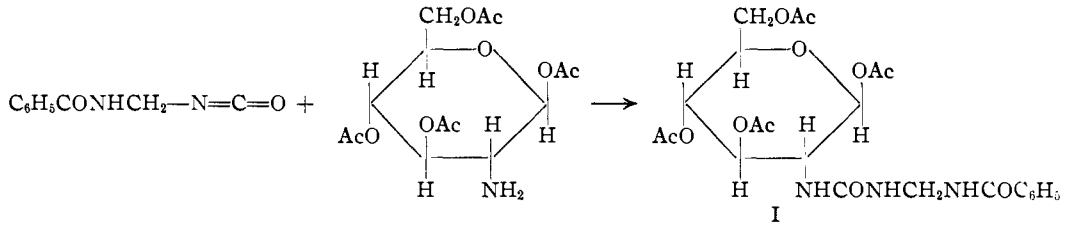
When an attempt was made to acylate 1,3,4,6-tetraacetyl-β-D-glucosamine using acyl amino acid azides, Curtius rearrangement took place in every case investigated. With 1,3,4,6-tetraacetyl-β-D-glucosamine, hippuryl azide gave N-(benzamidomethylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine, α,ε-dicarbobenzoxy-L-lysine azide gave N-(1,5-dicarbobenzoxyamido-*n*-pentylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine, and carbobenzoxy-L-methionine azide gave N-(1-carbobenzoxyamido-3-methylmercapto-*n*-propylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine. With carbobenzoxy-DL-serine azide in the presence of 1,3,4,6-tetraacetyl-β-D-glucosamine the only product isolated was DL-4-carbobenzoxyamidoöxazolidone-2. The tetraacetyl glucosamine in these reactions appeared to catalyze the Curtius rearrangement.

In a previous communication[3] several derivatives of D-glucosamine were reported in which acyl amino acid residues were linked to D-glucosamine or its tetraacetate through the nitrogen atom on carbon two in an amide type linkage. This was accomplished by reaction of an acyl amino acid chloride with 1,3,4,6-tetraacetyl-β-D-glucosamine in anhydrous chloroform or ethyl acetate in the presence of an excess of pyridine. In this way good yields of the peptide-like derivatives were obtained. In two cases these derivatives could be deacetylated to give crystalline acyl "glucopeptides."

Acid azides of amino acid derivatives have frequently been used with success in peptide syntheses. Here the azide group reacts as does the halogen of acid halides, acylating amines with the elimination of hydrazoic acid. The use of acid azides is preferable to the use of acid chlorides in many peptide syntheses, particularly with the carbobenzoxy amino acids, since their acid chlorides are notably unstable, decomposing even below room temperature to benzyl chloride and an amino acid N-carboxy anhydride.[4] During the course of the work cited above, attempts were made to employ acyl amino acid azides in place of the acid chlorides. In no case, however, was the expected product obtained. Instead urea derivatives were produced which would result from Curtius rearrangement of the azide followed by addition of the 1,3,4,6-tetraacetyl-β-D-glucosamine to the resulting isocyanate. For example, hippuryl azide and 1,3,4,6-tetraacetyl-β-D-glucosamine gave a 73% yield of N-(benzamidomethylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine (I) instead of the expected N-hippuryl-1,3,4,6-tetraacetyl-β-D-glucosamine.

$$C_6H_5CONHCH_2CON_3 \longrightarrow C_6H_5CONHCH_2{-}N{=}C{=}O$$

Curtius, *et al.*,[5-8] employed acid azides in the amino acid series for the preparation of a large number of peptides and other derivatives. Hippuryl azide was employed in the preparation of hippurylglycylglycine ethyl ester from glycylglycine ethyl ester under reaction conditions very similar to those used in the present study. Rearrangement generally took place only by heating at 50–100°, and usually in the presence of alcohols to form urethans. The necessity for using heat to bring about rearrangement of acyl amino acid azides has also been observed by others.[9,10]



I

Curtius also prepared the anilide and *p*-toluide of hippuric acid by allowing the base to stand at room temperature with hippuryl hydrazide in ethanol solution. Although dicarbobenzoxy-L-lysine azide has been used by Bergmann, *et al.*,[11] in the synthesis of lysine peptides under conditions essentially the same as those used here, with 1,3,4,6-tetraacetyl-β-D-glucosamine it gave N-(1,5-dicarbobenzoxy-*n*-pentylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine (IIa). Similarly carbobenzoxy-L-methionine azide gave rise to N-(1-carbobenzoxyamido-3-methylmercapto-*n*-propylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine (IIb).

That the products formed by reaction with acid azides were substituted ureas was originally suspected from the elemental analysis. For proof of structure two of these products were hydrolyzed with sulfuric acid and the aldehyde produced by decomposition of the unstable *gem*-diamine was identified. Use was made of the fact that formal-

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(2) Junior Fellow, National Institutes of Health, 1946–1948.

(3) D. G. Doherty, E. A. Popenoe and K. P. Link, THIS JOURNAL, **75**, 3466(1953).

(4) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(5) T. Curtius, *J. prakt. Chem.*, **52**, 243 (1895).

(6) T. Curtius and R. Wüstenfeld, *ibid.*, **70**, 73 (1904).

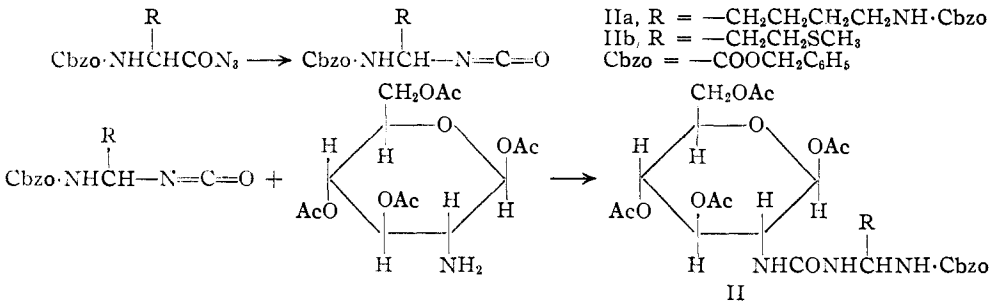
(7) T. Curtius and L. Levy, *ibid.*, **70**, 89 (1904).

(8) T. Curtius and E. Lambotte, *ibid.*, **70**, 109 (1904).

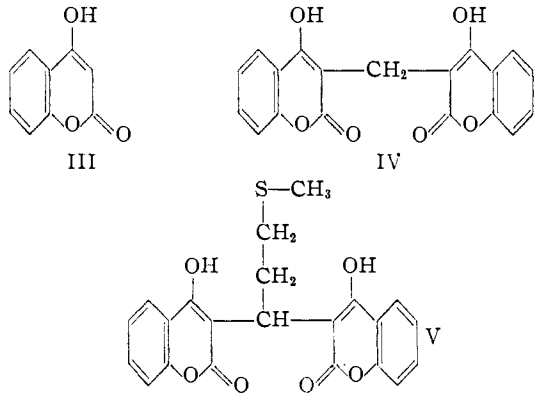
(9) M. Bergmann, L. Zervas and F. Schneider, *J. Biol. Chem.*, **113**, 341 (1936).

(10) J. S. Fruton, *ibid.*, **146**, 463 (1942).

(11) M. Bergmann, L. Zervas and J. P. Greenstein, *Ber.*, **65**, 1692 (1932).

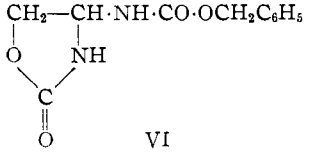


dehyde reacts instantly with 4-hydroxycoumarin (III) in hot water with the formation of 3,3′-methylenebis(4-hydroxycoumarin) (IV) (Dicumarol®).[12] By formation of IV with 4-hydroxycoumarin, formaldehyde was identified as one of the products of acid hydrolysis of I. Since 4-hydroxycoumarin has been shown to react with a variety of aldehydes in an analogous manner,[13] it was used for the identification of β-methylmercaptopropionaldehyde as one of the hydrolysis products of IIb. 3,3′ - β - Methylmercaptopropylidinebis - (4 - hydroxycoumarin) (V) prepared from the aldehyde obtained by acid hydrolysis was compared with that obtained by reaction of authentic β-methylmercaptopropionaldehyde[14] with 4-hydroxycoumarin. The details of these conversions are given in the experimental section.



The structures of I and IIb were thus established. The product from dicarbobenzoxy-L-lysine azide, IIa, was not so degraded, but from the elemental analysis and reasoning by analogy it may be presumed to have the structure given.

When an attempt was made to condense carbobenzoxy-DL-serine azide with 1,3,4,6-tetraacetyl-β-D-glucosamine the only product isolated was DL-4-carbobenzoxyamidoöxazolidone-2 (VI), which



would arise from Curtius rearrangement of the azide followed by cyclization to form the cyclic urethan. By contrast, Fruton[10] was able to use carbobenzoxy-L-serine azide as an acylating reagent in the preparation of several serine peptides.

There seems to be no explanation for the fact that 1,3,4,6-tetraacetyl-β-D-glucosamine reacts with acyl amino acid azides through a Curtius rearrangement. In the last example given it would appear to be functioning to catalyze the rearrangement. The solvents used in the present work are those used previously in many successful acylations with acid azides. Allison and Hixon[15] give for glucosamine pK_b = 6.19. This must not be very different from the value for 1,3,4,6-tetraacetyl-β-D-glucosamine and it is quite close to that for several amino acid and peptide esters. The anomaly noted here would appear to merit further investigation.

Experimental

N-(Benzamidomethylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine (I).—To 10.2 g. of 1,3,4,6-tetraacetyl-β-D-glucosamine dissolved in 100 ml. of ethyl acetate was added 6.4 g. of hippuryl azide, prepared according to Curtius.[5] Crystallization of the product began within about one minute. After keeping the reaction mixture at room temperature overnight it was cooled in ice, and the product was filtered off and washed with cold ethyl acetate; yield 11.3 g., 73%. For analysis it was recrystallized three times from methanol, m.p. 222–223°, $[\alpha]^{24}D$ +13.9 (*c* 5, pyridine).

Anal. Calcd. for $C_{23}H_{29}O_{11}N_3$: C, 52.77; H, 5.58; N, 8.02. Found: C, 52.79; H, 5.65; N, 8.09.

Identification of Formaldehyde as a Product of the Acid Hydrolysis of I.—A mixture of 0.5 g. of I and 20 ml. of 5% sulfuric acid was heated under reflux for two hours. About 15 ml. of liquid was then distilled off and to the distillate was added a solution of 27 mg. of 4-hydroxycoumarin (III) in 5 ml. of hot water. After heating the mixture 15 minutes in a boiling water-bath, the product was filtered off and washed with several portions of boiling water; yield 16.4 mg. After one recrystallization from dioxane the product melted at 288–292° and showed no depression of the melting point when mixed with an authentic sample of 3,3′-methylenebis-(4-hydroxycoumarin) (IV).

N-(1-Carbobenzoxyamido-3-methylmercapto-*n*-propylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine (IIb).—Carbobenzoxy-L-methionine hydrazide has been described by Dekker, Taylor and Fruton,[16] who give a m.p. of 110–112°. The compound prepared by us from carbobenzoxy-L-methionine by esterification with diazomethane followed by treatment with 85% hydrazine hydrate melted at 121.5–122° and had $[\alpha]^{24}D$ −14.1 (*c* 3.5, glacial acetic acid).

Anal. Calcd. for $C_{13}H_{15}O_3N_3S$: C, 52.50; H, 6.44. Found: C, 52.62; H, 6.68.

The above hydrazide (4.0 g.) was dissolved in a mixture of 40 ml. of water, 2.7 ml. of glacial acetic acid and 1.3 ml. of concentrated hydrochloric acid, cooled in an ice-salt bath and treated with a solution of 1.4 g. of sodium nitrite in 15 ml. of water, added in three portions with vigorous shaking. The azide separated almost instantly as a very viscous oil which was quickly extracted with ethyl acetate. The ethyl acetate solution (total volume about 200 ml.) was dried over anhydrous sodium sulfate and added to a solution of 4.3 g.

(12) M. A. Stahmann, C. F. Huebner and K. P. Link, *J. Biol. Chem.*, **138**, 513 (1941).

(13) W. R. Sullivan, C. F. Huebner, M. A. Stahmann and K. P. Link, THIS JOURNAL, **65**, 2288 (1943).

(14) G. Barger and F. P. Coyne, *Biochem. J.*, **22**, 1417 (1928).

(15) J. B. Allison and R. M. Hixon, THIS JOURNAL, **50**, 168 (1928).

(16) C. A. Dekker, S. P. Taylor, Jr., and J. S. Fruton, *J. Biol. Chem.* **180**, 155 (1949).

of 1,3,4,6-tetraacetyl-β-D-glucosamine in 75 ml. of ethyl acetate. The mixture was kept at room temperature for 24 hours. During this time 2.0 g. of the product (IIb) separated from the reaction mixture as needles which, after two recrystallizations from 95% ethanol, melted at 201–202° and had $[\alpha]^{22}D$ +14.85 (*c* 5, pyridine). The filtrate was extracted twice with *N* hydrochloric acid, twice with a 5% solution of potassium bicarbonate and twice with water, filtered through a dry filter paper and evaporated to dryness *in vacuo*. Recrystallization of the residue from 95% ethanol gave an additional 3.0 g. of the product, bringing the total yield to 64%.

Anal. Calcd. for $C_{27}H_{37}O_{12}N_3S$: C, 51.66; H, 5.94; N, 6.70. Found: C, 51.69; H, 5.97; N, 7.12.

Identification of β-Methylmercaptopropionaldehyde as a Product of the Acid Hydrolysis of IIb.—A mixture of 0.5 g. of IIb and 10 ml. of 5% sulfuric acid was boiled under reflux for one hour. Liquid was then distilled from the mixture until it was apparent that no more oil phase was distilling over (total distillate about 5 ml.). This distillate was added to a mixture of 260 mg. of 4-hydroxycoumarin and 10 ml. of water in a boiling water-bath. After 15 minutes the reaction mixture was cooled, scratched with a glass rod, then reheated to boiling, and filtered with suction. The product (38 mg.) was washed on the funnel with several portions of boiling water. After four recrystallizations from 95% ethanol it melted at 151–154°. The melting point was not depressed by mixture of this substance with a sample of 3,3′-β-methylmercaptopropylidinebis-(4-hydroxycoumarin) (V) prepared from authentic β-methylmercaptopropionaldehyde in a like manner.

Anal. Calcd. for $C_{22}H_{18}O_6S$: C, 64.38; H, 4.42. Found: C, 64.50; H, 4.67.

N-(1,5-Dicarbobenzoxyamido-*n*-pentylcarbamyl)-1,3,4,6-tetraacetyl-β-D-glucosamine (IIa).—A solution in chloroform of dicarbobenzoxy-L-lysine azide, prepared from 13 g. of the hydrazide by the method of Bergmann, Zervas and Greenstein,[11] was added to a cold solution of 10.4 g. of 1,3,4,6-tetraacetyl-β-D-glucosamine in 100 ml. of chloroform. Keeping the solution for 5 hours in an ice–salt-bath and 36 hours at room temperature, it was extracted twice with *N* hydrochloric acid, twice with 5% potassium bicarbonate solution and twice with water, dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue was dissolved in hot dioxane and a little water added. The product separated as a sirup which crystallized on cooling; yield 14.2 g., 63%. After one recrystallization from 95% ethanol, it melted at 190–191° and had $[\alpha]^{20}D$ +10.9 (*c* 4, pyridine). The analytical sample melted at 191–192°.

Anal. Calcd. for $C_{36}H_{45}O_{14}N_4$: C, 56.98; H, 6.10; N, 7.39. Found: C, 56.77; H, 6.15; N, 7.64.

Attempted Condensation of Carbobenzoxy-DL-serine Azide with 1,3,4,6-Tetraacetyl-β-D-glucosamine.—An excess of diazomethane in ether solution was added to a suspension of 15.3 g. of carbobenzoxy-DL-serine in 100 ml. of ethyl acetate. The acyl amino acid dissolved as the esterification proceeded. Evaporation of the solution *in vacuo* gave a sirup which was taken up in 200 ml. of absolute ethanol and 8 ml. of 85% hydrazine hydrate was added. Crystallization of the hydrazide began within an hour. After allowing it to stand overnight at room temperature some ether was added and the crystalline mass was broken up, filtered off and dried. Carbobenzoxy-DL-serine hydrazide, 14.9 g., melting at 153–154° was obtained.

A solution of 5.1 g. of this hydrazide in 50 ml. of water, 4.5 ml. of glacial acetic acid and 1.5 ml. of concentrated hydrochloric acid was cooled in an ice-bath and 2.2 g. of sodium nitrite dissolved in 15 ml. of water was added in portions with cooling and shaking over a period of ten minutes. The sirupy azide which formed was extracted with ethyl acetate and the solution was washed quickly with water, with 5% potassium bicarbonate and again with water, and dried over anhydrous sodium sulfate. The dried solution was added to 6.7 g. of 1,3,4,6-tetraacetyl-β-D-glucosamine dissolved in 100 ml. of ethyl acetate. After allowing the mixture to stand overnight, it was washed with dilute hydrochloric acid, with 5% potassium bicarbonate and with water, and evaporated to dryness *in vacuo*. The solid was taken up in ethyl acetate and precipitated by the addition of petroleum ether. A mixture of crystals was obtained which melted at 124–125°. Recrystallization yielded one fraction melting at 127–128°. The sirupy residue has not been crystallized. The analysis of the crystalline fraction indicated that it was DL-4-carbobenzoxyamidoöxazolidone-2, an isomer of which was prepared by Fruton[10] from L-serine.

Anal. Calcd. for $C_{11}H_{12}O_4N_2$: C, 55.92; H, 5.12. Found: C, 55.84; H, 5.21.

A few milligrams of the racemic oxazolidone was boiled with 10% hydrochloric acid for two minutes and cooled. Benzyl carbamate, melting point of 91°, in agreement with the value given by Fruton,[10] was obtained.

MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

Chemical Interactions of Amino Compounds and Sugars. VII.[1] *p*H Dependency[2]

BY M. L. WOLFROM, DORIS K. KOLB AND A. W. LANGER, JR.

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The *p*H dependency of the complex color-forming reaction between amino acids (glycine and alanine) and reducing sugars (D-xylose) has been investigated. The results indicate strong base catalysis between the initial *p*H values of 6.5 to 8.5, solvent or weak base catalysis between 3 and 5, and acid inhibition in the range 1 to 3. An evaluation of the temperature factor at *p*H 4 indicates an activation energy of 20.2 kcal.

Amino acids and reducing sugars react to form dark colored products. It is known in a qualitative sense that alkalinity favors and acidity retards the formation of these substances. Mohammad, Fraenkel-Conrat and Olcott[3] studied the rate of color formation between D-glucose and bovine serum albumin and found that the logarithm of the reaction rate rose linearly with *p*H in the range 4 to 10 with the apparent activation energy being 30.3 kcal. Frankel and Katchalsky[4] followed the *p*H drop occurring in the initial interaction between D-xylose (10.5 *M*) and glycine (0.5 *M*) at room temperature. They found that this change in *p*H passed through a maximum near *p*H 7.5 and dropped to approximately zero between the initial *p*H values 1 and 4 and also at the initial *p*H of 10.5. This *p*H change undoubtedly indicates reaction between the amino and carbonyl groups with a consequent increase in acidity due to the liberation of

(1) Previous communication in this series: M. L. Wolfrom, R. C. Schlicht, A. W. Langer, Jr., and C. S. Rooney, THIS JOURNAL, **75**, 1013 (1953).

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(3) A. Mohammad, H. Fraenkel-Conrat and H. S. Olcott, *Arch. Biochem.*, **24**, 157 (1949).

(4) M. Frankel and A. Katchalsky, *Biochem. J.*, **31**, 1595 (1937).