Stability of Hemiketal I in Acetic Acid under Various Conditions. A.—4-Oxo-4-dedimethylaminotetracycline 4,6-hemiketal (I, 207 mg, 0.499 mmole) was dissolved in 13 ml of glacial acetic acid, and the resulting solution was allowed to stand at room temperature for 1 hr. At the end of this time the solution was treated with hydrogen sulfide for a few minutes and then freeze dried overnight. The resulting amorphous, yellow powder was suspended in water for 2 hr. The mixture was adjusted to pH 2 with dilute hydrochloric acid and filtered to give an amorphous, yellow powder (159 mg, 77% recovery), $[\alpha]^{34}\mathrm{D}$ −111° (*c* 0.4, methanol). The ultraviolet and infrared spectra were identical with those of the starting material.

B.—The procedure in A was repeated except that mercuric acetate (160 mg, 0.5 mole/mole of hemiketal) was incorporated at the beginning also. The product in this case was on amorphous, yellow powder (142 mg, 69% recovery), $[\alpha]^{34}\mathrm{D}$ −113° (*c* 0.4, methanol). The ultraviolet and infrared spectra were identical with those of the starting material.

C.—The procedure in A was repeated except that mercuric acetate (160 mg, 0.5 mole/mole of hemiketal) and dimethylamine hydrochloride[5] (40 mg, 0.5 mole/mole of hemiketal) were also incorporated at the beginning. The product in this case was an amorphous, brown powder (147 mg). The infrared spectrum in this case was quite different from that of the starting hemiketal, having a strong γ-lactone band at 5.64 μ (mineral oil mull). Three crystallizations from methanol gave yellow crystal (27 mg, 13%). The ultraviolet and infrared spectra were identical with those of pure quinone VII.

D.—The procedure in A was repeated except that triethylamine (42 mg, 0.5 mole/mole of hemiketal) was also incorporated at the beginning. The product was an amorphous, orange-brown powder (147 mg). The infrared spectrum in this case was also quite different from that of the starting material, having a lactone band at 5.64 μ (mineral oil mull). Three crystallizations from methanol gave yellow crystals (29 mg, 14%), mp (evacuated capillary) 227.0–227.5° dec. The ultraviolet and infrared spectra were identical with those of pure quinone VII.

Stability of Tetracycline to Acetic Acid, Potassium Acetate, and Triethylamine.—Tetracycline (444 mg, 1 mmole), fused potassium acetate (98 mg, 1 mmole), and triethylamine (101 mg, 1 mmole) were dissolved in 7 ml of glacial acetic acid. After standing for 90 min at room temperature, the solution was sampled for paper chromatography (Whatman No. 1 paper buffered to pH 4.5 with McIlvaine's buffer, prewetted with 4:1 acetone–water, and developed with wet ethyl acetate). The only spot seen upon development was that of tetracycline (ultraviolet absorbing becoming ultraviolet green fluorescing on exposure to ammonia vapor).

Registry No.—Mercuric acetate, 1600-27-7; tetracycline, 60-54-8; VII, 7700-13-2; 8-hydroxy-1-tetralone, 7700-14-3; 2,5-dihydroxybenzoquinone, 615-94-1; dimethylamine salt of VII, 7700-15-4; VIII, 7703-94-8; I, 3764-23-6.

[5] Dimethylamine base is released in solution because mercuric ion complexes with chloride ion in preference to acetate ion thus effectively removing the hydrochloric acid.

Chemistry of Tetracyclines. II. Allotetracycline and Allooxytetracycline

J. S. PAUL SCHWARZ AND HAROLD E. APPLEGATE

The Squibb Institute for Medical Research,
New Brunswick, New Jersey

Received March 24, 1966

Compounds in the tetracycline family when treated with aqueous base under mild conditions are isomerized to phthalid derivatives called isotetracyclines.[1] This change is exemplified here by the isomerization of tetracycline (Ia) to isotetracycline (II) and is initiated by nucleophilic attack by base on C-11. We have found that, by changing the nature of the solvent and base, isomerization takes a different course.

When tetracycline and triethylamine were refluxed in dioxane, the tetracycline was slowly converted to an isomer which was named allotetracycline. The infrared band at 5.66 μ showed that this new compound possessed a γ-lactone function. Since the ultraviolet spectra in acid and in base (Table I) indicated the retention of the ring A chromophore and the presence of an 8-hydroxy-1-tetralone chromophore (especially compare with the ultraviolet spectra of V), the lactone must have been formed from the C-6 hydroxyl together with the carbonyl group at C-12, the only carbonyl group not involved in the chromophores. Allotetracycline is, therefore, IVa. Additional support for this structure is obtained by the fact that quinone V[2] is obtained from allotetracycline by mercuric acetate oxidation.

The ring cleavage and lactone formation that tetracycline undergoes to form allotetracycline is envisioned to proceed by the mechanism of base attack on tetracycline tautomer III analogous to that proposed for conversion of VI to VII.[2] The fact that more vigorous conditions are required to isomerize tetracycline to allotetracycline than to convert VI to VII is ascribed to the lack of developing aromaticity to promote the reaction in the former case.

When this reaction was extended to oxytetracycline (Ib), only a small yield of the expected allooxytetracycline was produced, the major portion of the product mixture being composed of nonbasic compounds. No explanation is offered at this time to explain the difference in reactivity between tetracycline and oxytetracycline in this reaction (see Scheme I).

Experimental Section

All melting points were taken in capillary tubes and are uncorrected. All infrared spectra were taken on a Perkin-Elmer Infracord and the significant bands between 2.4 and 6.5 μ are recorded. All ultraviolet spectra were taken on a Perkin-Elmer 202 spectrophotometer. The microanalyses were performed by the Microanalytical Laboratory of the Squibb Institute for Medical Research.

Allotetracycline (IVa).—Tetracycline (11.7 g, 26.3 mmoles) and triethylamine (4.0 ml, 30 mmoles) were dissolved in 60 ml of dioxane, and the resulting solution was refluxed under nitrogen for 18 hr. At this time paper chromatography[3] of an aliquot of the reaction mixture disclosed that the tetracycline had been completely isomerized.[4] The solvent was removed by distillation under vacuum, and the dark residue was dissolved in a minimum volume of boiling acetone. Fifty milliliters of benzene was added, and the solution was concentrated to about 25 ml to

---

[1] (a) C. W. Waller, B. L. Hutching, C. F. Wolf, A. A. Goldman, R. W. Broschard, and J. H. Williams, *J. Am. Chem. Soc.*, **74**, 4981 (1952); (b) J. H. Boothe, J. Morton, II, J. P. Petisi, R. G. Wilkinson, and J. H. Williams, *Antibiot. Ann.*, 46 (1953); (c) P. Sensi, G. A. DeFerrair, G. G. Gallo, and G. Rolland, *Farmaco* (Pavia) *Ed. Sci.*, **10**, 337 (1955); (d) J. R. D. McCormack, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Arigoni, W. H. Muller, R. Winterbottom, and A. P. Doerschuk, *J. Am. Chem. Soc.*, **79**, 2849 (1957); (e) C. R. Stephens, L. H. Conover, R. Pasternack, F. A. Hochstein, W. T. Moreland, P. P. Regna, F. J. Pilgrim, K. J. Brunnings, and R. B. Woodward, *ibid.*, **76**, 3568 (1954); (f) F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Brunnings, and R. B. Woodward, *ibid.*, **75**, 5455 (1953).

[2] See footnote *c* of Table I.

[3] Whatman No. 1 paper was treated with McIlvaine's buffer and dried. The paper was remoistened with 4:1 acetone–water, the acetone being allowed to evaporate prior to developing the chromatogram with wet ethyl acetate.

[4] Tetracycline is detected at $R_f \sim 0.4$ as an absorbing spot under ultraviolet light. Allotetracycline is detected at $R_f \sim 0.95$ as a blue-white fluorescing spot under ultraviolet light.

SCHEME I

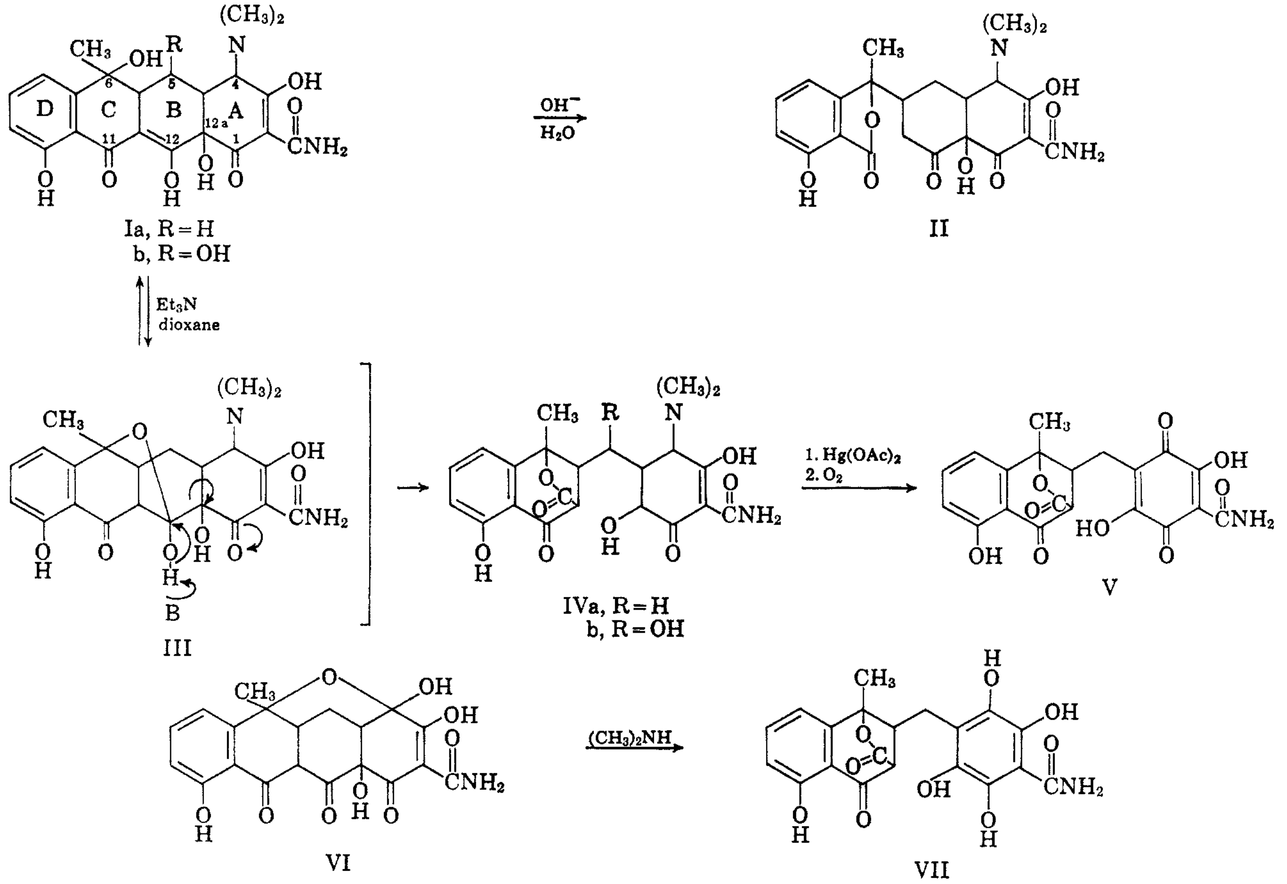

TABLE I

ULTRAVIOLET SPECTRA

Compd	λ_{max}, mμ (ε), in alcoholic H^+	λ_{max}, mμ (ε) in alcoholic OH^-	Ref.
Tetracycline (Ia)	269 (19,000), 365 (15,000)	247 (16,600), sh 263 (15,400), 381 (17,600)	
Isotetracycline (II)	243 (13,500), 274 (14,400)	246 (12,500), 285 (13,200), 335 (6100)	*a*
Allotetracycline (IVa)	264 (21,100), 340 (4500)	243 (19,200), 271 (16,300), 385 (6700)	
8-Hydroxy-1-tetralone	260 (9300), 335 (3100)		*b*
Quinone V	274 (29,600), 296 (18,300), 340 (5500)	271 (17,900), 308 (15,500), sh 314 (14,700), 389 (8600)	*c*

[a] Private communication from J. J. Hlavka, Lederle Laboratories. [b] Reference 1f. [c] See part I of this series: J. S. P. Schwarz, H. E. Applegate, J. L. Bouchard, and F. L. Weisenborn, *J. Org. Chem.*, **32,** 1238 (1967).

remove the bulk of the acetone. The wall of the flask was scratched, whereupon the product crystallized as light brown needles (7.66 g). This crude product was crystallized once from benzene to give pure white needles of benzene solvate (5.55 g, 40%): mp 132–133° dec; $[\alpha]^{31}D$ +23.0 (*c* 1.2, methanol); ultraviolet (ethanol) 259 mμ (ε 25,900), 335 mμ (ε 5700); infrared (mineral oil) 2.93 (s), 3.04 (m), 5.66 (s) 6.00–6.50 μ (s).

Anal. Calcd for $C_{22}H_{24}N_2O_8 \cdot C_6H_6$: C, 64.35; H, 5.79; N, 5.36; neut equiv, 522. Found: C, 64.95; H, 5.98; N, 5.05; neut equiv, 540.

The benzene of crystallization could not be removed by heating the solvate at 100° under vacuum. However, amorphous allotetracycline free of benzene could be prepared by precipitation from ethanol by water: mp 132–133° dec; $[\alpha]^{20}D$ +27.2° (methanol); ultraviolet (ethanol) 260 mμ (ε 22,700), 340 mμ (ε 5000); ultraviolet (0.01 *N* ethanolic hydrochloric acid) 264 mμ (ε 21,100), 340 mμ (ε 4500); ultraviolet (0.01 *N* ethanolic potassium hydroxide) 243 mμ (ε 19,200), 271 (16,300), 385 (6700); infrared (chloroform); 2.87 (m), 3.04 (w), 3.40 (w), 5.62 (s), 6.06 (s), sh 6.17, 6.39 μ (s).

Anal. Calcd for $C_{22}H_{24}N_2O_8$: C, 59.45; H, 5.44; N, 6.30. Found: C, 59.38; H, 5.44; N, 6.40.

Mercuric Acetate Oxidation of Allotetracycline.—Allotetracycline (100 mg of benzene solvate, 0.192 mmole) in 0.75 ml of glacial acetic acid was added to a solution of mercuric acetate (191 mg, 0.6 mmole) in 3.25 ml of the same solvent. A drop of water was added and the solution was allowed to stand at room temperature for 75 min. The precipitate of mercurous acetate was removed by centrifugation and the excess mercuric ion was removed with hydrogen sulfide. The mercury-free solution was freeze dried, and the residue was mixed with water. This mixture was adjusted to pH 2.1 with hydrochloric acid and stirred in the presence of air for 1 hr. The crude red product was recrystallized twice from methanol to give dark yellow crystals (27 mg 34%), mp (evacuated capillary) 226–228° dec. The infrared spectrum was identical with that of yellow quinone V.[2]

Allooxytetracycline.—Oxytetracycline (1.35 g, 2.94 mmoles) and triethylamine (0.5 ml, 3.7 mmoles) were dissolved in 20 ml of dioxane, and the resulting solution was refluxed under nitrogen for 8 hr. The solvent was distilled under vacuum, and the residue was taken up in chloroform. The bases were extracted from the chloroform with 0.1 *N* hydrochloric acid and back extracted into fresh chloroform after the pH was adjusted to 5.5. The chloroform was taken to dryness yielding 457 mg of crude bases. This mixture was preparatively chromatographed according to footnote 3 except that four sheets of buffered 3 MM paper were used and that the developing solvent was wet 3:1 hexane–ethyl acetate. The desired product moved as a band (R_f 0.95) which fluoresced blue-white under ultraviolet light. This band was eluted with ethyl acetate. The ethyl acetate was washed with saturated ammonium sulfate solution, dried with anhydrous sodium sulfate, and taken to dryness under vacuum (178 mg). This was crystallized from benzene to give a tan, microcrystalline powder (86 mg), mp 168–170° dec. The color was removed by precipitation from ethanol by the addition of water. The white, amorphous powder was twice crystallized from benzene to give white needles of benzene solvate (38 mg, 2.4%): mp 169–170° dec; $[\alpha]^{25}D$ +85.3° (MeOH); ultraviolet (ethanol) 262 mμ (ε 23,000), 340 mμ (ε 5700); infrared (mineral oil mull) 2.91 (m), 2.97 (m), 3.06 (w), 5.66 (s), 6.09 (s), sh 6.19, 6.38 μ (s).

Anal. Calcd for $C_{22}H_{24}N_2O_9 \cdot C_6H_6$: C, 62.42; H, 5.62; N, 5.20. Found: C, 62.35; H, 5.58; N, 5.40.

Registry No.—IVa, 7695-46-7; IVb, 7721-33-7; Ia, 60-54-8; II, 3811-31-2; 8-hydroxy-1-tetralone, 7695-97-8; V, 7695-48-9.

Amino Acids and Peptides. IX.[1] Synthesis of a Tetrapeptide Sequence (A_{13}–A_{16}) of Glucagon

BRIAN O. HANDFORD, THOMAS A. HYLTON, JOHN PRESTON, AND BORIS WEINSTEIN

Department of Chemistry, Stanford University, Stanford, California 94305

Received July 19, 1966

The central tetrapeptide sequence (A_{13}–A_{16}) of the hyperglycemic hormone glucagon[2] has been formed in part by several overlapping routes within the last several years. The first preparation afforded the tripeptide N-benzyloxycarbonyl-O-benzyl-L-tyrosyl-L-leucyl-α-methyl-β-*t*-butyl-L-aspartate, which was made by coupling N-benzyloxycarbonyl-O-benzyl-L-tyrosyl-L-leucine with α-methyl-β-*t*-butyl-L-aspartate.[3] Additionally, a homolog was obtained by an azide reaction between N-benzyloxycarbonyl-O-benzyl-L-tyrosyl-L-leucine hydrazide and α-ethyl-β-*t*-butyl-L-aspartate. The second synthesis described N-*t*-butyloxycarbonyl-L-leucyl-L-asparaginyl-L-serine hydrazide, constructed by a stepwise procedure from L-serine methyl ester.[4] The third approach mentioned the simple dipeptide *p*-methoxybenzyloxycarbonyl-O-benzyl-L-tyrosyl-L-leucine methyl ester that was used as an intermediate for subsequent work.[5]

A preparation of three related tetrapeptides constituting the sequence A_{13}–A_{16} of glucagon is given here. The work began by coupling N-benzyloxycarbonyl-β-*t*-butyl-L-aspartic acid α-dicyclohexylammonium salt (I)[6–8] with L-serine methyl ester hydrochloride (II)[9] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride[10] to yield N-benzyloxycarbonyl-β-*t*-butyl-L-aspartyl-L-serine methyl ester (III). Dipeptide III was formed in lower yield by the action of 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate.[11] Alternatively, crystalline N-benzyloxycarbonyl-β-*t*-butyl-L-aspartic acid (IV) was prepared[12] and condensed with serine salt II by use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, N,N′-dicyclohexylcarbodiimide,[13] and 2-ethyl-5-phenyloxazolium 3′-sulfonate.[14] Although dipeptide III was obtained in each case with the same physical constants as in the first preparation, the yields were uniformly disappointing. Later the dicyclohexylammonium salt I was treated with 2,4,5-trichlorophenol to prepare the corresponding α-2,4,5-trichlorophenyl ester (V). This particular compound is a valuable addition to the list of 2,4,5-trichlorophenyl esters[15] that have been widely employed in recent years to facilitate the construction of peptides.[16] The activated ester V on treatment with serine salt II afforded dipeptide III in good yield.

Hydrogenolysis of dipeptide III led to β-*t*-butyl-L-aspartyl-L-serine methyl ester (VI), which was combined with N-benzyloxycarbonyl-L-leucine 2,4,5-trichlorophenyl ester (VII) to form N-benzyloxycarbonyl-L-leucyl-β-*t*-butyl-L-aspartyl-L-serine methyl ester (VIII). Tripeptide VIII was obtained in higher yield by coupling N-benzyloxycarbonyl-L-leucine (IX) with amine VI in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. Reduction of tripeptide VIII gave L-leucyl-β-*t*-butyl-L-aspartyl-L-serine methyl ester (X), which was treated with N-benzyloxycarbonyl-L-tyrosine 2,4,5-trichlorophenyl ester (XI)[15] to yield N-benzyloxycarbonyl-L-tyrosyl-L-leucyl-β-*t*-butyl-L-aspartyl-L-serine methyl ester (XII), as an amorphous solid. A 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide coupling between N-benzyloxycarbonyl-L-tyrosine (XIII) and amine X afforded the same tetrapeptide (XII).

An alternative route to tetrapeptide XII is mentioned now. A 2-ethyl-5-phenyloxazolium 3′-sulfonate[14] coupling between N,O-dibenzyloxycarbonyl-L-tyrosine (XIV) and L-leucine methyl ester hydrochloride (XV)[9] afforded N,O-dibenzyloxycarbonyl-L-tyrosyl-L-leucine methyl ester (XVI). Dipeptide XVI on treatment with excess hydrazine formed N-benzyloxycarbonyl-L-tyrosyl-L-leucine hydrazide (XVII).[17] This particular reaction is of some interest because the protecting O-benzyloxycarbonyl group is simultaneously cleaved by hydrazine to give the corresponding mono-N-benzyloxycarbonyl derivative. An azide coupling between hydrazide XVII and 1-hydroxypiperidine[18] led to N-benzyloxycarbonyl-L-tyrosyl-L-leucine 1-hydroxypiperidine ester (XVIII). Activated ester XVIII and amine XI then gave tetrapeptide XII in moderate amount.

In an effort to improve the physical characteristics of tetrapeptide XII, N-benzyloxycarbonyl-O-benzyl-L-tyrosine *p*-nitrophenyl ester (XIX)[19] was treated with amine X to afford crystalline N-benzyloxycarbonyl-O-benzyl-L-tyrosyl-L-leucyl-β-*t*-butyl-L-aspartyl-L-serine methyl ester (XX). Treatment of N,O-dibenzyloxycarbonyl-L-tyrosine 2,4,5-trichlorophenyl ester (XXI) with amine X produced crystalline N,O-dibenzyloxycarbonyl-L-tyrosyl-L-leucyl-β-*t*-butyl-L-aspartyl-L-serine methyl ester (XXII). Alternatively, the same

---

(1) For the previous paper in this series, see A. A. Costopanagiotis, J. Preston, and B. Weinstein, *Can. J. Chem.*, **45**, in press (1967).

(2) P. P. Foà and G. Galansino, "Glucagon: Chemistry and Function in Health and Disease," Charles C. Thomas, Springfield, Ill., 1962.

(3) E. Wünsch and A. Zwick, *Chem. Ber.*, **97**, 3305 (1964).

(4) E. Schröder, *Ann.*, **681**, 241 (1965).

(5) F. Weygand and E. Nintz, *Z. Naturforsch.*, **20b**, 429 (1965).

(6) R. Schwyzer and H. Dietrich, *Helv. Chim. Acta*, **44**, 2003 (1961).

(7) E. Wünsch and A. Zwick, *Z. Physiol. Chem.*, **328**, 235 (1962).

(8) E. Schröder and E. Klieger, *Ann.*, **673**, 208 (1964).

(9) S. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).

(10) J. C. Sheehan, P. A. Cruickshank, and G. L. Boshart, *J. Org. Chem.*, **26**, 2525 (1961).

(11) J. C. Sheehan and J. J. Hlavka, *ibid.*, **21**, 439 (1956).

(12) After this study was completed, the same product was reported by other workers: E. Wünsch and A. Zwick, *Chem. Ber.*, **99**, 105 (1966).

(13) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(14) R. B. Woodward, R. A. Olofson, and H. Mayer, *ibid.*, **83**, 1010 (1961).

(15) J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1609 (1963).

(16) E. Schröder and K. Lübke, "The Peptides," Vol. 1, Academic Press Inc., New York, N. Y., 1965, p 97.

(17) H. Zahn and H. E. Messerknecht, *Z. Physiol. Chem.*, **315**, 228 (1959).

(18) B. O. Handford, J. H. Jones, G. T. Young, and T. F. N. Johnson, *J. Chem. Soc.*, 6814 (1965).

(19) Fluka AG, Buchs SG, Switzerland.