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Some Side Reactions of Nitro-L-arginine

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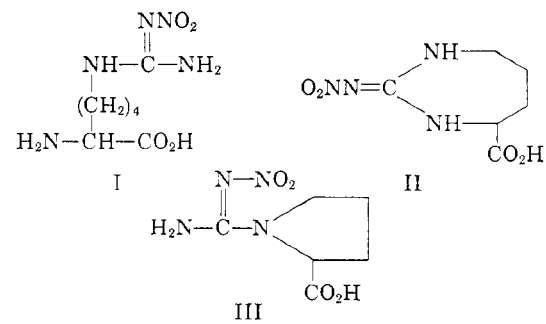
On treatment of nitro-L-arginine (I) with base, a side reaction occurred besides simple salt formation. The by-product was shown to be 2-nitrimino-4-carboxy-1,3-diazacycloheptane (II). *p*-Nitrophenyl carbobenzoxynitro-L-argininate (VII) was found to be unstable and the decomposition product, 1-nitroguanyl-3-carbobenzoxyamino-2-piperidone (VIII), identified. The reaction of the piperidone (VIII) with amino acid esters was studied and a shift of the nitroguanyl group from the piperidone to the amino acid ester found.

In the course of some reactions involving nitro-L-arginine[1] (I) and its derivatives several unexpected side reactions occurred. The first of these was encountered while attempting to prepare a *t*-butyloxycarbonyl[2] derivative. Nitro-L-arginine, aqueous sodium bicarbonate, *t*-butyl-*p*-nitrophenylcarbonate,[2] and *t*-butyl alcohol were heated under reflux for thirty minutes. After boiling off the *t*-butyl alcohol and extracting with ether at *p*H 6 to remove the *p*-nitrophenol, acidification gave a compound which did not evolve carbon dioxide on treatment with concentrated hydrochloric acid. Since this reaction is typical of *t*-butyloxycarbonyl compounds, the product was not the one desired. The material had an analysis agreeing with $C_6H_{10}N_4O_4$ and was optically active. The nitro-L-arginine ($C_6H_{13}H_5O_4$) had lost the elements of ammonia. Indeed in subsequent repetitions of the reaction the odor of ammonia could always be detected. The highest yield of the product was obtained by heating nitro-L-arginine on a steam bath with an excess of aqueous sodium carbonate for one and a half hours.

The unknown material was soluble in sodium bicarbonate and insoluble in *N* hydrochloric acid; neut. equiv. calculated 202, found 204. An infrared spectrum showed bands at 5.90 μ and 6.25 μ attributable to C=N and NO_2.[3] The ultraviolet spectrum showed a large peak at 270 mμ (ϵ = 14,000) and a small one at 217 mμ (ϵ = 4450). The starting nitro-L-arginine had a $\lambda_{max}^{H_2O}$ at 270 mμ (ϵ = 15,400). Kumler[4,5] has found 2-nitriminoimidazolidine to have λ_{max} 267 mμ (ϵ = 17,700). *N*-Nitromethylamine had λ_{max} 230 mμ (ϵ = 7000). The data seem to indicate that whatever the product is, no change occurred in the >C=N—NO_2 system of the starting material. A ninhydrin test was negative.

The unknown material was heated with *N* sodium hydroxide in a boiling water bath for an hour. A sample was acidified and chromatographed in *n*-butyl alcohol–acetic acid–water 4:1:5[6] (BAW). Two ninhydrin positive spots were found. One was very pronounced and this was identified as ornithine by R_f. The second spot was very faint, possibly a trace of partly reacted material. Nitro-L-arginine on undergoing the same treatment also gave ornithine and a lesser amount of a material identified as citrulline by R_f[7a] and by a positive Ehrlich test.[7b] Citrulline probably is an intermediate in the hydrolysis. Hence one may assume that the carbon skeleton was unchanged in the unknown from that of I. Reduction of the unknown with palladium on charcoal removed the nitro group to give $C_6H_{11}N_3O_2$, a crystalline amphoteric material.

At this point, two structures could be written for the unknown, II and III.



I II

III

N-Nitroguanyl-L-proline (III) was synthesized from L-proline and 2-methyl-1-nitro-2-thiopseudourea (IV).[8] The physical properties were quite different from those of the unknown. The infrared of the unknown had one band at 3.06 μ showing a secondary amine NH stretching.[9a] The spectrum of III, however, had two bands, 2.94 μ and 3.04 μ

(1) (a) A. Kossel and E. L. Kennaway, *Z. physiol. Chem.*, **72**, 487 (1911); (b) K. H. Hofmann, W. D. Peckham, and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).

(2) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957).

(3) W. D. Kumler, *J. Am. Chem. Soc.*, **76**, 814 (1954).

(4) W. D. Kumler and P. P. T. Sah, *J. Org. Chem.*, **18**, 669 (1953).

(5) W. D. Kumler, *J. Org. Chem.*, **18**, 676 (1953).

(6) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(7) (a) R. J. Block, E. L. Durrum, and G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis*. Academic Press, New York, 1955, p. 108. (b) Loc. cit. p. 92.

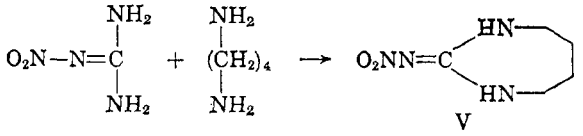
(8) L. Fishbein and J. A. Gallaghan, *J. Am. Chem. Soc.*, **76**, 1877 (1954).

(9) (a) L. J. Bellamy, *The Infrared Spectra of Complex Molecules*. Wiley, New York, 1958, p. 249; (b) Loc. cit. p. 213; (c) Loc. cit. p. 221.

showing a primary amine NH stretching. A mixed melting point of III with the unknown gave a depression. On basic hydrolysis, III gave proline as expected. The evidence indicates that the unknown is II.

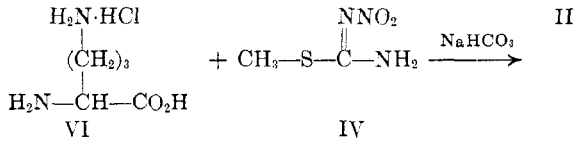
All attempts at the decarboxylation of 2-nitrimino-4-carboxy-1,3-diazacycloheptane II to give the known 2-nitrimino-1,3-diazacycloheptane[10] (V) failed.

There is precedent for this facile 7-membered ring formation. McKay and Wright[10] found that nitroguanidine reacted with 1,4-diaminobutane to give V in 64% yield.



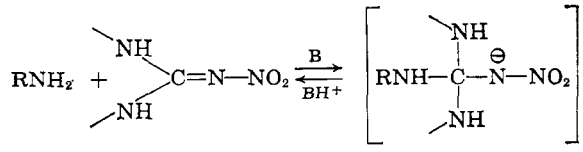
V

Attempts to synthesize II by the reaction of L-ornithine (VI) or methyl L-ornithinate with nitroguanidine failed. However, 2-nitrimino-4-carboxy-1,3-diazacycloheptane (II) was finally synthesized by the method of Hafner and Evans[11] from L-ornithine (VI) and IV. It is true, of course, that this synthesis could go through nitro-L-arginine as an intermediate.



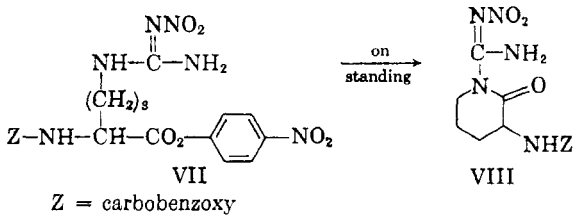
VI IV II

To test the reversibility of the ring closure, a small amount of II was heated with concentrated aqueous ammonia at 100° for one hour. On chromatographic analysis three ninhydrin positive spots were found which were identified as ornithine, nitroarginine, and a trace of arginine. *N*-Nitroguanyl-L-proline and ammonia gave proline. These results are readily understood if one assumes the >C=N—NO_2 acts as a carbonyl group. The intermediate from the addition of ammonia or an amine can theoretically decompose in four directions.



In connection with this side reaction, it was interesting to note that on two occasions cited in the literature recently[12a,b] solutions of nitro-L-arginine (I) in sodium hydroxide were used. We treated I with base, in the way described in each reference, but did not add any activated amino acid with which it could react. On acidification, less than 2% of II was found in each case. On neutralization, better than 90% of I was recovered in each case, hence there was probably little interference from this side reaction.

Another unexpected reaction was encountered by us and also by Bodansky.[13] The *p*-nitrophenyl ester of carbobenzoxynitro-L-arginine (VII), which we obtained as an oil that has not been characterized, on standing crystallized to give a cyclic lactam,



VII VIII

Z = carbobenzoxy

1 - nitroguanyl - 3 - carbobenzoxyamino - 2 - piperidone (VIII). A commercial sample[14] crystallized to give the same product.

The material was identified by analysis, infrared (5.78 μ imide carbonyl, 5.91 μ lactam, 6.25 μ NO_2)[3,9b,9c] ultraviolet $\lambda_{275\ m\mu}^{50\%\ alc.}$ (ϵ = 16,120) and mol. wt. 313 ± 31 calcd. 335. The compound is insoluble in dilute acid or sodium bicarbonate. An analogous condensation was described in the literature.[15] On the boiling of nitro-L-arginine in acetic anhydride and acetic acid N^{α}-acetylanhydro-DL-nitroarginine was obtained. Bergmann and Koster[16] converted L-arginine to triacetylanhydro-DL-arginine by boiling it in excess acetic anhydride. Zervas *et al*[17] found that N^{α},N^{ω}-dicarbobenzoxy-L-arginine gave N^{α},N^{ω}-dicarbobenzoxyanhydro-L-arginine as a by-product during a condensation with diethyl L-glutamate using acid chloride coupling or dicyclohexylcarbodiimide. In each of these cases a six-membered lactam formed.

An attempt to prepare a dipeptide by treating the lactam (VIII) with *t*-butyl glycinate[18] gave instead 3-carbobenzoxyamino-2-piperidone (IX). The compound had no ultraviolet absorption at 270 mμ. It was insoluble in dilute acid or sodium bicarbonate solution. The infrared showed only the peak at 5.90 μ. The 5.78 μ and 6.25 μ peaks have disappeared. Bodanszky[13] also reported this product.

(10) A. F. McKay and G. F. Wright, *J. Am. Chem. Soc.*, **70**, 430 (1948).

(11) L. S. Hafner and R. Evans, *J. Org. Chem.*, **24**, 1157 (1959).

(12) (a) L. T. Skeggs, Jr., K. E. Lentz, J. R. Kahn and N. P. Shumway, *J. Exp. Med.*, **108**, 283 (1958); (b) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957).

(13) M. Bodakszky and J. T. Sheehan, *Chemistry & Industry*, 1268 (1960). We thank Dr. Bodanszky for letting us see his manuscript prior to publication.

(14) Purchased from Cyclo Chemical Corp.

(15) S. M. Birnbaum and J. P. Greenstein, *Arch. Biochem. Biophys.*, **39**, 108 (1952).

(16) M. Bergmann and H. Koster, *Z. physiol. Chem.* **159**, 179 (1926).

(17) L. Zervas, T. T. Otani, M. Winitz, and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2878 (1959).

(18) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).

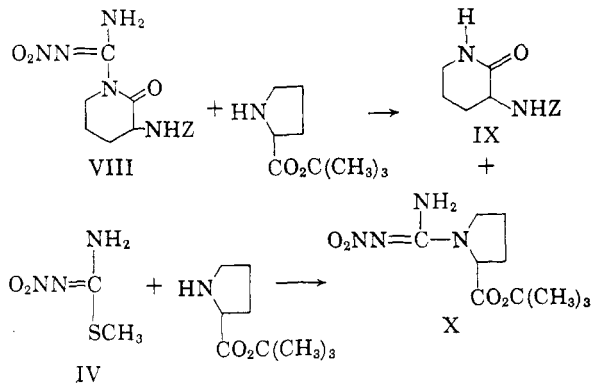
TABLE I

IDENTIFICATION OF REACTION PRODUCTS BY CHROMATOGRAPHY IN BAW

Compound	Treatment	Spotting Reagent	R_f Values: Orn	Arg	Cit	NO_2 Arg	Pro	Unidentified Other
1. Standard NO_2 | H arg OH	—	Ninhydrin	0.10	0.16		0.28	0.43[a]	
2. H arg OH (I)	*N* NaOH	Ninhydrin	0.12		0.22	0.27 (w)		
		Ehrlich	—		0.22	—		
3. II	*N* NaOH	Ninhydrin	0.09					0.44[b] (w)
4. O_2NN=C(NH$_2$)-pro·OH (III)	*N* NaOH	Ninhydrin					0.42[a]	
5. II	Concd. NH_3	Ninhydrin	0.14	0.20		0.33		
Simultaneous Standard		Ninhydrin	0.12	0.20		0.33		
6. O_2NN=C(NH$_2$)-pro·OH (IV)	Concd. NH_3	Ninhydrin					0.49[a]	

[a] = Yellow color. [b] = Blue color.

To give a further indication of the structure of IX and the course of the reaction, the nitroguanyl compound VIII was reacted with a different amine, *t*-butyl L-prolinate.[18] Again IX was isolated. In light of the earlier work we felt the nitroguanyl group should be found on the proline ester. Working up the mother liquors from the purification of IX gave *t*-butyl *N*-nitroguanyl-L-prolinate (X).



To prove the structure of X, it was synthesized from *t*-butyl-L-prolinate and 2-methyl-1-nitro-2-thiopseudourea (IV).

These findings may explain some of the difficulties encountered in the use of nitro-L-arginine. They certainly indicate care must be used in working with it.

EXPERIMENTAL

All melting points were obtained on a standardized Fisher-Johns block.

2-Nitrimino-4-carboxy-1,3-diazacycloheptane (II). A solution of 20 g. (0.38 mole) of sodium carbonate in 250 ml. of water was heated on a steam bath and 32.8 g. (0.15 mole) of nitro-L-arginine[1] added. Ammonia evolution was noted by its odor. The green solution was stirred occasionally and after 1.5 hr. cooled to room temperature. The *p*H was adjusted to 1. After 2 hr. at 0° colorless crystals appeared. These were collected and dried to give 16.0 g., m.p. 178–181° dec. Recrystallization from *ca.* 150 ml. of water gave 10.2 g. (34%) m.p. 181–182° dec. Neut. equiv. calcd.: 202. Found: 204. Infrared peaks 3.06 μ, 5.90 μ, 6.25 μ. $\lambda_{max}^{H_2O}$ 270 mμ (ϵ = 14,000), $\lambda_{max}^{H_2O}$ 217 mμ (ϵ = 4450), $[\alpha]_D^2$ + 48.5° (*c* 1, 0.5*N* potassium bicarbonate). This compares to nitro-L-arginine $\lambda_{max}^{H_2O}$ 270 mμ (ϵ = 15,400).

Anal. Calcd. for $C_6H_{10}N_4O_4$: C, 35.64, H, 4.99, N, 27.72. Found: C, 35.80, H, 5.20, N, 28.10.

2-Imino-4-carboxy-1,3-diazacycloheptane. With a slight amount of heating 3 g. (0.015 mole) of II was dissolved in 200 ml. of methanol and the solution cooled to room temperature. A 1.2-g. quantity of 10% palladium on charcoal[19] was added. The system was flushed with nitrogen, then hydrogen was bubbled through for 3 hr. During this time the mixture was occasionally warmed to dissolve material that came out of solution. The system was again flushed with nitrogen. The catalyst was filtered off, boiled with 50 ml. of water, and again filtered. The combined filtrates were taken to dryness, giving 1.75 g. of material melting above 300°. It was very difficult to remove the last traces of catalyst from the product. Several recrystallizations, by dissolving in water and adding 10 volumes of methanol gave a gray crystalline material melting at 323° dec. in 12% yield. Ninhydrin and Sakaguchi tests were negative. No ultraviolet absorption at 270 mμ was found, $[\alpha]_D^{25}$ +54° (*c* 2, water).

Anal. Calcd. for $C_6H_{11}N_3O_2$: C, 45.85, H, 7.05, N, 26.74. Found: C, 45.82, H, 7.16, N, 26.65.

Repetition of Skeggs and Schwarz's conditions.[12] Nitro-L-arginine was dissolved in two sodium hydroxide solutions in the manner described by each of the references. No activated amino acid was added, however, and after the specified time the solution was taken to *p*H 6. Nitro-L-arginine was recovered. The *p*H was adjusted to 1 and the solution extracted with ethyl acetate when no precipitate appeared. Evaporation to dryness of the organic layer gave whatever II was present.

	Skeggs Wt., g.	Skeggs M.p., °d.	Schwarz Wt., g.	Schwarz M.p., °d.
NO_2 | Starting H.arg.OH (*L*)[1]	2.63	254–263	3.30	254–273
NO_2 | Recovered H.arg.OH	2.40	248–256	3.19	240–254
	0.055	174–175	0.048	177–178

(19) Baker and Co., Inc. Catalysts.

N-Nitroguanyl-L-proline (III). Applying the method of Saroff and Evans,[20] 5.75 g. (0.05 mole) of L-proline was dissolved in 50 ml. of *N* sodium hydroxide. Portionwise 6.95 g. (0.05 mole) of 2-methyl-1-nitro-2-thiopseudourea[8] was added while the solution was shaken with a Narda Ultrasonic Generator Series 400. The odor of methyl mercaptan quickly became evident. After 30 min. of shaking the solution stood in a hood overnight. After cooling to 0°, the mixture was acidified with 6*N* hydrochloric acid. The product was collected, washed with ice water, and dried in a steam cabinet. The 8.15 g. quantity, (74%) m.p. 195–200° dec., browns at 190°, was recrystallized from 150 ml. of water (charcoal) to give 6.99 g. (63%) of product m.p. 198° d. brown. $[\alpha]_D^{25}$ −55.4° (*c* 2.4, 0.5*N* potassium bicarbonate). Infrared peaks 2.94 μ, 3.04 μ, 5.74 μ, 6.14 μ, 6.36 μ, 6.73 μ: U.V. $\lambda_{max}^{H_2O}$ 275 (ε = 12,300), $\lambda_{max}^{H_2O}$ 210 (ε = 3800). Mixed melting point with II depresses, 172–178° d.

Anal. Calcd. for $C_6H_{10}N_4O_4$: C, 35.64, H, 4.99, N, 27.72. Found: C, 35.49, H, 5.36, N, 27.65.

Degradation studies on I, II, and III. All compounds were treated 1 hr. at 100° with the reagent except the standards. The compounds were acidified, spotted on Whatman No. 1, and developed with BAW at 20 ± 2°.

Synthesis of II *from* L-*ornithine and 2-methyl-1-nitro-2-thiopseudourea.* To a solution of 3.37 g. (0.020 mole) of L-ornithine[21] in 40.0 ml. of *N* sodium hydroxide was added 2.92 g. (0.021 mole) of 2-methyl-1-nitro-2-thiopseudourea[8] following the general method of Hafner and Evans.[11] The mixture was shaken with a Narda Ultrasonic Generator and finally warmed on a steam bath. Methyl mercaptan was evolved. After standing over the weekend, the solution was acidified and cooled. Several days later 0.99 g. of product m.p. 165–175° dec. was collected. Two recrystallizations from 7-ml. quantities of water gave 0.51 g. (13%) m.p. 178–181° dec. A mixed melting point with II gave no depression. The infrared was identical with that of II. $[\alpha]_D^{25}$ +49° (*c* 1.1, 0.5*N* potassium bicarbonate).

1-Nitroguanyl-3-carbobenzoxyamino-2-piperidone (VIII). Carbobenzoxynitro-L-arginine[1b] (14.0 g., 0.040 mole), *p*-nitrophenol (8.0 g., 0.058 mole), and dicyclohexylcarbodiimide[22] (8.3 g., 0.40 mole) were added to dimethylformamide at 0°. After 30 min. at 0°, the reaction mixture was allowed to warm to room temperature. Two hours later the dicyclohexylurea was filtered off and the filtrate concentrated at 60°/0.02 mm. to a yellow oil. On standing in an open dish for 2 weeks, the material crystallized. Trituration with methyl isopropyl ketone left a white crystalline solid m.p. 144–145°. Recrystallization from isopropyl acetate yielded 4.5 g. (34%) of material, m.p. 146–147°. $[\alpha]_D^{25}$ −6.8° (*c* 3.1, dimethylformamide).

Anal. Calcd. for $C_{14}H_{17}N_5O_5$: C, 50.14; H, 5.11; N, 20.89. Found: C, 49.79; H, 5.32; N, 21.12.

The infrared spectrum showed peaks at 2.96 μ, 3.08 μ, 5.78 μ, 5.91 μ, 6.25 μ. Ultraviolet $\lambda_{max}^{50\%\ alc.}$ 275 mμ (ε = 16,120). Mol. wt., calcd., 335. Found: 313 ±31 (thermistor vapor pressure method).

A commercial sample[14] of *p*-nitrophenyl carbobenzoxynitro-L-argininate was also found to contain a large amount of lactam VIII.

3-Carbobenzoxyamino-2-piperidone (IX). An attempt to prepare *t*-butyl carbobenzoxynitro-L-arginylglycinate from 0.670 g. (0.0025 mole) of the lactam VIII and 0.960 g. (0.007 mole) of *t*-butyl glycinate[18] was made by mixing the two without solvent and permitting the resulting solution to stand for 3 days. A crystalline product was obtained which was purified by a recrystallization from ethyl acetate, to give 0.175 g. (28%) m.p. 174–175°. The ultraviolet at 270 mμ was negligible. The infrared had a broad peak at 5.94 μ and a narrow peak at 3.98 μ.

Anal. Calcd. for $C_{13}H_{16}N_2O_3$: C, 62.89; H, 6.50; N, 11.28. Found: C, 62.24; H, 6.68; N, 11.04.

t-Butyl N-nitroguanyl-L-prolinate (X). 1-Nitroguanyl-3-carbobenzoxyamino-2-piperidone (0.670 g., 0.0025 mole) and 1.94 g. (0.0113 mole) of *t*-butyl prolinate[18] were intimately mixed and permitted to stand at room temperature for 44 days. Then the slushy reaction mixture was shaken with 25 ml. of *N* hydrochloric acid and the mixture filtered through a sintered glass funnel. The residue on drying gave 0.490 g. (79%) m.p. 162–172°. Recrystallization from ethyl acetate gave 0.35 g. (58%) of IX, m.p. 176.5–177°. Mixed melting point with previous IX gave no depression.

The filtrate from above was shaken with 50 ml. of methylene chloride. The organic layer was evaporated to dryness yielding 0.550 g. of a crystalline product, m.p. 105–113°. Two recrystallizations from water gave 0.139 g. (22%) of crystalline material m.p. 156–157°. This was shown to be *t*-butyl *N*-nitroguanyl-L-prolinate $[\alpha]_D^{25}$ −68° (*c* 1.66, 95% ethanol).

Anal. Calcd. for $C_{10}H_{18}N_4O_5$: C, 46.50; H, 7.02; N, 21.69. Found: C, 46.85; H, 7.31; N, 21.72.

Synthesis of t-butyl N-nitroguanyl-L-prolinate (X). Heating together 0.695 g. (0.005 mole) of 2-methyl-1-nitro-2-thio-pseudourea (IV)[8] and 0.855 g. (0.005 mole) of *t*-butyl L-prolinate[18] (X) in 15 ml. of water on a steam bath for a few minutes gave a crystalline product on subsequent cooling. This product was collected by filtration and the residue washed with 5 ml. of water, yielding 0.870 g. (67%) of material m.p. 156–157°. A recrystallization from water gave 0.350 g. (27%) of product, m.p. 157–158°. On taking a mixed melting point with the *t*-butyl *N*-nitroguanyl-L-prolinate from the previous reaction, no depression was noted. $[\alpha]_D^{25}$ −72° (*c* 2, 95% ethanol); $\lambda_{max}^{H_2O}$ 275 mμ (ε = 16,200).

Anal. Calcd. for $C_{10}H_{18}N_4O_5$: C, 46.50; H, 7.02; N, 21.69. Found: C, 46.86; H, 7.46; N, 21.56.

Acknowledgment. The authors wish to thank Mr. L. Brancone and staff for the analyses and Mr. W. Fulmor and staff for the spectra and optical rotations.

PEARL RIVER, N. Y.

(20) H. A. Saroff and R. L. Evans, *Biochem. et Biophys. Acta*, **36**, 511 (1959).

(21) Mann Research Laboratories, Inc.

(22) Aldrich Chemical Co., Inc.