BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 43 2539—2543 (1970)

Bromodenitrogenation of N^2-Benzoylarginine Ethyl Ester with *N*-Bromosuccinimide

Kentaro ANZAI

The Institute of Physical and Chemical Research, Yamato-machi, Saitama

(Received February 3, 1970)

N^2-benzoylarginine ethyl ester (II) reacted with *N*-bromosuccinimide at pH's above 7 to yield, as the main product, N^2-benzoyl-N^5-cyanoornithine ethyl ester (VII), as well as N^3,N^6-bis(4-benzamido-4-ethoxycarbonylbutyl)*s*-tetrazine-3,6-diimine (VIII), *N*,*N'*-di(4-benzamido-4-ethoxycarbonylbutyl)urea (IX), and N^2-benzoylcitrulline ethyl ester (X) as minor products. On acid hydrolysis, VII afforded the following amino acids: ornithine (III), citrulline (IV), N^6-[(4-amino-4-carboxybutyl)-carbamoyl]arginine (V), and *N*,*N'*-di(4-amino-4-carboxybutyl)urea (VI). The mechanism of these reactions was also discussed.

Amino acids react with *N*-bromosuccinimide (NBS), with the evolution of carbon dioxide, to produce corresponding aldehydes.[1] According to Okuyama *et al.*,[2,3] arginine, acetyl arginine, and DNP-arginine as well as guanidine react with NBS, with the evolution of nearly equimolar amounts of nitrogen gas, at pH's above 7. Some proteins, *e. g.*, clupein, gelatin, and the crude urease extract, also suffer from NBS oxidation. On the basis of the above findings the chemical modification of a peptide antibiotic, triculamin,[4,5] which contains two arginine residues in a molecule, was attempted. However the product obtained was an intractable mixture which could hardly be separated chromatographically and this unfruitful result prompted

1) N. Konigsberg, G. Stevenson and J. M. Luck, *J. Biol. Chem.*, **235**, 1341 (1960).

2) N. Okuyama, N. Sato and M. Hirota, *Nippon Kagaku Zasshi*, **86**, 443 (1965).

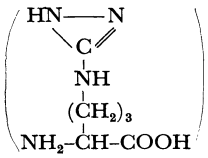
3) N. Okuyama, private communication, Feb. 16, 1968.

4) S. Suzuki, K. Asahi, J. Nagatsu, Y. Kawashima and I. Suzuki, *J. Antib.*, **20A**, 126 (1967).

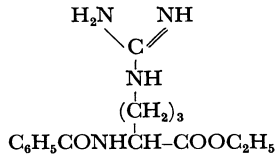
5) K. Anzai and S. Suzuki, *Agr. Biol. Chem.*, **33**, 1737 (1969).

us to study the reaction path of NBS oxidation on arginine.

Okuyama *et al.*[3,6] postulated an intermediate, I, from which, on further oxidation, nitrogen could evolve, leaving N^2-formyl ornithine. However, an attempt to isolate the latter compound failed, though ornithine was obtained upon the hydrolysis of the oxidation product.

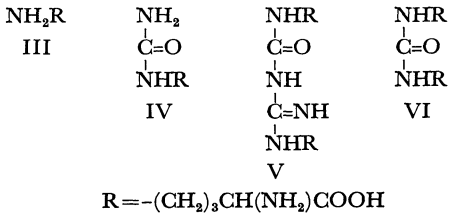


I



II

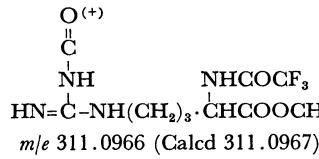
N^2-Benzoylarginine ethyl ester (II) was allowed to react with an excess amount of NBS under neutral conditions at room temperature. The product was extracted with ethyl acetate, and then hydrolysed with hydrochloric acid. CM Sephadex chromatography afforded the following five amino acids (yields): ornithine (III), 52%; citrulline (IV), 29%; N^6-[(4-amino-4-carboxybutyl)carbamoyl]arginine (V), 8%; N,N'-di(4-amino-4-carboxybutyl)urea (VI), 3%; arginine, 2%.



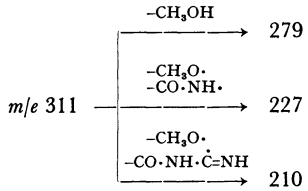
III IV V VI

R=$-(CH_2)_3CH(NH_2)COOH$

V is a strong base; it is eluted from CM Sephadex column closely after arginine, and it is obtained as an acetate. It showed a positive ferricyanide-nitropurside reaction (substituted guanidine). NMR shows the presence of two methylene and two methine groups: δ 3.24 (t, J=5 Hz, 2H), δ 3.35 (t, J=7 Hz, 2H), δ 3.71 (t, J=7 Hz, 1H), δ 3.75 (t, J=5 Hz, 1H). Though the trifluoroacetylated methyl ester of V does not give the parent peak, the



m/e 311.0966 (Calcd 311.0967)

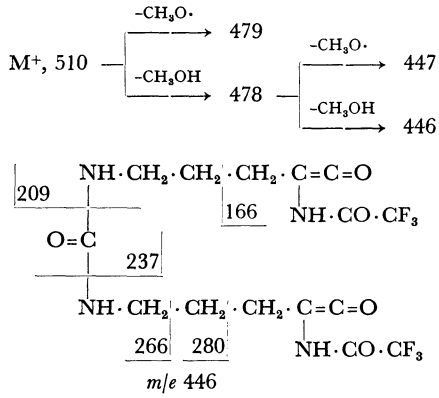


Scheme 1

6) K. Niijima and N. Okuyama, *Seikagaku*, **35**, 396 (1963).

fragmentations, shown in Scheme 1, can be reasonably explained on the basis of the structure V.

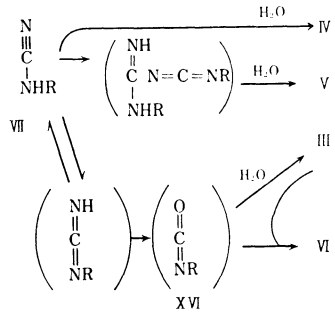
The NMR of VI shows one methylene (δ 3.15, t, J=6 Hz) and one methine (δ 3.75, t, J=6 Hz) proton signals, showing that it is a symmetrically-substituted compound. The trifluoroacetylated methyl ester of VI shows a very weak parent peak at *m/e* 510. However, the intense fragmentations shown in Scheme 2 can reasonably be explained on the basis of the structure VI.



Scheme 2

We attempted to isolate the NBS oxidation product of II using silicic acid chromatography. The main product was identified as N^2-benzoyl-N^5-cyanoornithine ethyl ester VII; three minor products were also obtained.

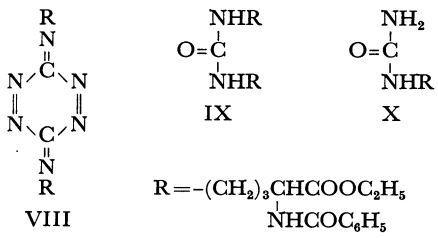
The IR of VII shows a strong band at 2210 cm^{-1} attributable to a cyano group. Though the analytical and NMR data support the structure VII, the mass spectrum shows some ambiguity. Though the operation conditions can be chosen so as to give the expected parent peak at *m/e* 289.1418 (calcd for $C_{15}H_{19}N_3O_3$ 289.1426), unexplicable peaks at higher mass are observed on an elevation of the temperature: These can possibly be ascribed to the polymerization of the sample. VII also afforded III, IV, V, and VI on acid hydrolysis; a reasonable reaction path from VII to the three amino acids is shown in Scheme 3:



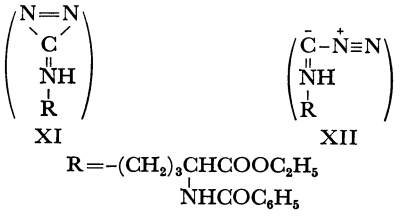
R=$-(CH_2)_3CHCOOC_2H_5$
$NHCOC_6H_5$

Scheme 3

The three minor products were identified as N^3,N^6-bis(4-benzamido-4-ethoxycarbonylbutyl)-*s*-tetrazine-3,6-diimine (VIII), *N,N'*-di(4-benzamido-4-ethoxycarbonylbutyl)urea (IX), and N^2-benzoyl-citrulline ethyl ester (X).



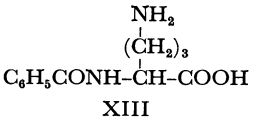
The intense UV absorption of VIII ($\lambda_{max}^{methanol}$ 222, ε 83000; 303 mμ, ε 7000), as well as the absence of IR absorption in the 1800—2900 cm^{-1} region, exclude other possible structures, XI and XII, which



could be expected to give the same analytical data and an NMR spectra similar to that of VIII. XI or XII was first postulated[7] as an intermediate through which N^2-benzoyl-N^5-formylornithine ethyl ester might be given, along with the evolution of nitrogen gas.

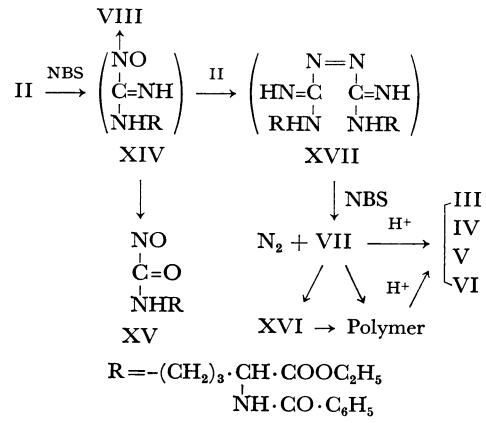
IX and X gave the corresponding amino acids, VI and IV, on acid hydrolysis; the analytical and spectral data are also consistent with the proposed structures.

After VII, VIII, IX, and X had been extracted with ethyl acetate, the aqueous layer was heated with sodium hydroxide. Subsequent CM Sephadex chromatography afforded N^2-benzoylornithine (XIII).



The product analysis leads to the possible reaction path outlined in Scheme 4; by postulating an intermediate, XIV, all the products mentioned above can reasonably be situated there.

The compound XV was once obtained; though its poor reproducibility made satisfactory identification difficult, the analytical data and the MS



Scheme 4

fragmentation pattern suggested it to be 2-benzamido-5-(2-nitrosoformamido)valeric acid ethyl ester (XV) (See Experimental section).

Possibly the main reaction proceeds *via* II, XIV, XVII, and VII, with the evolution of nitrogen from XVII. According to this process, 1.5 mol of NBS is needed for the oxidation of 1 mol of II. VII as well as its hydrolysed product, XVI, might have a tendency to polymerize easily under the present reaction conditions, resulting in an unexpectedly low yield of VII (20% after purification). A polymerized product, which was separated roughly by means of silicic acid chromatography, also gave four amino acids, *i. e.*, III, IV, V, and VI, on acid hydrolysis.

VII was still hydrolysed in a neutral solution, the reaction might proceed in a way similar to that shown in Scheme 3, giving IX, X, and the ethyl ester of XIII.

According to the reaction path proposed here, the two atoms of a nitrogen molecule do not originate from the same guanidine residue, as was first postulated.[7] Ammonia, liberated from VII by hydrolysis, may also be oxidized to a nitrogen molecule.

Experimental

Reaction Procedure. In 25 m*l* of a 0.1N phosphate buffer solution at pH 7, 171 mg (5×10^{-3}M) of the hydrochloride of N^2-benzoylarginine ethyl ester (II) were dissolved. A solution of 178 mg (10^{-2}M) of NBS in 25 m*l* of acetone was added dropwise with stirring and the reaction proceeded with an evolution of nitrogen gas accompanying the decrease in the pH. If the insoluble product separated, acetone was added until a homogenous solution was obtained; the pH of the solution was kept at 7—8 with sodium hydroxide throughout the reaction. The decrease in the pH ceased before the addition of NBS was completed. Five grams of ammonium sulfate were added to diminish the excess NBS.

Acid Hydrolysis of the Reaction Product Yielding Ornithine (III), Citrulline (IV), N^6-[(4-Amino-4-carboxybutyl)carbamoyl]-arginine (V), and *N,N'*-Di(4-amino-4-carboxybutyl)urea (VI). The reaction

7) In a letter to Dr. N. Okuyama the present author suggested the reaction path to be II→I→XI or XII $\xrightarrow{H_2O}$ N^2-benzoyl-N^5-formylornithine ethyl ester+N_2. This mechanism can be rejected on the basis of this paper.

mixture described in the preceding section was extracted with ethyl acetate; then, after the solvent had been evaporated, the residue, which weighed 1.15 g, was dissolved in a mixture of acetic acid and conc. HCl (1 : 1) and the solution was heated at 105°C for 14 hr. The acids were then evaporated, and the residue was dried over sodium hydroxide *in vacuo*. Water was added, and the water-insoluble part, from which 130 mg of benzoic acid was obtained after crystallization from ethanol and water, was excluded by filtration; we thus obtained a mixture of amino acids, which were separated chromatographically as will be described below.

The amino acid mixture was charged on a column (2×90 cm) of CM Sephadex and developed with a 0.4N pyridine solution buffered at pH 5.0 with acetic acid. Three fractions, all of which showed a positive ninhydrin reaction, were thus obtained. The first one (a fraction at 100—190 ml) consisted of two amino acids as the main components; they were separated further chromatographically on a cellulose column using a mixture of propanol, pyridine, acetic acid, and water (15, 10, 3, 10) as the developing solvent. The one which was eluted first was identified as citrulline (IV) by comparing it with an authentic sample; yield, 250 mg.

The latter one (yield, 20 mg) was crystallized from water and ethanol and was identified as *N,N'*-di(4-amino-4-carboxybutyl)urea (VI). It gradually decomposed above 240°C and did not show a definite mp; $[\alpha]_D$= +2.3 (20°C, *c* 1, H_2O).

Found: C, 44.62; H, 7.55; N, 20.05%. Calcd for $C_{11}H_{22}N_4O_5$: C, 45.50; H, 7.63; N, 19.30%. NMR (D_2O) δ 3.15 (t, J=6 Hz, $-NH\cdot C\underline{H}_2\cdot CH_2-$), δ 3.75 (t, J=6 Hz, $CH_2-C\underline{H}(NH_2)-COOH$): IR, ν_{max}^{KBr} 3350, 2940, 1625, 1583, 1417, 1347 cm^{-1}.

The second fraction (420—530 ml), which was eluted from the CM Sephadex column, consisted mainly of one amino acid; this amino acid was purified further chromatographically on a cellulose column and was identified as the acetate of ornithine (III) by comparing it with an authentic sample.

The third fraction (540—750 ml) consisted of two amino acids which were separated chromatographically on a cellulose column (2×90 cm; propanol, pyridine, acetic acid and water; 15, 10, 3, 10). The one which was eluted first was crystallized from water and ethanol and was identified as arginine acetate; yield, 29 mg.

The second one, which was eluted later, was identified as the acetate of N^6-[(4-amino-4-carboxybutyl)carbamoyl]arginine (V); yield, 78 mg, mp 178—183°C.

Found: C, 42.64; H, 7.03; N, 20.62%. Calcd for $C_{14}H_{28}N_6O_7$: C, 42.85; H, 7.19; N, 21.42%.

NMR (D_2O) δ 1.91 (s, $C\underline{H}_3COO^-$), δ 3.24 (t, J=5 Hz, $-NH\cdot C\underline{H}_2\cdot CH_2-$), δ 3.35 (t, J=7 Hz, $-NH\cdot C\underline{H}_2\cdot CH_2-$), δ 3.71 (t, J=7 Hz, $-CH_2\cdot C\underline{H}(NH_2)COOH$), δ 3.75 (t, J=5 Hz, $-CH_2\cdot C\underline{H}(NH_2)COOH$).

Isolation of N^2-Benzoyl-N^5-cyanoornithine Ethyl Ester (VII), N^3,N^6-Bis(4-benzamido-4-ethoxycarbonylbutyl)s-tetrazine-3,6-diimne (VIII), *N,N'*-Di(4-benzamido-4-ethoxycarbonylbutyl)urea (IX), and N^2-Benzoylcitrulline Ethyl Ester (X). The ethyl acetate extract of the reaction mixture was dried *in vacuo*, and the residue was chromatographed on a silicic acid column (2×50 cm) using a mixting of ethyl acetate and benzene as the developing solvent, while the ratio of the former to the latter was gradually increased. The fractions, all of which showed single spots on TLC with a Greig-Leaback reagent, were then collected.

The main product, N^2-benzoyl-N^5-cyanoornithine ethyl ester (VII), was eluted with a mixture of benzene and ethyl acetate (2 : 3). After the solvent had been evaporated, the solidified residue was washed with benzene; yield, 290 mg; mp 85—85.5°C.

Found: C, 61.79; H, 6.59; N, 14.31%. Calcd for $C_{15}H_{19}N_3O_3$: C, 62.26; H, 6.62; N, 14.52%.

NMR ($CDCl_3$), δ 1.30 (t, J=7 Hz $-OCH_2C\underline{H}_3$), δ 1.85 (m, $-C\underline{H}_2\cdot C\underline{H}_2\cdot CH_2-$), δ 3.08 (m, $-NH\cdot C\underline{H}_2\cdot CH_2-$), δ 4.22 (q, J=7 Hz, $OC\underline{H}_2CH_3$), δ 4.70 (m, $-CH_2\cdot C\underline{H}\cdot NH-$), δ 5.30 (broad $N\equiv C\cdot N\underline{H}\cdot CH_2-$), δ 7.2—8.0 (complex, $-C_6\underline{H}_5$): ν_{max}^{KBr} 3300, 2930, 2210, 1735, 1640, 1535, 1490, 1215, 1030, 720, 700 cm^{-1}.

The molecular weight determination by the V.P.O. method gives much larger values (chloroform, 757; ethyl acetate, 839) than that calculated (289.33), suggesting that some molecules associate in the solvent.

After VII and two other minor products (one of which was identified as succinimide (yield, 90 mg), and the other of which was not yet been identified because of its low yield) had been eluted, the solvent for the development was changed to ethyl acetate. Two products were thus obtained.

The one which was eluted first was crystallized from ethyl acetate and ligroin and was identified as N^3,N^6-bis(4-benzamido-4-ethoxycarbonylbutyl)s-tetrazine-3,6-diimine (VIII); yield, 31 mg; mp 157—158°C.

Found: C, 59.30; H, 6.10; N, 17.99%. Calcd for $C_{30}H_{36}N_8O_6$: C, 59.59; H, 6.00; N, 18.53%.

IR, ν_{max}^{KBr} 3280, 3050, 2920, 1740, 1633, 1604, 1580, 1540, 1210, 1193 cm^{-1}: $\lambda_{max}^{methanol}$ 222 (ε, 83000), 303 mμ (ε, 7000).

The other product, which was eluted later than VIII with ethyl acetate, was crystallized from ethanol and ligroin and was identified as *N,N'*-di(4-benzamido-4-ethoxycarbonylbutyl)urea (IX); yield, 32 mg; mp 196—197°C.

Found: C, 61.78; H, 7.06; N, 10.26%. Calcd for $C_{29}H_{28}N_4O_7\cdot\frac{1}{2}H_2O$: C, 61.78; H, 6.97; N, 9.94%.

M^+, 554 (weak): IR, ν_{max}^{KBr} 3360, 3260, 1740, 1648, 1605, 1570, 1540, 1490, 1470, 1340, 1260, 1035, 715 cm^{-1}: the acid hydrolysis (acetic acid-conc. HCl (1 : 1), 105°C, 16 hr) of IX gave VI, which was identified by comparison with a sample obtained as has been described in the second section.

When the developing solvent was changed to a mixture of ethyl acetate and methanol (9 : 1), a brownish-colored fraction was rapidly eluted; yield of the residue, 160 mg. No crystalline material was obtained from this dirty fraction. It exhibited a tailing aspect on TLC and may be a mixture of the polymerized products. As in the case of VII, it afforded four amino acids, *i. e.*, III, IV, V, and VI, on acid hydrolysis.

Another product was eluted with the same solvent mixture after the brownish fraction had been eluted and crystallized from ethanol and ligroin. The analytical and spectral data of the compound show that it is N^2-benzoylcitrulline ethyl ester (X), with one mole of succinimide as an adduct; yield, 90 mg; mp 196—197°C.

Found: C, 56.04; H, 6.27; N, 13.79%. Calcd for $C_{19}H_{26}N_4O_6$: C, 56.14; H, 6.45; N, 13.79%.

NMR (DMSO-d_6): δ 1.18 (t, J=7Hz, $-O\cdot CH_2\cdot C\underline{H}_3$), δ 1.7 (m, $NH\cdot C\underline{H}_2\cdot C\underline{H}_2\cdot CH_2-$), δ 2.4 (partially overlapped with the peak of DMSO, $2C\underline{H}_2$ of succinimide), δ 3.2 (m, $-NH\cdot C\underline{H}_2\cdot CH_2-$), δ 4.07 (q, J=7 Hz, $-OC\underline{H}_2$

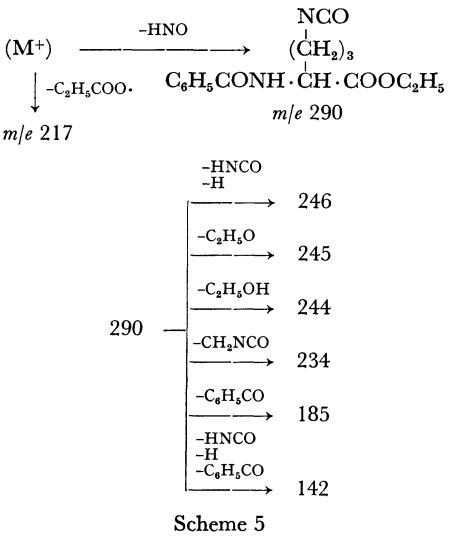
CH_3), δ 4.25 (m, $-CO\cdot NH\cdot C\underline{H}\cdot CH_2-$), δ 6.75 (s, N$\underline{H}$ of succinimide), 7.2—8.0 (complex, $C_6\underline{H}_5CO$), δ 8.30 (t, J=6 Hz, $-NH_2\cdot CO\cdot N\underline{H}\cdot CH_2-$), δ 8.70 (d, J=8 Hz, $C_6H_5\cdot CO\cdot N\underline{H}\cdot CH<$), δ 10.25 (s, $N\underline{H}_2\cdot CO\cdot NH-$): $\lambda_{max}^{methanol}$ 223 mμ (ε, 14200): IR, ν_{max}^{KBr} 3400, 3290, 3060, 2920, 1757, 1688, 1667, 1638, 1550, 1520, 1385, 1250, 1218, 1183, 1160 cm^{-1}: M^+, 307 (weak), M^+-NH_3, 290.1260 (Calcd for $C_{15}H_{18}N_2O_3$, 290.1267), M^+ of succinimide, 99 (intense). On acid hydrolysis (using a mixture of acetic acid and conc. HCl (1 : 1), 105°C, 20 hr) X afforded citrulline, which was identified by comparison with an authentic sample.

The Isolation of N^2-Benzoylornithine (XIII). After the reaction mixture described in the first section had been extracted with ethyl acetate, the volume of the aqueous layer was diluted to 100 m*l* and 4 g of sodium hydroxide were added. After the solution had been heated at 100°C for 2 hr, it was passed through an IR 120 (H-cycle) column (3×50 cm). With 1N aqueous ammonia a ninhydrin-positive fraction was eluted; it was shown to be almost homogeneous on TLC. Further purication was effective with chromatography on a CM Sephadex column (2×90 cm) using a 0.4N pyridine solution buffered at pH 5.0 with acetic acid. After the solvent had been evaporated, the residue was crystallized from water and ethanol; yield, 33 mg; mp 227—228°C.

Found: C, 60.48; H, 6.44; N, 11.76%. Calcd for $C_{12}H_{16}N_2O_3$: C, 61.00; H, 6.83; N, 11.86%. NMR (D_2O) δ 1.85 (complex, $-CH_2\cdot C\underline{H}_2\cdot C\underline{H}_2\cdot CH<$), δ 3.02 (t, J=6 Hz, $-NH\cdot C\underline{H}_2\cdot CH_2-$), δ 4.38 (t, J=6 Hz, $-CH_2\cdot C\underline{H}\cdot NH\cdot CO-$) δ 7.6 (complex, $-C_6\underline{H}_5$): IR, ν_{max}^{KBr} 3350, 2900, 1652, 1633, 1590, 1532, 1495, 1465, 1410, 1370, 1345, 1307, 1299 cm^{-1}. The methyl ester of XIII, prepared by the method of McLaffaty *et al.*[8], afforded the expected parent peak at 250. The acid hydrolysis of XIII (6N HCl, 105°C, 16 hr) afforded ornithine, which was identified by comparison with an authentic sample.

2-Benzamido-5-(2-nitrosoformamido)valeric Acid Ethyl Ester (XV). The reaction mixture described in the first section was extracted with ethyl acetate, the solvent was evaporated, and the residue, after dried *in vacuo*, was dissolved in hot benzene. Usually on standing in a refrigerator only an oily substance was separated. However, in a few cases a small amount of crystals was obtained; yield 10 mg; mp 183—184°C. The effort to create conditions which would give a good reproducibility in obtaining the compound was unfruitful. The attempt to separate the same compound by means of silicic acid chromatography was also unsuccessful. Though the sample obtained was too scanty to make a full identification, the analytical and spectral data suggested it to be 2-benzamido-5-(2-nitrosoformamido)valeric acid (XV).

Found: C, 53.23; H, 5.93; N, 12.53%. Calcd for $C_{15}H_{19}N_3O_5\cdot H_2O$: C, 53.09; H, 6.24; N, 12.39%. The parent peak was not observed: M^+-HNO, 290.1258 (calcd for $C_{15}H_{18}N_2O_4$ 290.1267). Other fragmentations, the compositions of which were confirmed by high-resolution MS, are shown in Scheme 5. IR, ν_{max}^{KBr} 3440, 3340, 3200, 3120, 3000, 1733, 1687, 1640, 1615, 1580, 1537, 1443, 1345, 1222, 1203, 1028, 710 cm^{-1}.



Scheme 5

The author wishes his hearty thanks to Dr. S. Suzuki, the chief of the Laboratory of Antibiotics, this institute, for his kindly giving him a chance to study on this subject and for his encouragement, and to Dr. S. Emoto, this institute, and Dr. N. Okuyama, Tokyo Metropolitan University, for their useful suggestions.

8) M. Senn, R. Venkataraghavan, and F. W. McLafferty, *J. Amer. Chem. Soc.*, **88**, 5593 (1966).