bon side as a gas. A polarization curve for cyclopropane is shown in Fig. 6, and it can be seen that it hydrogenates readily. A static run was also attempted with cyclopropane in a manner similar to the experiment used to obtain the data of Table I. Reaction was initiated at 0.1 v. and about 35 ma. After 4 hr., the operating cell voltage fell from 0.08 to 0.035 v. and volume began to increase in the cyclopropane compartment signalling the transfer of hydrogen. Mass spectral analysis showed the hydrogenation to be 89% complete and hydrogen present. It appears that some hydrogen was transferred (no more than 0.3 ml.) to the cyclopropane chamber because of concentration cell effects and this was sufficient to terminate the hydrogenation reaction. A comparable chemical hydrogenation experiment was performed in the apparatus of Fig. 2 by first filling the buret, C, with hydrogen and proceeding as described for propylene. Hydrogen was consumed (from volume measurements) at the rate of 0.02 ml./min. This was less than one-tenth of the rate of the comparable electrogenerative process.

It appears, then, that electrogenerative hydrogenation is useful for studying mechanisms of conventional catalytic hydrogenation as well as electrochemical hydrogenation. Since open circuit potentials can also be measured,[2] it is possible to study olefin–metal interactions with regard to metal and olefin type.[2] Measurement of hydrogenation rate at given potentials provides additional information on surface interaction, especially poisoning. Thus, electrogenerative hydrogenation provides a new and novel approach to the study of electrode reactions and mechanisms.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND 20014]

New Methods for Nonenzymatic Peptide Cleavage. Electrolytic, Differential, and Solvolytic Cleavage of the Antibiotic Cyclopeptide Rufomycin[1]

BY H. IWASAKI[2] AND B. WITKOP

RECEIVED APRIL 4, 1964

The antibiotic cycloheptapeptide rufomycin A contains the following "bifunctional" amino acids possessing reactive centers which are amenable to cleavage of adjacent peptide groups: 3-nitrotyrosine, N,4-dimethylglutamic-γ-semialdehyde, Δ^4-norleucine (2-amino-4-hexenoic acid), and a substituted tryptophan. The following new principles for selective cleavage of peptide bonds were elaborated: (i) electrolytic cleavage of the 3-nitrotyrosyl bond resulted in liberation of up to 38% of the adjacent alanine NH_2-terminal; (ii) solvolytic cleavage of the O-mesylate of O-methyldihydrorufomycin A (VII) resulted in 53% selective cleavage to *seco*-O-methyldihydrorufomycin A-δ-lactone (XI) with release of the adjacent leucyl unit; (iii) differential oxidation with N-bromosuccinimide cleaved selectively the peptide bond next to tryptophan and dehydronorleucine, when 3-nitrotyrosine was protected by O-methylation—this twofold cleavage with NBS released a novel substituted (5-bromo)spirodioxindole lactone XX characterized as its DNP derivative and convertible by the action of mineral acid to the known spirolactone XXI from DNP-tryptophan; (iv) differential oxidative cleavage with bromocarbamide cleaved only next to tryptophan but not dehydronorleucine; and (v) oxidative cleavage of DNP-*seco*-O-methyldihydrorufomycin XI by NBS yielded the dipeptide DNP-leucyl-2-amino-4-hydroxy-5-bromohexanoic acid lactone (XXII). Rufomyin A is conveniently purified and isolated as the sodium borohydride reduction product, dihydrorufomycin A, which readily crystallizes from ethanol.

Homogeneity and Purification of Rufomycin A.—Rufomycin A is a cyclic peptide antibiotic specifically active aganst Mycobacteria including the strains resistant to isonicotinic acid hydrazide, streptomycin, and kanamycin. It was isolated from the culture of a *Streptomyces* strain together with the less active rufomycin B. Both antibiotics (I, II) are cyclic heptapeptides.[3]

Crude rufomycin A was obtained as a yellow amorphous powder by evaporation of the solvent from the mother liquor of rufomycin B which crystallized first from ethanolic solution.

The homogeneity of crude rufomycin A was studied by thin layer chromatography on silica gel G. Among many solvent systems, benzene–dimethylformamide–acetone (76:4:20) and benzene–methanol–acetic acid (95:10:5) gave a satisfactory two-dimensional thin layer chromatogram. Crude rufomycin A thus contained at least one major and three minor components.

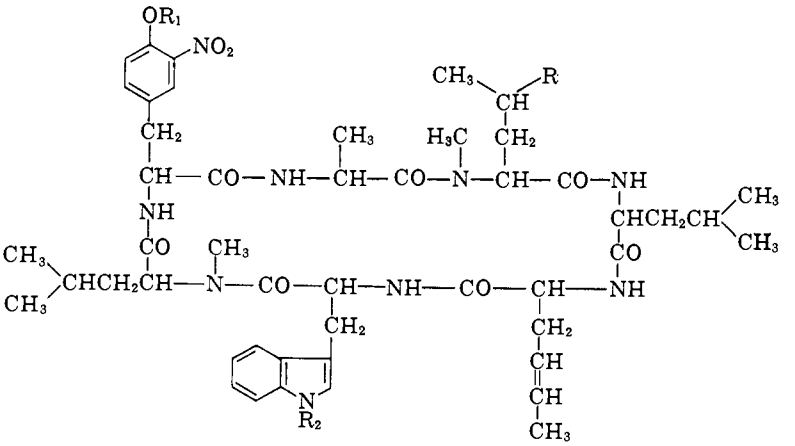
For the purification of crude rufomycin A, silica gel column chromatography with benzene–methanol (95:5) and benzene–ethanol (95:5) were promising but not good enough for preparative purposes. Good separation was achieved by the application of gradient elution of 2 to 7% methanol in benzene. Crude rufomycin A consists of five components. Component I is identical with pure rufomycin B. Component II, as yet unknown, is present as an impurity both in rufomycin A and B. From 500 mg. of crude rufomycin A, 148 mg. of the main component (III) was isolated. The four components II, III, IV, and V all contain a group sensitive to reduction by sodium borohydride. This group is not present in rufomycin B (I) according to the study of the reduction with sodium borohydride by thin layer chromatography.

Convenient Purification of Rufomycin A as the Dihydro Derivative.—Rufomycin A contains 1 mole of N,4-dimethylglutamic acid γ-semialdehyde and is reduced by sodium borohydride to dihydrorufomycin A (III). The R_f value of the main component of crude dihydrorufomycin A is remarkably different from

(1) Presented in part at the IUPAC Symposium on the Chemistry of Natural Products, Kyoto, Japan, April, 1964; *cf.* H. Iwasaki, Y. Fujita, J. Ueyanagi, and B. Witkop, Abstracts, p. 172 (1964).

(2) Associate in the Visiting Program of the USPHS, 1963–1964.

(3) (a) J. Ueyanagi, M. Fujino, T. Kamiya, H. Iwasaki, A. Miyake, and S. Tatsuoka, presented before the 6th Symposium of the Chemistry of Natural Products, Kyushu University, Japan, Oct. 17, 1963. (b) While this investigation was in progress the similarity and eventual identity of rufomycin and its congeners with the ilamycins became apparent through the studies of H. Umezawa and his group: *cf.* T. Takita and H. Naganawa, *J. Antibiotics* (A), **16**, 246 (1963).



I, rufomycin A
R = CHO, R_1 = H
II, rufomycin B
R = CH_3, R_1 = H
III, dihydrorufomycin A
R = CH_2OH, R_1 = H
IV, O-methyldihydrorufomycin A
R = CH_2OH, R_1 = CH_3
V, O,O-diacetyldihydrorufomycin A
R = CH_2OCOCH_3, R_1 = $COCH_3$
VI, O-methyl-O-nisyldihydrorufomycin A
R = $CH_2O{\cdot}SO_2$-*p*-$C_6H_4NO_2$, R_1 = CH_3
VII, O-methyl-O-mesyldihydrorufomycin A
R = $CH_2OSO_2CH_3$, R_1 = CH_3

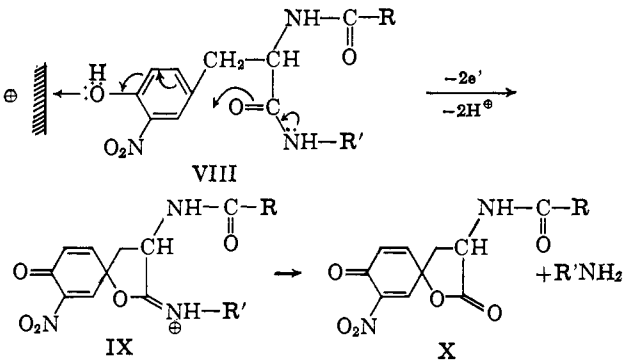
that of (unreducible) rufomycin B (silica gel thin layer chromatogram with benzene–ethanol 9:1). Moreover, dihydrorufomycin A crystallizes readily from ethanol, while the purification of rufomycin A by crystallization so far has not been possible.[4]

The purified dihydrorufomycin A was homogeneous by thin layer chromatography with benzene–ethanol (9:1) and 2-butanone–pyridine (7:3). The O-methyl-nitrotyrosyl, O-acetylnitrotyrosyl-O-acetylhydroxy-leucyl derivatives of dihydrorufomycin A, IV and V, were obtained in crystalline form and characterized; IV was made by O-methylation of dihydrorufomycin A in 1,2-dimethoxyethane with excess ethereal diazomethane and V by acetylation with boiling acetic anhydride. The ultraviolet absorption spectrum of the O-methyl ether IV had λ_{max} 331 mμ compared with 355 mμ for dihydrorufomycin A. This peak disappeared completely in the spectrum of the diacetate V.

Electrolytic Cleavage of the 3-Nitrotyrosyl Peptide Bond.[5]—Efforts to increase selectivity in the nonenzymatic cleavage of tyrosyl peptide bonds[6a] have stimulated an exploration of techniques such as electrolytic oxidation: at a platinum anode, phloretic acid is converted to its dienone lactone in 20% yield.[6b] Under somewhat modified conditions, phloretylglycine is cleaved to the dienone lactone and glycine to the extent of 30–50%.

The electrolyses were carried out at 25° with catholyte and anolyte in separate compartments connected by an agar bridge. Initially spectral changes were studied with a simple model, N-acetyl-3-nitro-L-tyrosine, which was dissolved in 35% aqueous ethanol solution containing triethylammonium acetate as anolyte.

The changes of the ultraviolet absorption spectrum of the reaction mixture were observed each hour. The absorption at 280 and 360 mμ almost disappeared after 8 hr. of electrolysis (*cf.* Experimental). Freeze-drying of the reaction mixture failed to produce a product with the properties of the expected nitrodienonespirolactone X (R = CH_3), a molecule probably too reactive for isolation. The complete absence of a peak at >250 mμ during and after electrolysis is indication enough that IX (or X) in the process of formation must undergo secondary transformation and addition reactions too fast to be measurable.



VIII

IX

X

The electrolytic cleavage of N-benzoyl-3-nitrotyrosylalanine (VIII, R = C_6H_5, R′ = $CH(CH_3)$-COOH) was carried out in a more acidic electrolyte, *viz.*, triethylammonium acetate–trifluoroacetate (0.2 *M*, pH 2.2). The higher acidity of the medium was meant to protect the released amino acid from further oxidation.

In order to raise the oxidation efficiency of the anode, a cylindrical platinum electrode with a wide area (7.5 × 10 cm.) was used.

The electric current was 100–130 ma. at a potential of 40–50 v. The absorption maximum at 275 mμ came to a minimum after 5 hr. of electrolysis (Fig. 1 and 2). The cleavage yield, based on the trinitrophenylation method,[7] reached a maximum of 25% after 4 hr., followed by a decrease thereafter. Anodic destruction of the liberated alanine apparently competes with anodic cleavage.

(4) The group of Prof. Umezawa used sodium borohydride reduction of rufomycin A (ilamycin) to demonstrate the presence of δ-hydroxy-N-methyleucine in hydrolysates of dihydrorufomycin A: T. Takita, *J. Antibiotics* (A), **16**, 175 (1963).

(5) *Cf.* H. Iwasaki, L. A. Cohen, and B. Witkop, *J. Am. Chem. Soc.*, **85**, 3701 (1963).

(6) (a) J. G. Wilson and L. A. Cohen, *ibid.*, **85**, 564 (1963); (b) A. I. Scott, P. A. Dodson, F. McCapra, and M. B. Meyers, *ibid.*, **85**, 3702 (1963).

(7) K. Satake, T. Okuyama, M. Ohashi, and T. Shinoda, *J. Biochem.* (Tokyo), **47**, 654 (1960).

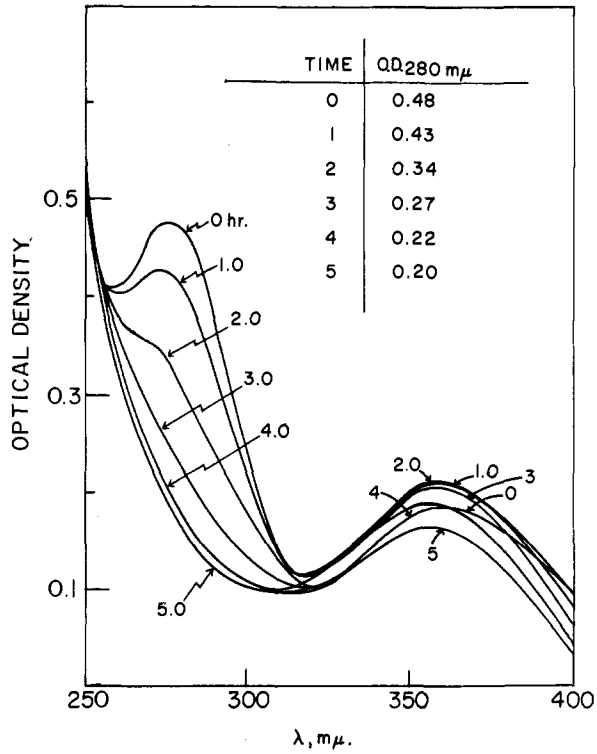


Fig. 1.—The changes in ultraviolet absorption spectrum of N-benzoyl-NO_2-Tyr → Ala following electrolysis.

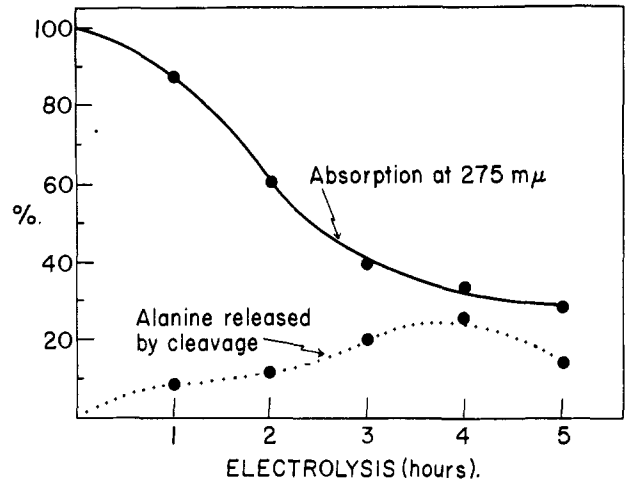


Fig. 2.—The release of alanine in the anodic cleavage of N-benzoyl-3-nitrotyrosylalanine as a function of decrease of absorption at 275 mμ.

This assumption was supported by the fact that an independent chromatographic assay for intact alanine indicated a yield after 4 hr. of only 13.5% of free alanine, a value obtained by quantitative chromatography of the DNP-alanine methyl ester.[8] The liberated alanine could be protected by the addition of acidic ion exchange resin. Thus, the addition of Dowex 50 resin (H^+ form) to the electrolysis mixture (10% acetic acid as electrolyte) raised the cleavage yield to 33% by removing the released alanine selectively from solution. The recovery yield of alanine according to gas chromatography of DNP-alanine methyl ester was 15.4% (Table I).

The oxidation and cleavage of N-benzoyl-3-nitrotyrosylalanine by N-bromosuccinimide (NBS) parallels the observation made during electrolysis. The phenol

(8) S. Ishii and B. Witkop, *J. Am. Chem. Soc.*, **85**, 1832 (1963).

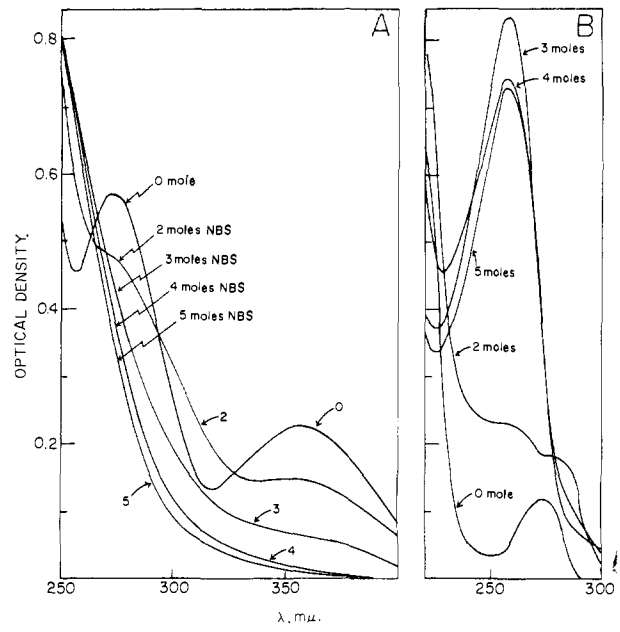


Fig. 3.—A: The disappearance of the absorption peaks of N-benzoyl-3-nitrotyrosylalanine methyl ester on oxidation with 2–5 moles of NBS. B: The maximal appearance of the dienenone band at 259 mμ on oxidation of phloretylglycine after oxidation with 3 moles of NBS.

chromophore as well as the C_6H_5–NO_2 peak (360 mμ) disappear completely after the addition of 5 moles of NBS (Fig. 3A). By contrast, phloretylglycine shows the rapid appearance of the high-extinction spirodienone lactone band at 259 mμ after the addition of 3 moles of NBS (Fig. 3B).

TABLE I
OXIDATIVE CLEAVAGE OF 3-NITROTYROSINE PEPTIDES

Method of cleavage	TNP assay of total NH_2-terminal, %
N-Benzoyl-3-nitrotyrosylalanine	
Anodic oxidation without ion-exchange resin	25.6
Anodic oxidation with ion-exchange resin	33.3
N-Bromosuccinimide	37.8
Rufomycin A	
Anodic oxidation with ion-exchange resin	38.0

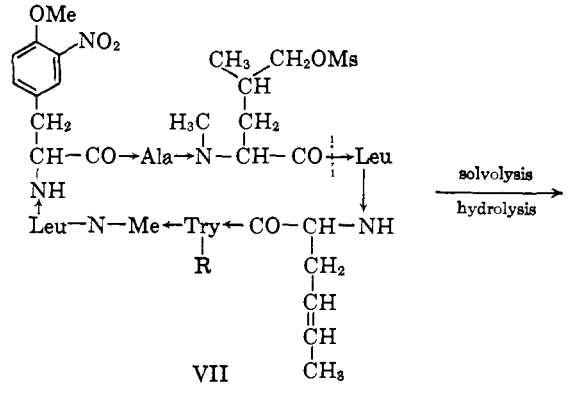
The yields of NH_2-terminal alanine (methyl ester) released from the nitrotyrosyl peptide as assayed by the trinitrophenylation method are in very good agreement.

Rufomycin A, purified by silica gel chromatography, was submitted to electrolysis under the same conditions. After 6 hr., the absorption at 280 mμ decreased to about half of the original value in agreement with the fact that rufomycin A contains both 1 mole of 3-nitrotyrosine and 1 mole of tryptophan (Fig. 4). The liberation of NH_2-terminal groups in the anodic oxidation of rufomycin A was 38% as assayed by trinitrophenylation.

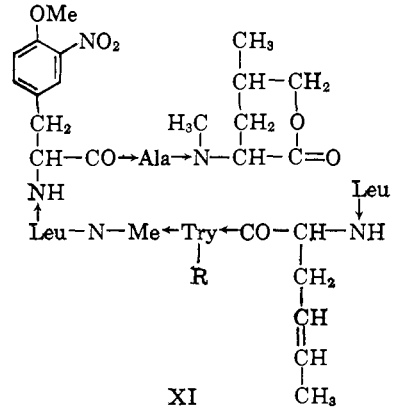
In control experiments, alanylleucine was submitted to electrolysis under the same conditions and rufomycin A was stirred for 5 hr. with Dowex 50 in 10% acetic acid containing ethanol. In neither case was there any release of leucine or alanine. In contrast to N-bromosuccinimide, which has been shown to cleave tryptophyl–peptide bonds in preference to those of

tyrosine,[9] electrolytic oxidation has no discernible effect on tryptophyl peptide bonds under the conditions cited above.

Solvolytic Cleavage of O-Methyldihydrorufomycin A O-Mesylate (VII).—The γ-hydroxyleucine group in dihydrorufomycin A would be expected to assist in any acid-catalyzed preferential cleavage of dihydrorufomycin.[10] The degree of participation of the neighboring peptide group and the selectivity of this cleavage should be much greater with an appropriate leaving group under conditions of solvolysis in a medium of low nucleophilic character. Initially *p*-nitrophenylsulfonation ("nisylation") was attempted with dihydrorufomycin A. Thin layer chromatography separated the O-nisyl product from the O,O-dinisyl compound in which the tyrosine hydroxyl had been sulfonated. Preliminary solvolysis experiments in 50% acetic acid clearly showed the liberation of leucine in a selective fashion. The next improvement was the use of O-methyldihydrorufomycin A (IV) which furnished a mono-O-nisyl derivative VI which, however, was too unstable for isolation and characterization. A homogeneous, easily crystallizable starting material for solvolysis was the O-mesyl derivative VII, which permitted the preparation of *seco*-dihydrorufomycin A lactone XI in good yield.



VII



XI

The solvolytic cleavage proceeds in two steps: a δ-iminolactone XIII is formed first in the presumably concerted intramolecular displacement pictured in XII. Although some spontaneous hydrolysis of this iminolactone occurs during solvolysis in a mixture of methyl ethyl ketone, acetic acid, and water, additional leucine is released by subsequent hydrolysis (XIII → XIV) in aqueous acid. Table II summarizes

(9) B. Witkop, *Advan. Protein Chem.*, **16**, 272 (1961).
(10) *Cf.* L. Zürn, *Ann.*, **631**, 56 (1960).

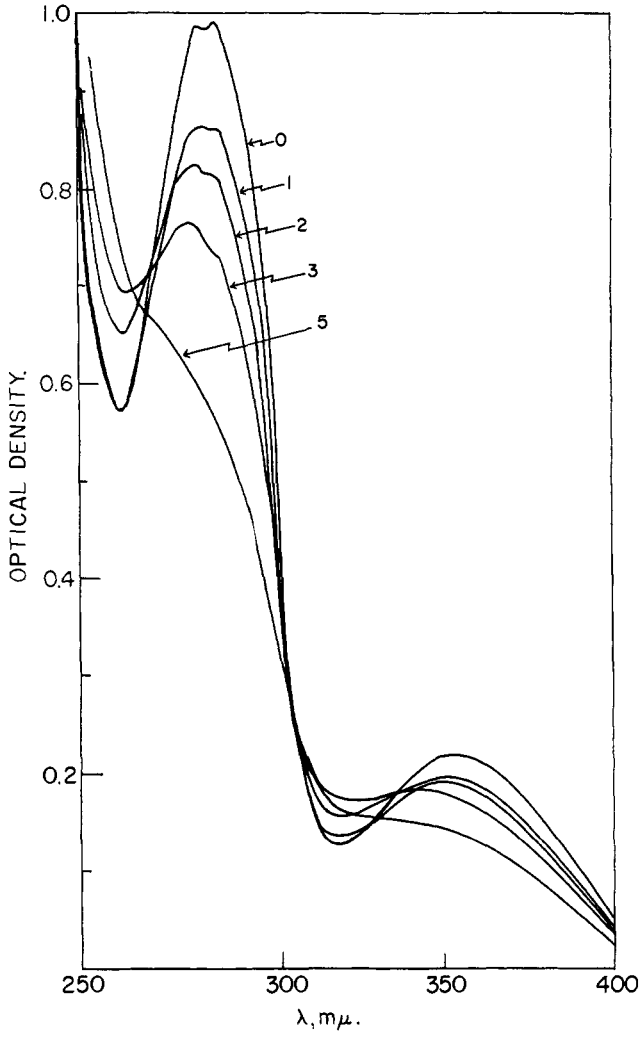
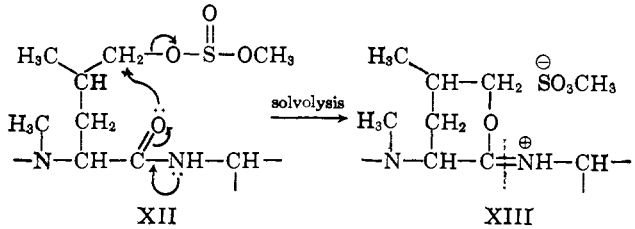
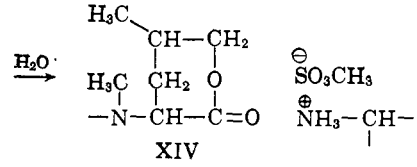


Fig. 4.—Changes in the ultraviolet spectrum of rufomycin A following electrolysis.

the conditions that led to the optimal conditions, *viz.*, methyl ethyl ketone as medium for solvolysis.



XII XIII



XIV

Considerable improvements in the yield of liberated NH_2-terminal leucine were achieved when 10% water and 5% acetic acid were added to the methyl ethyl ketone (Table III). The optimal time for solvolytic cleavage was 9–13 hr. (Table IV); 17 hr. of hydrolysis raised the release of NH_2-terminal from 53 (Table IV) to 57%.

Other higher boiling solvents either failed to reach the optimal cleavage yields achieved in methyl ethyl

TABLE II

INFLUENCE OF THE SOLVENT ON THE SOLVOLYTIC PEPTIDE CLEAVAGE AND RELEASE OF A NEW NH_2–TERMINAL IN O-METHYLDIHYDRORUFOMYCIN A

Solvent	Hydrolysis	% of NH_2 released
AcOH–AcONa·$3H_2O$	AcOH–1.0 *N* HCl (4:1), 20°	8.1
AcOH–AcOK (anhydr.)	AcOH–1.0 *N* HCl (4:1), 20°	4.6
AcOH–AcOAg	AcOH–1.0 *N* HCl (4:1), 20°	0
Me Et ketone (anhydr.)	AcOH–1.0 *N* HCl (4:1), 20°	32.2
Dimethyl sulfoxide (room temp.)	AcOH–1.0 *N* HCl (4:1), 20°	3.8
...	AcOH–1.0 *N* HCl (4:1), 20°	0.8
...	AcOH–H_2O (4:1), 20°	0.8
...	AcOH–H_2O–AcOK (reflux)	18.4

TABLE III

THE EFFECT OF WATER AND ACETIC ACID ON THE SOLVOLYTIC PEPTIDE CLEAVAGE IN ME ET KETONE. IN EACH EXPERIMENT HYDROLYSIS WAS ACHIEVED IN AcOH–1.0 *N* HCl FOR 17 HR. AT 20°

Time, hr.	Addition to methyl ethyl ketone	% of NH_2 released
5	None	18.4
	10% water	26.8
9	10% water	37.6
	10% water, 5% AcOH	50.0
15	None	18.4
	10% water	37.6
	10% water, 5% AcOH	42.0

TABLE IV

RELATIONSHIP BETWEEN TIME OF SOLVOLYSIS AND RELEASE OF NH_2–GROUP IN 10% WATER–ME ET KETONE. IN EACH CASE HYDROLYSIS WAS ACHIEVED IN HOAc–1.0 *N* HCl (4:1) FOR 10 HR.

Time of solvolysis, hr.	% of NH_2 released
5	26.8
7	31.4
9	37.9
13	53.0
17	41
24	52

TABLE V

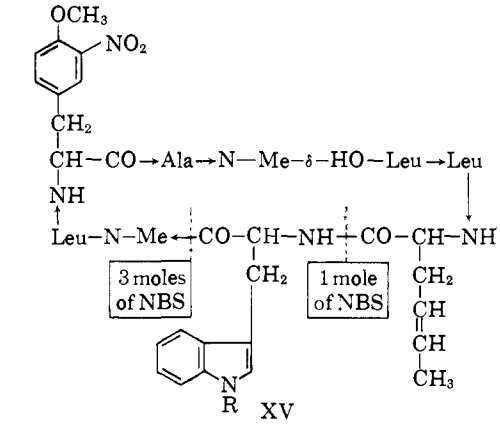
SOLVOLYTIC CLEAVAGE YIELDS (13 HR. REFLUX) IN OTHER SOLVENT SYSTEMS, FOLLOWED BY HYDROLYSIS AT 20° FOR 17 HR. IN ACETIC ACID–1.0 *N* HCl (4:1)

Solvent	% of NH_2 released
2-Pentanone (anhydr.)	22.2
2-Pentanone, 5% water	30.2
2-Pentanone, 5% water, 5% AcOH	50.5
Acetonitrile (anhydr.)	52.0
Acetonitrile, 10% water	43.8

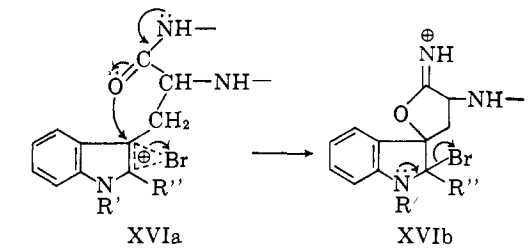
ketone or released additional NH_2–terminals; *e.g.*, that of tryptophan (Table V).

Seco-O-methyldihydrorufomycin A lactone (XI) was easily purified *via* its yellow dinitrophenylation product which permitted an easy separation from intact dihydrorufomycin A derivatives in which O-mesyl had been replaced by hydroxyl or acetoxyl in the course of solvolysis through intermolecular competition with the solvent. Purification of the DNP-secopeptide XIa by preparative thin layer and silica gel chromatography yielded a homogeneous product which furnished on hydrolysis DNP-leucine as the only NH_2–terminal amino acid.

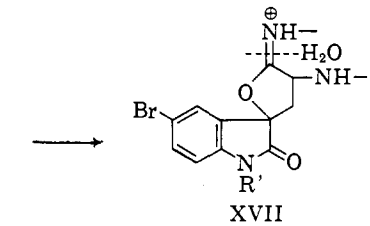
Oxidative Cleavages with N-Bromosuccinimide.—In the O-methylated dihydrorufomycin A (IV) the amino acids sensitive to NBS oxidation have been reduced to 2-amino-4-hexenoic acid and what was originally thought to be tryptophan. Cleavage of the peptide bonds adjacent to dehydronorleucine and "tryptophan" (XV) should liberate free 5-bromospirodioxindole lactone (*cf.* XXI) *via* the presumed intermediates XVIa, XVIb, and XVII.



XV

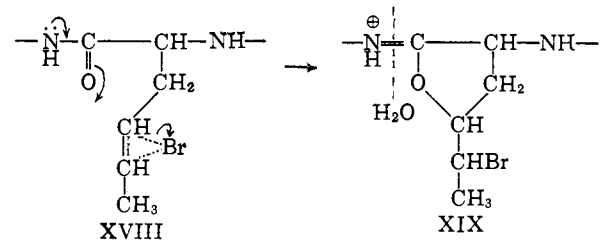


XVIa XVIb



XVII

A dehydronorleucine-peptide XVIII should undergo oxidative cleavage *via* the iminolactone XIX, by the same mechanism established for peptides of (methyl)-



XVIII XIX

allylglycine,[11] and for complete cleavage should require 1 mole of oxidant, whereas cleavage of the tryptophan peptide bond requires 3 moles of NBS.[12]

The action of NBS on O-methyldihydrorufomycin A was first studied at spectroscopic concentration. The changes in the ultraviolet spectrum after the addition of 0.5–5 moles of NBS were observed after a reaction

(11) N. Izumiya, J. E. Francis, A. V. Robertson, and B. Witkop, *J. Am. Chem. Soc.*, **84**, 1702 (1962).

(12) A. Patchornik, W. B. Lawson, E. Gross, and B. Witkop, *ibid.*, **82**, 5923 (1960).

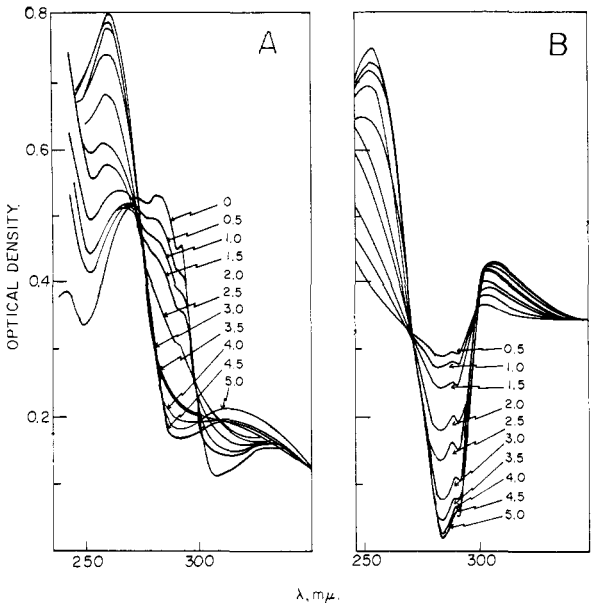


Fig. 5.—The changes in the ultraviolet spectrum of O-methyldihydrorufomycin A on oxidation and cleavage with NBS as observed directly (A) and as difference spectrum (B). The numbers express equivalents of NBS.

time of 20 min. at room temperature (Fig. 5A). In order to obtain a homogeneous reaction medium 80% acetic acid was used as solvent.

The difference spectrum is shown in Fig. 5B. The reference cell contained an equivalent amount of the starting material. Maximum decrease in the absorption at 285 mμ, observed after the consumption of 5 moles of NBS, was identical with the value calculated for one tryptophan.

The extent of cleavage of the peptide bond following 2-amino-4-hexenoic acid was followed by the trinitrophenylation method. Trinitrobenzenesulfonic acid is known to react only with primary amino groups. The secondary amino group of any liberated N-methylleucine following "tryptophan" would not be assayed by this reagent.

Neither could the liberation of NH_2-terminal N-methylleucine be determined after dinitrophenylation since the major part of DNP-N-methylleucine was destroyed in the process of hydrolysis.

The oxidative cleavage between 2-amino-4-hexenoic acid and "tryptophan" reached a maximum value of 45% after the consumption of 4 moles of NBS (Fig. 6). The decrease in optical density at 285 mμ parallels the release of NH_2-terminal "tryptophan" (Fig. 6); *i.e.*, 2-amino-4-hexenoic acid and "tryptophan" are oxidized more or less simultaneously by NBS.

N-Bromocarbamide, *i.e.*, NBS in concentrated urea solution, oxidizes tryptophan selectively without attacking tyrosine.[13] The same differentiation is shown in Fig. 6: maximal decrease of optical density at 285 mμ and negligible release of NH_2-terminal "tryptophan" in urea solution is observed with 3 moles of NBS. Therefore, N-bromocarbamide selectively oxidized and cleaved next to tryptophan, and not dehydronorleucine.

When the liberated spirodioxindole lactone XX was isolated and purified as the DNP-derivative XXa by repeated preparative thin layer chromatography it was

(13) M. Funatsu, N. M. Green, and B. Witkop, *J. Am. Chem. Soc.*, **86**, 1846 (1964).

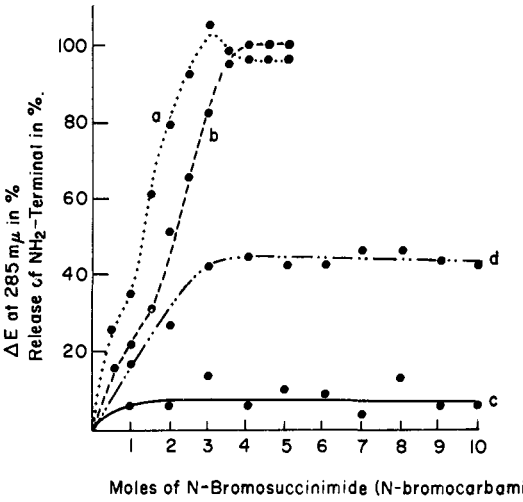
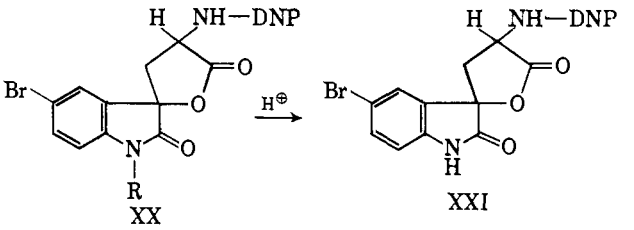


Fig. 6.—Oxidative cleavage of O-methyldihydrorufomycin A by NBS in water and in urea solution: a, ·●····●·, decrease in optical density at 285 mμ in urea; b, –●– – –●–, decrease in optical density at 285 mμ without urea; c, –●——●–, release of NH_2 group of "Try" (TNP-method) in urea; d, ··●——··●–, release of NH_2 group of "Try" without urea.

not identical with the lactone prepared from DNP-tryptophan XXI.[14] Its infrared absorption spectrum was definitely different although the peaks at 5.6 and 5.74 μ were identical and showed the presence of a γ-lactone and oxindole. Its ultraviolet absorption showed the same maximum at 344 mμ as XXI but only a shoulder at 245–260 mμ.



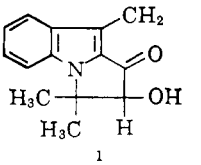
The yield of this substance was low, possibly because of some steric inhibition of 1,5-interaction in a cyclic peptide. In addition, the conditions for dinitrophenylation (pH 7.6) lead to 60% opening of the lactone.

The unknown lactone XXa was converted to the known lactone XXI by 2.0 *N* HCl at 100° in the course of 5 hr. This easy acid-catalyzed loss of a residue, stable to base, is indicative of a substituent at the indole nitrogen. The substituent, probably $C_5H_{11}O_3$,[15,15a] dehydrates to an allylic system prior to hydrolysis. The structure of the lactone XXa is now under investigation by mass spectrography.

(14) L. K. Ramachandran and B. Witkop, *ibid.*, **81**, 4028 (1959).

(15) T. Ueyanagi, M. Fujino, and T. Kamiya, personal communication.

(15a) NOTE ADDED IN PROOF.—The latest findings of the Takeda group support structure i for the substituted tryptophan in rufomycin A. If this expression is correct, the oxidation by NBS would involve both cleavage of the tryptophan C-peptide bond and the carbonyl α-substituent. The latter question is under investigation.



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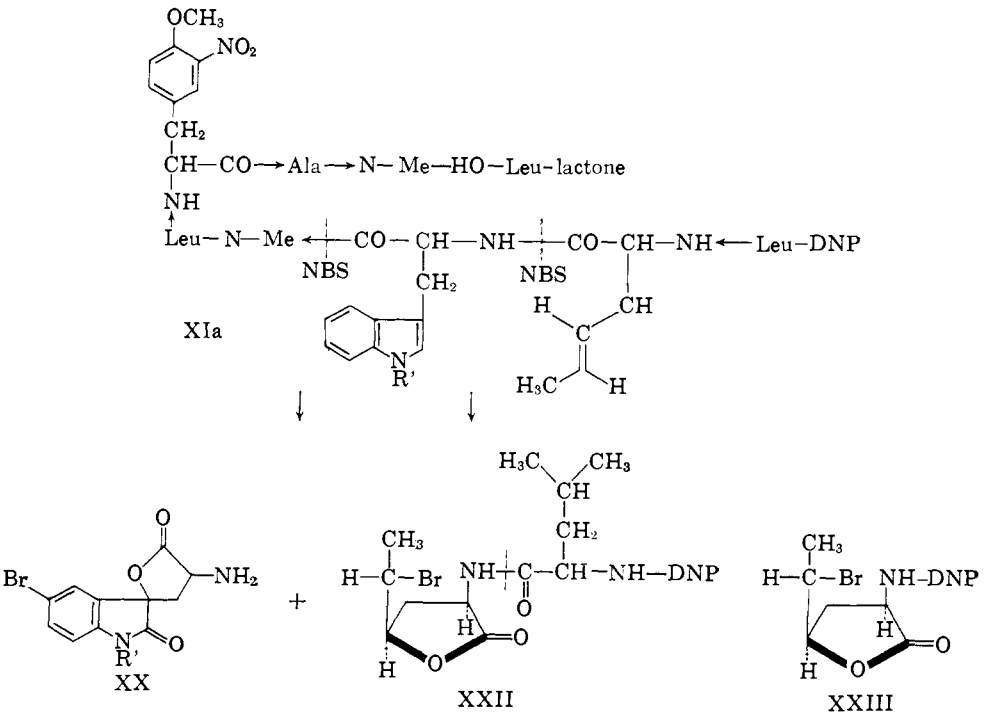

In addition to lactone XXa there were also isolated two further fragments, one, presumably the linear peptide N-MeLeu→Me-NO_2-Tyr→Ala→N-Me-δ-HO-Leu→Leu→δ-Br-δ-OH-norleucine lactone, the other the dinitrophenyl derivative of the tripeptide Leu-δ-Br-γ-HO-nor-Leu-"Try"-lactone.

Oxidative Cleavage of DNP-seco-O-methyldihydrorufomycin A by NBS.—The oxidative cleavage of DNP-seco-O-methyldihydrorufomycin A (XIa) was expected to yield the dipeptide DNP-leucyl-2-amino-4-hydroxy-5-bromohexenoic acid lactone (XXII) in addition to the dioxindole-spirolactone XX. The action of NBS on the DNP-secopeptide (XIa), was first studied at spectroscopic concentration. The changes of O.D. at 285 mμ were recorded after oxidation for 20 min. at room temperature in 80% acetic acid (Fig. 7A). Maximum decrease in the absorption at 285 mμ was again observed after consumption of 5 moles of NBS (Fig. 7B) and agreed with the value calculated for one tryptophan.

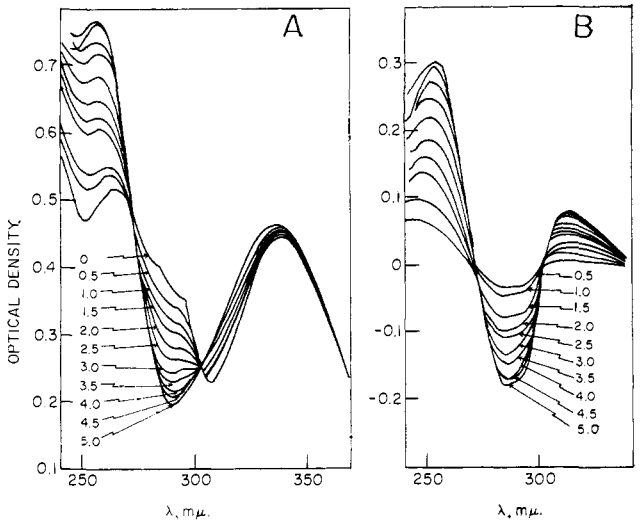

Fig. 7.—Changes in the ultraviolet absorption of DNP-*seco*-O-methyldihydrorufomycin A in the course of selective oxidation and cleavage by NBS as observed directly (A) and as difference spectrum (B). The numbers express equivalents of NBS.

The extent of cleavage between 2-amino-4-hexenoic acid and "tryptophan" was determined by trinitrophenylation (Fig. 8). Maximal cleavage amounted to 48% after consumption of 7 moles of NBS. The rate of liberation of new NH_2–terminal as a function of added NBS was slower than with the cyclopeptide (Fig. 6), but the final value was almost the same.

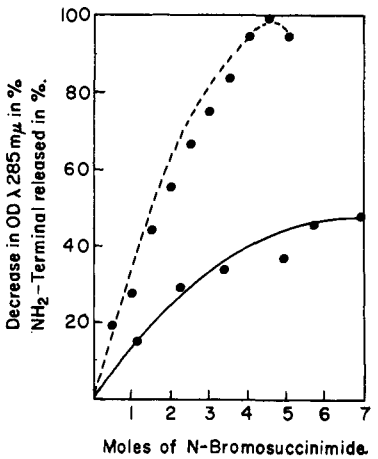

Fig. 8.—Oxidative cleavage of DNP-*seco*-O-methyldihydrorufomycin A: – – – –, decrease in optical density at 285 mμ; ———, release of NH_2 group of "Try" (TNP method).

DNP-leucyl-2-amino-4-hydroxy-5-bromohexanoic acid lactone, purified by repeated preparative thin layer chromatography, was finally isolated as a homogeneous compound. It was originally accompanied by three minor DNP derivatives which had all DNP-leucine as NH_2–terminal. The infrared absorption of this dipeptide at 5.58 and 5.93 μ indicated the presence of a γ-lactone and a peptide bond. By comparison, DNP-isoleucylglycine methyl ester showed peaks at 5.7 (methyl ester) and 5.93 μ (peptide). The ultraviolet spectrum showed λ_{max} 335 and 255 mμ

(ethanol solution). DNP-leucine was obtained as the only NH_2-terminal after hydrolysis with HCl–AcOH (1:1) at 105° for 18 hr. 2-Amino-4-hydroxy-5-bromohexanoic acid lactone, which remained in the acidic aqueous phase during the ether extraction of DNP-leucine, was then dinitrophenylated to XXIII. It was purified and characterized by thin layer chromatography in two different systems. The stereochemistry of XXII and XXIII, still a matter of conjecture, rests on the observation of preferential formation of *cis*-disubstituted bromolactones,[16] and the assumption of *trans* opening of the intermediate bromonium adduct to the *trans*-ethylene of Δ^4-norleucine to give the γ-lactone of a γ,δ-*threo*-bromohydrin.

Experimental

Gradient Elution Column Chromatography of Crude Rufomycin A.—A suspension of 470 g. of silicic acid (Bio-Rad Laboratories) in benzene containing 2% methanol was packed in a 3.5 × 110 cm. column. The mixing chamber (magnetic stirrer) contained 3 l. of 2% methanol–benzene. The volume of the solvent in the mixing chamber was kept constant by balancing outflow and inflow from a storage flask, which contained 7% methanol–benzene. A solution of 500 mg. of crude rufomycin A (lot No. 1525) in 10 ml. of 2% methanol–benzene was poured onto the column. Fractions of 10 ml. were collected. Each fraction was assayed for rufomycin by the characteristic ultraviolet absorption peak at 350 mμ. The 15 tubes corresponding to the theoretical peak were pooled and evaporated *in vacuo*. The two-dimensional thin layer chromatogram of III, the main component, showed a single spot.

Individual Reductions of Fractions I–V with Sodium Borohydride.—A small amount (2–5 mg.) of each fraction was treated with 10 mg. of sodium borohydride in methanol for 1 hr. at room temperature. The solvent was removed by evaporation *in vacuo* and the residue was dissolved in 10 ml. of ethyl acetate, then washed several times with water. The ethyl acetate solution was concentrated and submitted to thin layer chromatography in benzene–ethanol (9:1).

Dihydrorufomycin A (III).—A solution of 3 g. of crude rufomycin A in 30 ml. of methanol was reduced by the slow addition of 0.5 g. of sodium borohydride (*ca.* 20 mole equiv.). After complete addition the reaction mixture was stirred for 2 hr. at room temperature, then evaporated *in vacuo*. To the residue was added 200 ml. of ethyl acetate and 50 ml. of 1.0 *N* HCl. The ethyl acetate layer was washed several times with dilute HCl and water, and dried over anhydrous magnesium sulfate. The solvent was evaporated *in vacuo* and the residue, 3.2 g., was dissolved in 100 ml. of benzene–ethanol (9:1) and poured on a silica gel column (4.0 × 35 cm.). Benzene–ethanol (9:1) was used as the developing solvent. Two minor bands and one major yellow band were visible after development. The major band was eluted last and this fraction evaporated *in vacuo*. The residue, 2 g., was recrystallized three times from ethanol, from which dihydrorufomycin A appeared as yellow needles, 1.2 g., m.p. 165–170°, $[\alpha]^{25}D$ −69.1 ± 1.0° (methanol).

Anal. Calcd. for $C_{54}H_{79}N_9O_{14}$: C, 60.15; H, 7.39; N, 11.69; NCH_3, 2.79. Found: C, 60.54; H, 7.53; N, 11.66; NCH_3, 2.07; (OCH_3, 0.22).

O-Methyldihydrorufomycin A (IV).—A solution of 200 mg. of dihydrorufomycin A in 20 ml. of 1,2-dimethoxyethane was methylated with excess diazomethane (10 mmoles) in ether (15 ml.). The reaction mixture was allowed to stand for 2.5 hr. at room temperature. The solvent was removed by evaporation *in vacuo* and the residue was recrystallized three times from ethanol. The O-methyl ether IV crystallized as almost colorless needles, 100 mg., m.p. 159–165°.

Anal. Calcd. for $C_{55}H_{81}N_9O_{14}$: C, 60.47; H, 7.48; N, 11.54; OCH_3, 2.84. Found: C, 60.67; H, 7.79; N, 10.90; OCH_3, 2.96.

O,O-Diacetyldihydrorufomycin A (V).—A solution of 200 mg. of dihydrorufomycin A in 10 ml. of acetic anhydride was refluxed for 20 min. The reaction mixture was evaporated *in vacuo* and the residue was dissolved in ethyl acetate. The ethyl acetate solution was washed several times with dilute bicarbonate solution and dried over anhydrous magnesium sulfate. The solvent was evaporated *in vacuo* and the residue recrystallized three times from ethanol. The diacetate V formed colorless needles, 120 mg., m.p. 146–150°.

Anal. Calcd. for $C_{58}H_{83}N_9O_{16}$: C, 59.93; H, 7.20; N, 10.85; CH_3CO, 7.41. Found: C, 60.24; H, 7.22; N, 10.87; CH_3CO, 7.61.

N-Benzoyl-3-nitro-L-tyrosine.—The sodium salt of 2.5 g. (11 mmoles) of 3-nitro-L-tyrosine (Cyclo Chemical Co.) was prepared in 50 ml. of water by the addition of 500 mg. of sodium hydroxide (12 mmoles). To this ice-cold solution was added, alternately for 30 min., portions of 1.5 g. (37.5 mmoles) of sodium hydroxide and of 1.3 ml. of benzoyl chloride (11 mmoles). The mixture was kept alkaline and at 0 to 5°. After stirring for an additional 30 min., the reaction mixture was acidified with dilute HCl and extracted three times with ethyl acetate. The ethyl acetate solution was washed with water, dried over sodium sulfate, and evaporated *in vacuo*. The residue was dissolved in a small amount of ethanol and stored in the refrigerator overnight. The yellow needles (3.2 g., m.p. 182–182.5°) were collected. Three recrystallizations from ethanol raised the m.p. to 187.5–188°.

Anal. Calcd. for $C_{16}H_{14}N_2O_6$: C, 58.17; H, 4.27; N, 8.43. Found: C, 58.25; H, 4.34; N, 8.29.

N-Acetyl-3-nitro-L-tyrosine.—The N-acetyl derivative was prepared in analogy to the N-benzoyl derivative. After recrystallization from acetone–hexane there were obtained yellow needles, m.p. 182.5°.

Anal. Calcd. for $C_{11}H_{12}N_2O_6$: C, 49.25; H, 4.51. Found: C, 49.47; H, 4.40.

N-Benzoyl-3-nitro-L-tyrosylalanine Methyl Ester.—To a solution of 140 mg. (1 mmole) of alanine methyl ester hydrochloride and 0.14 ml. (1 mmole) of triethylamine in 6 ml. of methylene chloride was added 330 mg. (1 mmole) of N-benzoyl-3-nitro-L-tyrosine and 207 mg. (1 mmole) of N,N'-dicyclohexylcarbodiimide. The mixture was allowed to stand for 5 hr. at room temperature. N,N'-Dicyclohexylurea formed immediately, precipitated, and was removed by filtration. The filtrate was washed with hydrochloric acid, bicarbonate, and water. The methylene chloride solution was dried over anhydrous sodium sulfate and the solvent was removed *in vacuo* at room temperature. The residue was crystallized and recrystallized from acetone–hexane to yield 213.6 mg. of yellow crystals, m.p. 172–173°.

Anal. Calcd. for $C_{20}H_{21}N_3O_7$: C, 57.82; H, 5.10. Found: C, 57.83; H, 5.25.

The dipeptide prepared by hydrolysis with 2 equiv. of aqueous methanolic sodium hydroxide was crystallized and recrystallized three times from methanol–water to yield 105 mg. of yellow crystals, m.p. 192–193°.

The Cleavage of Tyrosyl-Peptide Bonds by Anodic Oxidation. Acrylamide Gel Bridge.—As a conducting bridge between anode cell and cathode cell, an agar gel filled U-type glass tube has commonly been used. However, at higher current density, the agar gel tends to warm up and forms cracks. By contrast it was found that an acrylamide gel bridge is resistant to cracking by heat.

Acrylamide gel was made by mixing "Cyanogum 41," dimethylaminopropionitrile (DMAPN), and ammonium persulfate[17] at room temperature. A difficulty in the preparation of the gel was the failure of the solution to harden at and close to the surface. Therefore, the following procedure was adopted: the U-tube was extended on each side by glass tubes of the same diameter, 2–3 cm. long, with the help of rubber tubing. The mixture of "Cyanogum 41" (2.5 g. in 5 ml. of water), DMAPN (0.5 ml., 10%), ammonium persulfate (0.5 ml., 10%), and electrolyte (25 ml. of 1.0 *M* in water) was poured in this tube and allowed to harden overnight. After removal of the rubber tubing the extension tubes were cut off with a razor blade and both ends covered with gauze.

This acrylamide gel bridge worked well without softening or cracking at high current density. However, when the reaction mixture required assay by ninhydrin or trinitrophenylation, the bridge could not be used, because the components of the gel seeped into the anolyte and reacted with these reagents. In this case it was not possible to obtain reliable control values. Therefore, an ordinary agar bridge was used with dialysis membrane covering both ends of the U-tube. This protected the gel from cracking to some extent by holding the agar in place.

(16) N. Izumiya and B. Witkop, *J. Am. Chem. Soc.*, **85**, 1835 (1963); *cf.* M. F. Ansell and M. H. Palmer, *Quart. Rev.* (London), **18**, 212 (1964).

(17) S. Raymond and Y. Wang, *Anal. Biochem.*, **1**, 391 (1960).

Anodic Oxidation of N-Acetyl-3-nitro-L-tyrosine.—To a solution of 100 mg. of N-acetyl-3-nitro-L-tyrosine in 70 ml. of ethanol was added 130 ml. of 0.1 *M* triethylammonium acetate (pH 5.2). This solution was put into the anode compartment. The catholyte was 200 ml. of 0.1 *M* triethylammonium acetate. The bridge was an inverted U-type glass tube filled with acrylamide gel or with gel made from 1% agar in 0.1 *M* triethylammonium acetate. Two pieces of platinum sheet (3.0 × 7.5 cm.) were used as electrodes. The solution in both cells was stirred magnetically and cooled with water during electrolysis. The electric current was 35–37 ma. at a potential of 50 v. The changes at 280 mμ in the ultraviolet spectrum were recorded each hour as

Time	0	1	2	3	4	5	8
$O.D._{280\ m\mu}$	0.34	0.31	0.26	0.21	0.17	0.15	0.08

The reaction mixture after 8 hr. of electrolysis was lyophilized to yield a dark brown oil. Attempted purification of the labile nitrodienone or its transformation products by chromatography on Florisil was not successful.

Electrolytic Cleavage of N-Benzoyl-3-nitrotyrosylalanine.—The buffer system, 0.4 *M* triethylammonium acetate–trifluoroacetate, was made up as follows: to the mixture of 27.8 ml. of distilled triethylamine, 11.4 ml. of acetic acid, and 15 ml. of trifluoroacetic acid, water was added to make a total volume of 500 ml. (pH 2.2). The anolyte contained an aliquot of a solution of 22 mg. of N-benzoyl-3-nitrotyrosylalanine (0.05 mmole) in 5 ml. of ethanol, 50 ml. of 0.4 *M* triethylammonium acetate buffer, and 45 ml. of water (pH 2.2). The catholyte contained 5 ml. of ethanol, 50 ml. of 0.4 *M* triethylammonium acetate buffer, and 45 ml. of water. In the following experiments a cylindrical platinum electrode (7.5 × 10 cm.) was used as anode and a platinum sheet (3.0 × 7.5 cm.) as cathode. The bridge was filled with gel made from 1% agar in the same buffer solution as the cathode. Both ends of the U-tube were covered with dialysis membranes. Both catholyte and anolyte were stirred and cooled during electrolysis. During the 5 hr. of electrolysis the current varied from 50–40 v. and 100–180 ma. Every hour 0.5 ml. of the reaction mixture was withdrawn, neutralized with 0.22 ml. of 4% bicarbonate, diluted with 4.5 ml. of water (pH 5.5), and the change of ultraviolet absorption recorded (see below). The alanine released in the course of electrolysis was examined by silica gel thin layer chromatography (*sec*-butyl alcohol–formic acid–water, 75:15:10) and electrophoresis (pH 1.9 and 3.6, acetic acid–pyridine buffer).

Assay of Liberated Alanine by Trinitrophenylation.—After each hour of electrolysis an aliquot of 2 ml. of the anolyte after extraction with ethyl acetate was trinitrophenylated. From the absorption at 340 mμ, the total cleavage after each hour was determined.

Time, hours	0	1	2	3	4	5
Absorption at 275 mμ, %	100	87.4	60.7	39.2	33.9	28.5
Cleavage yield, %	0	8.8	12.0	20.0	25.6	14.8

Assay of Liberated Alanine by V.p.c. of DNP Derivative.—To the reaction mixture was added a known amount of isoleucine as internal standard[8]; the mixture was then dinitrophenylated, and methylated with diazomethane. The DNP-alanine methyl ester containing DNP-isoleucine methyl ester was assayed by gas chromatography. From the comparison of the areas of the peaks between alanine and isoleucine, the quantity of alanine was calculated (Table II).

Protection of Liberated Alanine by Dowex 50 Resin.—Electrolysis of 21 mg. of N-benzoyl-3-nitrotyrosylalanine, dissolved in 5 ml. of ethanol, was repeated as described. This time the anolyte contained 10% acetic acid instead of triethylammonium acetate buffer and 1.5 ml. of Dowex 50W X8 (H^+ form, 50–100 mesh). The catholyte contained 5 ml. of ethanol, 50 ml. of 20% acetic acid, and 45 ml. of water. In the course of 5 hr. the current stayed at 100 v. and sank from 45 to 36 ma. After 5 hr. of electrolysis, the resin was separated by filtration and washed with ethanol and water. The absorbed alanine was eluted with 6% ammonia. The eluate and washings were evaporated *in vacuo*. By electrophoresis and thin layer chromatography, alanine was identified with an authentic sample.

Total alanine liberated in solution and in the resin, according to the absorption at 340 mμ of the TNP derivative, was 33.3%, a figure which probably comprises not only alanine but also degradation products such as ammonia (which this time was not present in the buffer system). The intact alanine liberated was 15.4% according to gas chromatography of the DNP-alanine methyl ester.

Oxidation and Cleavage of N-Benzoyl-3-nitrotyrosylalanine Methyl Ester by NBS.—The solution of 41.5 mg. of N-benzoyl-nitrotyrosylalanine methyl ester (0.1 mmole) in a mixture of 5 ml. of acetic acid and 5 ml. of water was divided into 5 aliquots of 2 ml. each, to which was added, respectively, 7.2 (2 mole equiv.), 10.7 (3 mole equiv.), 14.3 (4 mole equiv.), and 17.8 mg. (5 mole equiv.) of N-bromosuccinimide. For comparison, 22.3 mg. of phloretylglycine (0.1 mmole) was treated in the same way. Samples of 0.1 ml. of each aliquot were diluted with 10 ml. of water and the changes in ultraviolet absorption were observed. The results are presented in Fig. 3A and B.

Each aliquot, after the addition of 2 ml. of water, was washed twice with 2 ml. of ethyl acetate. Quantitative analysis by gas chromatography was not possible because two unknown peaks appeared as shoulders next to the peaks of alanine and isoleucine (internal standard).

Quantitative Analysis by Trinitrophenylation.—The other half of the respective aliquot was diluted with water to 5 ml. out of which 0.5 ml. (1 mole equiv.) was trinitrophenylated.[7] From the absorption at 340 mμ, the amount of released alanine methyl ester was calculated as

NBS, moles	0	2	3	4	5
Alanine methyl ester, %	0	14.1	29.6	37.8	35.2

By comparison, the amount of glycine released by NBS oxidation of phloretylglycine was:

NBS, moles	0	2	3	4	5
Glycine, %	0	19.6	88	88	77.7

The amount of NH_2-terminal released by NBS oxidation of the nitrotyrosine peptide corresponds very closely with the amount released by electrolysis.

Electrolysis of Rufomycin A.—The anolyte contained an aliquot of a solution of 20 mg. of pure rufomycin A in 40 ml. of ethanol, 55 ml. of 20% acetic acid, 15 ml. of water, and 2 ml. of Dowex 50W X4 (H^+ form, 200–400 mesh). The final pH of the solution was 2.4. The catholyte cell was an aliquot of the mixture of 40 ml. of ethanol, 55 ml. of 20% acetic acid, and 15 ml. of water (pH 2.4). The bridge was filled with gel made from 1 g. of agar, 5 g. of sodium acetate, and 50 ml. of water. The voltage was kept constant at 100 v.; the electric current dropped from 90 to 32 ma. in the course of 6 hr. Each hour λ_{max} 280 mμ was measured on an aliquot of 1.5 ml. of the reaction mixture diluted with 1.5 ml. of 50% ethanol. After 6 hr., the absorption at 280 mμ had decreased to about half the original value. After the reaction, the pH of the anolyte was 2.3, that of the catholyte 3.8. The reaction mixture was filtered and the ion-exchange resin washed with 50% ethanol–water. The absorbed substance on the resin was eluted with 3% ammonia in 50% ethanol–water (20 ml.).

The eluate was evaporated *in vacuo* and dried to yield 8 mg. of residue. The filtrate of the reaction mixture and the washings were combined and evaporated *in vacuo* to yield 36 mg.

The residues were dissolved, separately in 1 ml. of 50% ethanol, and 50 λ of them was used for quantitative analysis (the rest of the solution was used for dinitrophenylation). To 50 λ of the sample solution was added 1 ml. of 2-methoxyethanol and 1 ml. of 0.1% trinitrobenzenesulfonic acid (TNBS) in 0.1 *M* phosphate buffer (pH 7.66). After 3 hr. at 40° 1 ml. of 1.0 *N* HCl in 2-methoxyethanol was added. Two blank solutions were treated similarly; in the one, the sample solution was replaced with the same volume of 50% ethanol, and in the other the 0.1 *M* phosphate buffer did not contain TNBS. The ultraviolet absorption between 300 and 400 mμ was recorded and the absorption of the two blank solutions subtracted. The eluted substance from the resin showed maximum optical density of 0.88 at 335 mμ, and the substance from the filtrate 0.4 at 330 mμ. The optical density of a TNP-peptide in 1 μmole/ml. is about 11 according to Satake, *et al.*[7] The amount of NH_2-terminal present in the eluted substance from the resin and in the substance from the filtrate was calculated as 0.48 and 0.22 μmole, respectively. Accordingly, the molar ratio of NH_2-terminal to rufomycin A was 38%.

The rest of the ethanol solution (0.9 ml., each) of the eluate and filtrate from the resin was each divided into two equal volumes, respectively. To one of them was added 0.5 μmole of valine as an internal standard for subsequent dinitrophenylation with 1 ml. of 5% fluorodinitrobenzene (FDNB) and 0.5 ml. of 2% triethylamine. The mixture was stirred at room temperature

for 3 hr. and evaporated *in vacuo* to dryness. Dinitrophenol was removed by sublimation. The residue was dissolved in 2 ml. of hydrochloric acid–acetic acid (1:1) and hydrolyzed at 105° for 14 hr. in a sealed tube. The hydrolysate was diluted with three volumes of water and extracted with ether. The ether solution was washed three times with aqueous bicarbonate and, after acidification, the DNP derivatives were extracted three times with ether from the aqueous solution. The ether solution was dried over anhydrous magnesium sulfate and evaporated *in vacuo*. The residue was analyzed by thin layer chromatography in $CHCl_3$–MeOH–AcOH (95:5:1). The presence of DNP-alanine identical with an authentic sample was confirmed. In addition, a small amount of DNP-leucine was also present.

O-Methyldihydrorufomycin A O-Mesylate (VII).—To a solution of 500 mg. of O-methyldihydrorufomycin A (IV) in 20 ml. of pyridine (stirring, ice-cooling) was added 3.5 ml. of methanesulfonyl chloride in 35 ml. of pyridine. After 1 hr. at 0–5°, the reaction mixture was stirred at room temperature for an additional 5 hr., concentrated to about 5 ml. by evaporation *in vacuo*, and extracted with 150 ml. of ethyl acetate and 20 ml. of water. The ethyl acetate extract was washed several times with 1.0 *N* HCl and water, dried over anhydrous magnesium sulfate, evaporated *in vacuo*, and the residue crystallized from ethanol. Recrystallization from ethanol furnished 300 mg. of colorless crystals of VII, m.p. 155–157°.

Anal. Calcd. for $C_{56}H_{83}N_9O_{16}S$: C, 57.47; H, 7.15; N, 10.77; S, 2.74. Found: C, 58.03; H, 7.13; N, 10.55; 10.67; S, 2.47.

The homogeneity of VII was confirmed by thin layer chromatograms in two different solvent systems (benzene–EtOH, 9:1; benzene–EtOH–AcOH, 80:15:5).

Optimal Conditions for Solvolysis and Hydrolysis of the Mesylate VII.—A solution of 3.0 mg. of the mesylate VII in methyl ethyl ketone–acetic acid–water (85:5:10) was refluxed for 13 hr. The reaction mixture was evaporated *in vacuo* and the residue was distributed between 10 ml. of ethyl acetate and 5 ml. of water. The mixture was shaken well and the ethyl acetate layer was washed several times with water, dried over anhydrous magnesium sulfate, evaporated *in vacuo*, and the residue dissolved in 4 ml. of AcOH–1.0 *N* HCl (4:1). The mixture was allowed to stand for 17 hr. for hydrolysis. The solvent was removed by evaporation *in vacuo*. Half of the residue was used for the qualitative determination of the NH_2-terminal. It was dinitrophenylated with a 5% solution of FDNB in dimethoxyethane and 2% triethylamine, then hydrolyzed with HCl–AcOH (1:1) for 17 hr. at 105°. The selective release of DNP-leucine was confirmed by thin layer chromatography.

The other half was used for the quantitative assay of free NH_2-terminals released by solvolysis and hydrolysis. The cleavage yield was 57% as determined by the trinitrophenylation method. A control experiment, in which the O-mesylate VII was not solvolyzed but only treated with AcOH–1.0 *N* HCl (4:1) for 17 hr. at room temperature, showed the liberation of only 0.8% of NH_2-terminal.

Seco-O-methyldihydrorufomycin A Lactone (XI).—A solution of 420 mg. of the O-mesylate VII in 40 ml. of methyl ethyl ketone–water–acetic acid (85:10:5) was refluxed for 13 hr. Methyl ethyl ketone was evaporated *in vacuo* and the residue distributed between 100 ml. of ethyl acetate and 20 ml. of water. The ethyl acetate layer was washed three times with water, dried over anhydrous magnesium sulfate, and evaporated *in vacuo* to yield 340 mg. of a tan powder. This material was dissolved in 100 ml. of acetic acid–1.0 *N* hydrochloric acid (4:1) and allowed to stand for 17 hr. at room temperature. The solvent was removed by evaporation *in vacuo*, the residue dissolved in 10 ml. of ethanol, and the solution evaporated *in vacuo*. This process was repeated in order to remove completely acetic acid and hydrogen chloride. The residue, 335 mg., according to assay by trinitrophenylation, contained 52.7% of secopeptide XI. The infrared absorption at 5.8 μ showed the presence of a six-membered lactone band. This residue was dissolved in 25 ml. of dimethoxyethane, and 9 ml. of a solution of 5% DNFB in dimethoxyethane and 10 ml. of a 10% triethylamine solution was added. The mixture was allowed to stand at room temperature for 16 hr. The solvent was removed by evaporation *in vacuo* and the residue washed twice with ether and dissolved in 100 ml. of ethyl acetate. This solution was washed twice with acid and water, and dried over anhydrous magnesium sulfate. The solvent was removed by evaporation *in vacuo*. From the residue (650 mg.) dinitrophenol and dinitrofluorobenzene were removed by sublimation *in vacuo*. The crude DNP-secopeptide XIa was dissolved in 10 ml. of benzene–ethyl acetate–acetic acid (9:3:1) and purified on a silica gel column (3.5 × 36 cm.). First dinitrophenol was eluted with 600 ml. of benzene–ethyl acetate–acidic acid (9:3:1), then the DNP-seco derivative XIa with benzene–ethanol–acidic acid (80:15:5). It still contained minor impurities according to thin layer chromatography. It was further purified by silica gel chromatography and preparative thin layer chromatography as follows. For the second silica gel chromatography (2.5 × 33 cm.) chloroform–methanol–acetic acid (95:5:1) was used for development and elution. The main fraction was collected and evaporated *in vacuo*. The residue was submitted to preparative thin layer chromatography (20 × 20 cm., 1 mm. thick). From the main yellow band, the DNP-secopeptide XIa, 70 mg. of a yellow powder, was eluted with ethanol; infrared spectrum 5.77 (lactone), 5.9–6.1 (–CONH–), 6.7, 7.5 (DNP–), 10.3 (*trans* F) μ; ultraviolet spectrum (ethanol solution) 340 (DNP-peptide), 280–295 mμ shoulder (Try).

Anal. Calcd. for $C_{61}H_{83}N_{11}O_{18}$: C, 58.22; H, 6.65; N, 12.25. Found: C, 58.20; H, 6.49; N, 11.89.

About 1 mg. of XIa was hydrolyzed with HCl–HAc (1:1) at 105° for 17 hr. in a sealed tube. The reaction mixture was diluted with water, then extracted with ether. The ether-soluble substance was DNP-leucine as confirmed by thin layer chromatography.

The homogeneity of XIa was checked by two thin layer chromatograms (chloroform–methanol–acetic acid, 95:5:1; and benzene–ethanol–acetic acid, 80:15:5)

Oxidative Cleavage of O-Methyldihydrorufomycin A with N-Bromosuccinimide. NBS Titration of Tryptophan.—The solution of 3.5 mg. of O-methyldihydrorufomycin A in 1.2 ml. of 80% acetic acid was divided into 11 aliquots of 0.1 ml. each (*i.e.*, 0.3 μmole of IV). The standard solution of oxidant contained 5.4 mg. of freshly recrystallized NBS in 10 ml. of 80% acetic acid. Each of the 11 aliquots was allowed to react, respectively, with the following volumes of NBS solution: 0.0 (blank), 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50 ml., corresponding to the addition of 0 to 5.0 equiv. of NBS. After 20 min., each was brought up to a volume of 0.6 ml. by the addition of 80% acetic acid, and to a final volume of 4 ml. by the addition of ethanol. The changes in the extinction at 285 mμ were recorded with this final solution (Fig. 5, 6). From the difference spectrum, the maximum decrease of O.D. at 285 mμ was determined as 0.32. The value calculated for one unit of tryptophan is (0.3/4) × (5.5/1.31) = 0.315.

Correlation between NBS Oxidation and Release of New NH_2-terminal "Tryptophan."—The solution of 11.7 mg. (12 μmoles) of O-methyldihydrorufomycin A in 3.0 ml. of 80% acetic acid was divided into 11 aliquots of 0.25 ml. (1 μmole). The oxidant solution was 17.8 mg. (100 μmoles) of NBS (freshly recrystallized) in 10 ml. of 80% acetic acid. Each aliquot was treated, respectively, with the following volumes of the NBS solution: 0.0 (blank), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 ml., corresponding to the addition of 0.0 to 10 equiv. of NBS. For the determination of ammonia, possibly released in the course of degradation of NBS, the same procedure was applied to a control experiment with 80% acetic acid without the antibiotic IV.

After 20 min., 0.1 ml. of formic acid was added to the reaction mixture in order to destroy excess NBS. For the completion of hydrolysis 1/4 volume of 1.0 *N* HCl was added and the mixture was allowed to stand for 12 hr. at room temperature. Each aliquot was divided into two halves and evaporated *in vacuo*. Trinitrobenzenesulfonic acid reagent was added to one-half; the other half served as blank. The amount of released NH_2-terminal was calculated as described in connection with the electrolytic cleavage. The results are presented in Fig. 6. The amount of ammonia released by the degradation of NBS itself was less than 3% in all samples.

Oxidation of O-Methyldihydrorufomycin by Bromocarbamide (NBS in Urea Solution).—The determination of the changes in the ultraviolet spectrum and the quantitative assay of released NH_2-terminal were carried out as described in the preceding experiment with the sole difference that O-methyldihydrorufomycin was dissolved in 8.0 *M* urea in 80% acetic acid (instead of 80% acetic acid). Since the presence of large amounts of urea interferes with the trinitrophenylation assay, the residue obtained by evaporation of each aliquot was dissolved in 5 ml. of ethyl acetate and washed twice with 1 ml. of water to remove urea. The ethyl acetate was evaporated *in vacuo* and the residue subjected to assay by the trinitrophenylation method. The results are presented in Fig. 6.

Isolation of Lactone XXa.—To a solution of 194.8 mg. (200 μmoles) of O-methyldihydrorufomycin A in 6 ml. of 80% acetic acid was added the solution of 250 mg. of freshly recrystallized NBS in 6 ml. of 80% acetic acid. After 20 min. at room temperature 0.5 ml. of formic acid was added in order to decompose excess NBS. The solution was evaporated *in vacuo*. The residue was dissolved in 10 ml. of 1.0 *N* HCl–AcOH (1:4) and allowed to stand for 12 hr. at room temperature in order to permit complete hydrolysis of the intermediate iminolactone XVII. This additional hydrolytic step increased the yield of released NH_2–terminal "tryptophan" by 11–13% without affecting other peptide bonds. The reaction mixture was evaporated *in vacuo* and the residue dinitrophenylated with 6 ml. of a 5% solution of DNFB in dimethoxyethane, 3 ml. of 0.2 *M* phosphate buffer (pH 7.66), and 200 mg. of bicarbonate for 5 hr. at room temperature. The solvent was removed by evaporation *in vacuo* and the residue was dissolved in ethyl acetate and washed several times with dilute acid, then dried over anhydrous magnesium sulfate. Ethyl acetate was evaporated *in vacuo* and the residue was submitted to sublimation to remove dinitrophenol and DNFB as much as possible. The residue (*ca.* 500 mg.) was purified by thin layer chromatography on silica gel plates (20 × 20 cm., 1 mm. thick) and developed with benzene–ethyl acetate–acetic acid (8:2:1) to yield fractions I–V as yellow bands of varying width. Each yellow band was extracted with ethanol and the solvent was removed by evaporation *in vacuo*. Infrared and ultraviolet spectra helped to identify the fractions: I, 34 mg., was dinitrophenol, identified by spectrum and thin layer chromatography; II, III, IV, and V were again submitted to preparative thin layer chromatography in chloroform–methanol–acetic acid (95:5:1). Fraction II, 1.3 mg., is a dioxindole-spirolactone (XXa); its infrared spectrum showed the presence of a five-membered lactone and oxindole, and the absence of peptide bonds. Its ultraviolet spectrum was typical of that of a DNP-amino acid.

Fraction III is a mixture of 3 mg. of the tripeptide DNP-Leu-δ-Br-γ-OH-norLeu-[O]"Try"-lactone and 5 mg. of dinitroaniline. From IV three DNP-peptides were isolated. Two of them are probably N-Me-Leu→O-Me-NO_2-Tyr→Ala→N-Me-δ-HO-Leu→Leu→δ-Br-γ-OH-norLeu-lactone and the corresponding open acid. Terminal DNP-N-methylleucine could not be isolated by hydrolysis at 105° for 12 hr. After hydrolysis of the hexapeptide, the water-soluble amino acids were identified as N-Me-Leu, NO_2-Tyr (by hydrolysis of O-Me-NO_2-Tyr), Ala, N-Me-δ-HO-Leu, Leu, and δ-Br-γ-OH-norLeu-lactone whose identity was concluded from the fact that it differed from the δ-chloronorleucine which has been reported to form from 2-amino-4-hexenoic acid with HOAc–HCl (1:1) on refluxing.[3a,18]

An additional peptide turned out to be impure and was not further investigated.

The unknown dioxindole-spirolactone XXa was converted to the known lactone XXI by refluxing with 2.0 *N* HCl at 100° for 3–4 hr.

NBS Titration of DNP-seco-O-dihydrorufomycin A δ-Lactone.—The solution of 4.1 mg. of the dinitrophenylated secopeptide XIa in 1.2 ml. of 80% acetic acid was divided into eleven aliquots of 0.1 ml. (0.3 μmole) each. From a solution of 5.4 mg. of freshly recrystallized NBS in 10 ml. of 80% acetic acid were added, respectively, the following volumes: 0.0 (blank), 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50 ml., corresponding to 0 to 5.0 equiv. of NBS. After 20 min., each tube was diluted with 80% acetic acid to 0.6 ml. and with 3.4 ml. of ethanol to a total of 4.0 ml. The spectral changes were recorded with this solution (Fig. 7A and B). The difference spectrum showed a maximum decrease at 285 mμ of 0.18, calculated value (0.3/8) × (5.5/1.31) = 0.16.

Correlation between NBS Oxidation and Release of New NH_2–terminal "Tryptophan."—The solution of 6.2 mg. (5.34 μmoles) of DNP-seco-O-methyldihydrorufomycin A in 1.55 ml. of 80% acetic acid was divided into six fractions of 0.25 ml. each. The oxidant solution contained 17.8 mg. (100 μmoles) of freshly recrystallized NBS in 10 ml. of 80% acetic acid. To each aliquot, respectively, was added the following volume of the standard NBS solution: 0.0 (blank), 0.1, 0.2, 0.3, 0.4, and 0.5 ml., corresponding to 0.0, 1.14, 2.28, 3.42, 4.57, and 5.71 equiv. of NBS. After 20 min. 0.1 ml. of formic acid was added to the reaction mixture. For complete hydrolysis one-fourth volume of 1.0 *N* HCl was added to the reaction mixture and the solution was allowed to stand for 12 hr. at room temperature. Each reaction mixture was divided into two portions and evaporated *in vacuo*. The trinitrobenzenesulfonic acid reagent was added to one-half and the other half served as blank. The release of NH_2–terminal is summarized in Fig. 8.

Isolation of DNP-leucyl-2-amino-4-hydroxy-5-bromohexanoic Acid Lactone (XXII).—To a solution of 160 mg. (140 μmoles) of DNP-secopeptide XIa in 4 ml. of 80% acetic acid was added the solution of 175 mg. (7 equiv.) of freshly recrystallized NBS in 4 ml. of 80% acetic acid. After 20 min. at room temperature, 0.5 ml. of formic acid was added. The solution was evaporated *in vacuo*. The residue dissolved in 10 ml. of 1.0 *N* HCl–HAc (1:4) and allowed to stand for 12 hr. at room temperature for complete hydrolysis of the iminolactone. The reaction mixture was evaporated *in vacuo*. The residue was distributed between 60 ml. of ethyl acetate and 20 ml. of dilute hydrochloric acid. The ethyl acetate layer was washed several times with dilute acid, 1% bicarbonate solution, dried over anhydrous magnesium sulfate, and evaporated *in vacuo* to give 136 mg. of a yellow powder which was purified by thin layer chromatography on a silica gel plate (20 × 20 cm., 1 mm. thick) in benzene–ethyl acetate–acetic acid (8:2:1). Two yellow bands were separated and extracted with ethanol. The solvent was removed by evaporation *in vacuo*.

The residues were again purified by preparative thin layer chromatography in the system chloroform–methanol–acetic acid (95:5:1). From the second chromatogram, four DNP derivatives were separated, among which the lactone fraction (3 mg.) with infrared absorption bands at 5.58 (five-membered lactone) and 5.93 μ (peptide) had the properties of the expected DNP-leucyl-2-amino-4-hydroxy-5-bromohexanoic acid lactone (XXII). The other three DNP derivatives weighed 4, 1.6, and 1.3 mg. Complete hydrolysis of XXII with HCl–HAc (1:1), at 105° for 12 hr. furnished NH_2–terminal DNP-leucine. The substance soluble in aqueous acid was dinitrophenylated with DNFB in dimethoxyethane and 2% triethylamine. The thin layer chromatogram gave a homogeneous compound different from all controls and presumed to be DNP-2-amino-4-hydroxy-5-bromohexanoic acid lactone XXIII.

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