ability to enhance the expulsion of either dinitroaniline or ethanol in the hydrolysis of IV.

If the reaction of alcohol and water with nitro-substituted anilides proceeds *via* the formation of tetrahedral intermediates similar to those presumed to occur in the hydrolyses of II–IV, it may be expected that the mechanism of general acid–base catalysis of these reactions will depend both on the nature of the substrate and of the catalyst. For example, it would be anticipated that phosphate and acetate buffers would catalyze the hydrolysis of *p*-nitroformanilide mainly in the pH region of rate-determining breakdown of intermediates. Amine buffers, on the other hand, should exert their catalytic influence mainly in pH regions of rate-determining formation of intermediates, since these monofunctional catalysts appear best capable of increasing the rate of expulsion of alcohol from the tetrahedral intermediate (and hence the rate of addition of alcohol or water to the anilide carbonyl group).

Experimental Section

The preparation or purification of the imidate esters and buffers has been described.[1a]

The hydrolysis of the formimidates I–IV was carried out in 2% CH_3CN–H_2O (v/v), at 30°, and the ionic strength was 0.5 (maintained with added KCl) in all cases except a few reactions at high phosphate buffer concentrations, which are noted in Table III. With the iminolactone V, the solvent was 10% CH_3CN–H_2O. All reactions were allowed to proceed to completion (6–10 half-lives), using the kinetic data obtained in an earlier study.[1a] Reactions were initiated by the addition of 0.1 ml of an acetonitrile solution of the substrate to 5 ml of aqueous buffer solution, previously equilibrated at 30°, followed by vigorous mixing on a Vortex shaker. Final concentrations of imidate esters were generally 0.5–1.5 × 10^{-4} *M*, except for the *m*-nitroformimidate II, where substrate concentration was about 10^{-3} *M*, owing to the small extinction coefficients of the products at the wavelength of measurement.

Analysis for products was carried out by a diazotization assay for aniline (with I) or by direct spectrophotometric examination of reaction mixtures (with II–V), using described procedures.[1a] The identity of the reaction products was confirmed in several cases by comparison of the complete ultraviolet spectra of reaction mixtures to those of synthetic mixtures of the aniline and corresponding formanilide, at the concentration determined by analysis.[1a] Owing to the resemblance of the ultraviolet spectra of 2,4-dinitroaniline (λ_{max} 347 mμ (ε 14,000), 263 (9000), in 1% acetonitrile–water) to that of 2,4-dinitrophenolate ion (λ_{max} 359 mμ (ε 15,000), 256 (7900)),[21] complete spectra were determined of the hydrolysis products of IV under the following conditions: acetate buffer, pH 4.95, 0.01 and 0.50 *M*; phosphate buffer, pH 5.95, 0.01 and 0.1 *M*. The yields of 2,4-dinitroaniline were, respectively, 64.1, 22.3, 79.3, and 60.9%. In all cases, the spectra were identical in the wavelength region 220–450 mμ to those of the corresponding mixtures of 2,4-dinitroaniline and 2,4-dinitroformanilide. Also, no change in spectrum occurred on acidification of the reaction mixture to pH 1; the presence of significant quantities of 2,4-dinitrophenolate ion would have led to a large hypsochromic shift on acidification to 2,4-dinitrophenol (pK_a *ca.* 4, λ_{max} 260 mμ (ε 12,700), shoulder at *ca.* 290 mμ).

(21) *Cf.* D. V. Parke, *Biochem. J.*, **78**, 262 (1961).

Structure of the Peptide Antibiotic Amphomycin[1]

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Abstract: The structure of amphomycin, elucidated mainly by selective acid degradation, is represented in Figure 1.

Elucidation of the structure of amphomycin, an antibiotic useful in medicine, was suggested to us by Professor David Perlman (of the University of Wisconsin). It is the first described[2] member of a series of closely related peptide antibiotics. Of these, two alternative structures were tentatively proposed[3] for glumamycin,[4–7] but only the amino acid and fatty acid constituents are known in the cases of zaomycin,[8] crystallomycin,[9] aspartocin,[10–12] laspartomycin,[13] and tsushimycin.[14] The structural studies described in this paper point to the identity of glumamycin with amphomycin and also indicate that aspartocin and tsushimycin are different from amphomycin only in respect to the fatty acid constituent, but not in the peptide part of their molecules.

Fatty Acid Constituents and Heterogeneity. Countercurrent distribution of the amphoteric form of amphomycin,[2] in a system of 1-butanol–pyridine–acetic acid–water (4:2:1:7), resulted in a distribution curve that coincided with the curve calculated for the experimentally found distribution coefficient (k = 3.0).[15] Samples taken from different parts of the distribution band were hydrolyzed and analyzed for amino acids; the same ratios of amino acids were found in all of them.

(1) This paper is dedicated to Professor Theodor Wieland to honor him on his 60th birthday.

(2) B. Heinemann, M. A. Kaplan, R. D. Muir, and I. R. Hooper, *Antibiot. Chemother. (Washington, D. C.)*, **3**, 1239 (1953).

(3) M. Fujino, *Bull. Chem. Soc. Jap.*, **38**, 517 (1965).

(4) M. Shibata, T. Kanzaki, K. Nakazawa, M. Inoue, H. Hitomi, K. Mizuno, M. Fujino, and M. Akira, *J. Antibiot., Ser. A*, **15**, 1 (1962).

(5) (a) M. Inoue, *Bull. Chem. Soc. Jap.*, **35**, 1249 (1962); (b) *ibid.*, **35**, 1255 (1962).

(6) M. Inoue, *ibid.*, **35**, 1556 (1962).

(7) M. Fujino, M. Inoue, J. Ueyanagi, and A. Miyake, *ibid.*, **38**, 515 (1965).

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(9) G. F. Gauze, T. P. Preobrazhenskaya, V. K. Kuvalenkova, N. P. Ilicheva, M. G. Brazhnikova, N. N. Lomakina, I. N. Kouscharova, U. A. Shorin, I. A. Kunrat, and S. P. Shapovalova, *Antibiotiki*, 2, 9 (1957).

(10) A. J. Shay, J. Adam, J. H. Martin, W. K. Hausman, Ph. Shu, and N. Bohonos, *Antibiot. Annu.*, 194 (1960).

(11) W. K. Hausman, A. H. Struck, J. H. Martin, R. H. Baritt, and N. Bohonos, *Antimicrob. Ag. Chemother.*, 352 (1964).

(12) W. K. Hausman, D. B. Borders, and J. E. Lancaster, *J. Antibiot.*, **22**, 207 (1969).

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(14) J. I. Shoji, S. Kozuki, S. Okamoto, R. Sakazaki, and H. Otsuka, *ibid.*, **21**, 439 (1968); *cf.* also J. I. Shoji and H. Otsuka, *ibid.*, **22**, 473 (1969).

(15) M. Bodanszky, N. C. Chaturvedi, J. A. Scozzie, R. K. Griffith, and A. Bodanszky, *Antimicrob. Ag. Chemother.*, 135 (1970).

Nevertheless, from our previous studies[16] on the fatty acid constituents of amphomycin, it was obvious that a mixture was at hand. The individual members of the amphomycin family contain the same amino acids in identical ratios but are different in respect to their fatty acid constituents. The major components of the fatty acid mixture, (+)-3-anteisotridecenoic acid and (+)-3-isododecenoic acid, are not sufficiently different to provide the individual members with solubility properties that can lead to separation in the distribution. The structure elucidation had to be carried out on the mixture.

Amino Acid Constituents. From acid hydrolysates of amphomycin, L-aspartic acid (3 mol), glycine (2 mol), L-*threo*-β-methylaspartic acid,[17] L-proline, L-valine, D-pipecolic acid, L-*threo*-α,β-diaminobutyric acid,[18] and D-*erythro*-α,β-diaminobutyric acid[16] (1 mol each) were isolated and identified.[15]

End Groups. Rearrangements. Treatment of amphomycin with ninhydrin,[19] followed by hydrolysis and quantitative amino acid analysis, revealed that the single free amino group[2] belongs to the D-*erythro*-α,β-diaminobutyric acid residue. The action of nitrous acid on the antibiotic produces a threonine moiety. Therefore the β-amino group of the diaminobutyric acid must be free.

The four free carboxyl groups[2] are the β-carboxyls of the aspartyl residues and of the β-methylaspartic acid. Titration curves gave no indication for the presence of free α-carboxyl groups. Furthermore, on esterification with methanol followed by reduction with $LiBH_4$,[20] hydrolysis, and amino acid analysis, the pattern of products was quite similar to the pattern obtained on analogous treatment of α-aspartylglycine and different from the pattern produced from β-aspartylglycine as starting material.

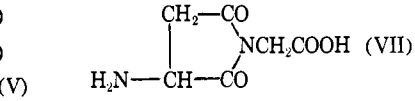
On prolonged standing of aqueous solutions of amphomycin at room temperature, a transformation of the antibiotic can be observed. On thin-layer chromatograms in the system 1-butanol–acetic acid–water (3:1:1), in addition to the original ninhydrin positive spot at R_f 0.4, a second spot at about R_f 0.5 appears and gradually increases with time, while the spot at 0.4 decreases and finally disappears. A concomitant decrease in titration values shows that the number of free carboxyl groups is diminished in this process. Ring closures involving the side chains of aspartyl residues and resulting in the formation of aminosuccinimide moieties were not unexpected.[21]

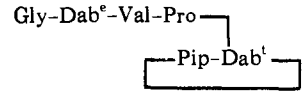
Partial Acid Hydrolyses (*cf.* **Chart I**). The presence of aspartyl residues in amphomycin prompted the application of the selective cleavage conditions established by Partridge and Davis.[22] After brief boiling of a solution of the antibiotic in 0.25 *N* acetic acid, a fragment consisting of aspartic acid acylated by the fatty acids (FA–Asp, I) could be extracted with organic solvents. On prolonged hydrolysis with the same weak acid, aspartic acid and β-methylaspartic acid were gradually liberated, together with one of the two glycine residues. This glycine had to be located, therefore, between two aspartyl residues, or between an aspartyl moiety and the β-methylaspartic acid in the sequence. A larger fragment was isolated from the hydrolysis mixture by preparative paper chromatography or by ion-exchange chromatography. More substantial quantities of the same material could be secured by countercurrent distribution in a Craig apparatus. This peptide did not contain aspartic acid; it consisted of one residue each of glycine, proline, valine, pipecolic acid, L-*threo*-α,β-diaminobutyric acid, and D-*erythro*-α,β-diaminobutyric acid. The sequence of the hexapeptide was elucidated mainly through the mass spectra of its acetyl derivative and the mass spectra observed on the permethylated and perdeuteriomethylated derivatives. Structure II, derived[23,24] from these spectra, was supported by data of elemental analysis, titration with acid and base, and the unusual mobility of the hexapeptide on paper chromatograms and thin layer chromatograms.

Chart I. Fragments Formed in Partial Acid Hydrolyses of Amphomycin

FA–Asp (I)
Asp–Gly (III)
Gly–Asp (IV)
MeAsp–Asp (V)

MeAsp–Asp–Gly (VI)





FA = fatty acid(s), including (+)-3-isododecenoic and (+)-3-anteisotridecenoic acids
MeAsp = L-*threo*-β-methylaspartic acid
Dab^e = D-*erythro*-α,β-diaminobutyric acid
Dab^t = L-*threo*-α,β-diaminobutyric acid
Pip = D-pipecolic acid

Hydrolysis of amphomycin with 6 *N* hydrochloric acid at 105° for 80 min[3] yielded a complex mixture from which the acidic peptides were isolated by ion-exchange chromatography and purified by high-voltage paper electrophoresis. In addition to the dipeptides Asp–Gly (III), Gly–Asp (IV), and MeAsp–Asp (V), a tripeptide MeAsp–Asp–Gly (VI) and the aminosuccinimide derivative (VII) were secured. The N-terminal residues of these peptides were determined by the ninhydrin method.[19] From the products of this hydrolysis, the partial sequence MeAsp–Asp–Gly–Asp could be deduced (*cf.* also ref 3).

Structure of Amphomycin. A comparison of the peptides formed on partial acid hydrolysis of glumamycin[3] with the fragments obtained from amphomycin leaves little doubt about the identity of the two antibiotics. Some apparent differences stem from the nomenclature erroneously used for the fatty acid constituent[5b] of glumamycin and from the fact that racemization of the diaminobutyric acid residues during acid hydrolysis of glumamycin[7] was overlooked. The alternative sequences A and B tentatively proposed by Fujino[3] for glumamycin have to be considered, there-

(16) M. Bodanszky, N. C. Chaturvedi, and J. A. Scozzie, *J. Antibiot.*, 22, 399 (1969).
(17) M. Bodanszky and G. G. Marconi, *ibid.*, 23, 238 (1970).
(18) A. A. Bodanszky and M. Bodanszky, *ibid.*, 23, 149 (1970).
(19) M. Bodanszky and D. F. Dyckes in "Progress in Peptide Research," S. Lande, Ed., Gordon and Breach, New York, N. Y., 1972, p 169.
(20) Y. Burstein, M. Fridkin, and A. Patchornik, *Isr. J. Chem.*, 5, 64 (1971).
(21) M. A. Ondetti, A. Deer, J. T. Sheehan, J. Pleščec, and O. Kocy, *Biochemistry*, 7, 4069 (1968).
(22) S. M. Partridge and H. F. Davis, *Nature (London)*, 165, 62 (1950).
(23) M. Bodanszky, A. A. Bodanszky, C. A. Ralofsky, R. C. Strong, and R. L. Foltz, *J. Antibiot.*, 24, 294 (1971).
(24) R. C. Strong, A. A. Bodanszky and M. Bodanszky, *Antimicrob. Ag. Chemother.*, 42 (1971).

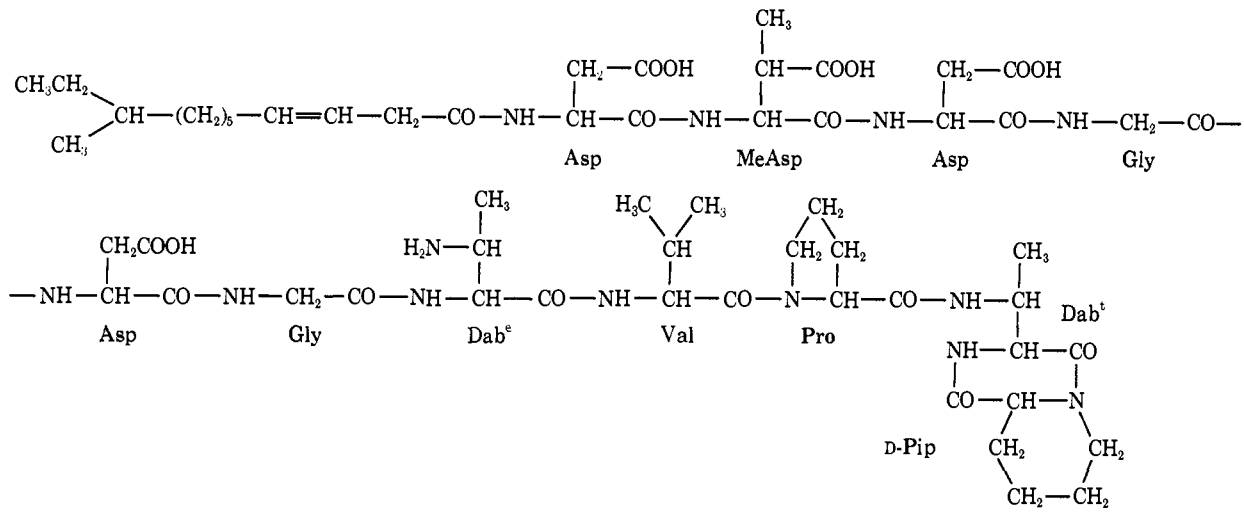
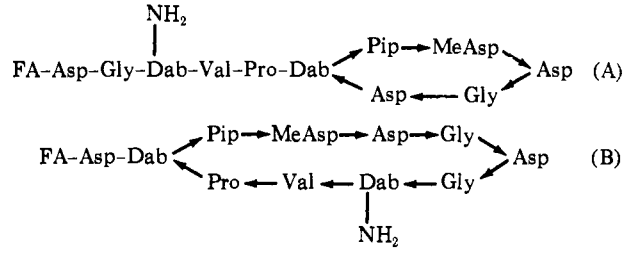
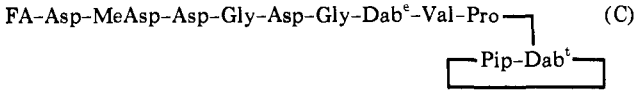


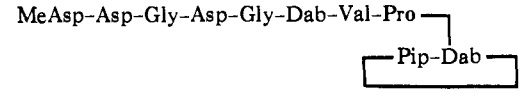
Figure 1. The structure of amphomycin. Only one member of the amphomycin family, with (+)-3-anteisotridecenoic acid at its N terminal, is depicted. The second major member contains (+)-3-isododecenoic acid. For abbreviations, *cf.* Chart I.



fore, as potentially valid for amphomycin as well. These structures, however, offer no explanation for the ease of cleavage of FA–Asp (I) from the antibiotic. In fact, the bonds around the Dab residues show a rather unusual resistance to hydrolysis.[25] The ready cleavage of I from the parent molecule, together with the presence of a diketopiperazine partial structure in hexapeptide II, point to a third possible sequence, C. The ir spectrum



of amphomycin (and of the hexapeptide II) provides indication, but no clear proof, for the presence of a diketopiperazine. However, conclusive evidence supporting sequence C was obtained through the isolation and study of a new fragment that slowly forms in aqueous solutions of amphomycin at room temperature. The acidity provided by the free carboxyls is apparently sufficient for cleaving off FA–Asp (I), leaving a decapeptide VIII. Subsequently, VIII was isolated also as a product of brief hydrolysis of amphomycin with 0.25 *N* acetic acid. Deamination of VIII with nitrous acid destroyed its MeAsp residue and converted the *erythro*-α,β-diaminobutyric acid residue into threonine. Dansylation[26] of VIII followed by hydrolysis gave the dansyl derivatives of Dab and of *threo*-β-methylaspartic acid. The crucial fact that the β-methylaspartic acid is indeed the only N-terminal residue was confirmed by the ninhydrin method[19] as well. Since neither pipecolic acid nor the *threo*-α,β-diaminobutyric acid was found to be N-terminal, they must form in VIII, as in the hexapeptide II, a diketopiperazine. The evidence of the N-terminal residue in VIII, together with the already established partial sequence MeAsp–Asp–Gly–Asp and the structure of II, allow only the following sequence for VIII



This sequence cannot be reconciled with structures A and B but is fully compatible with sequence C.[27] The structure of amphomycin is shown in Figure 1.

Experimental Section

Precoated silica gel plates (Merck) were used for thin layer chromatography (tlc) with the solvent systems (A) 1-propanol–H_2O, 7:3, and (B) 1-butanol–AcOH–H_2O, 3:1:1. For quantitative amino acid analysis, samples were hydrolyzed with 6 *N* HCl in evacuated, sealed ampoules at 110° for 16 hr. After evaporation of the acid, ratios of the amino acid components were determined on a Beckman-Spinco 120C instrument. For paper electrophoresis, the "flat plate" of a Savant apparatus was used.

Titrations were carried out with a Radiometer autotitrator TTT1, recorder SBR2, titrator assembly TTA3, and a 2.5-ml buret; N_2 was bubbled through the solutions during titrations.

N-Terminal residues were determined by the ninhydrin[19] method by applying a sample (2–3 mg) of a peptide as a streak to a silica

(25) R. C. Strong, A. A. Bodanszky, and M. Bodanszky, *J. Antibiot.*, **23**, 257 (1970).

(26) W. R. Gray and B. S. Hartley, *Biochem. J.*, **89**, 379 (1963).

(27) The possibility that the diketopiperazine part of VIII is not a genuine feature of the molecule of amphomycin, but forms under the mild conditions of degradation, has not been overlooked. A similar concern was already expressed in connection with the hexapeptide fragment II (ref 23). Possible precursors containing the -Asp-Pip-Dab- partial sequence could be ruled out by reduction[28] of the antibiotic with sodium in liquid ammonia in the presence of methanol: only the valine and diaminobutyric acid content of the hydrolysate was diminished; the aspartyl residues remained intact. Since the partia lsequence -Asp-Dab-Pip-, which could similarly yield the diketopiperazine ⌐Dab–Pip¬, was not excluded in this experiment, a model dipeptide, L-prolylglycine, was exposed to the conditions leading to the liberation of II and to those resulting in the formation of VIII. On long refluxing in dilute acid, the dipeptide indeed produced some diketopiperazine, but practically none was found after the brief heating period that was used in the preparation of VIII. Especially, because an Asp–Dab bond would need to be broken before ring closure could begin, the rapid formation of the diketopiperazine in VIII is quite unlikely and its presence in the parent molecule was not further questioned.

(28) M. Wilchek, S. Sarid, and A. Patchornik, *Biochim. Biophys. Acta*, **104**, 616 (1965).

Table I. Acidic Peptides from Partial Hydrolysis with 6 *N* HCl

Fraction no.	R_f *n*-PrOH–H_2O (7:3)	R_f BuOH–HOAc–H_2O (3:1:1)	Electrophoretic mobility at pH 6.4[a]	Amino acid analysis—Untreated peptide	Amino acid analysis—Ninhydrin treated	Deduced structure
93–111	0.20	0.10	0.54	Asp 1.0 Gly 1.0	Asp 0.5 Gly 1.0	Asp–Gly[e]
101–111	0.20	0.12	0.80	Asp 1.1 Gly 1.0	Asp 1.0 Gly 0.4	Gly–Asp
111–130	0.17	0.11	0.87	Asp 1.0 Gly 1.0	Asp 0.3 Gly 1.0	α-Asp–Gly
130–153	0.19	0.10	1.00	Asp only		Free Asp
153–180	0.23	0.09	0.71	Asp + MeAsp 1.9[b,c] Gly 1.0	Asp 0.9[d] Gly 1.0	MeAsp–Asp–Gly
153–180	0.35	0.16	0.90	Asp + MeAsp[c]	Asp only[d]	MeAsp–Asp

[a] Relative to free aspartic acid under the same conditions. [b] Calculated using a color constant for aspartic acid. Standard analyses on authentic samples of aspartic acid and β-methylaspartic acid show a negligible difference in their color constants, and the two were determined together. [c] Methylaspartic acid was identified on electropherograms as a spot of approximately equal intensity as aspartic acid and lower mobility (0.90 relative to Asp at pH 6.4). [d] MeAsp absent on electropherograms. [e] Aminosuccinimidoacetic acid was identified by comparison (tlc) with an authentic sample.

gel plate (10 × 20 cm). The plate was developed with solvent system A or B and thoroughly dried in an air stream. One-half of the plate was sprayed with a 3% solution of ninhydrin in acetone. After about 6 hr at room temperature, the stained band and the corresponding untreated area were cut out. The excess ninhydrin was removed with acetone; the peptides were eluted with 1-butanol–ethanol–H_2O (1:1:1), hydrolyzed, and analyzed for amino acids.

Ir spectra were recorded on a Cary 12 spectrophotometer. Samples were applied as KBr disks.

Analysis of Amphomycin. A sample (100 mg) of the Ca salt of the antibiotic (obtained from H. Lundbeck and Co., Copenhagen-Valby, Denmark) was suspended in methanol (25 ml) and the suspension stirred until complete solution occurred. On slow evaporation at room temperature, crystals deposited; these were collected, washed with a small volume of methanol, and dried at 60° *in vacuo*.

Anal. Calcd for $C_{58}H_{88}N_{13}O_{20}\cdot{}^3/_2Ca\cdot 7H_2O$: C, 47.3; H, 7.0; N, 12.4; Ca, 4.1; H_2O, 8.6. (This calculation is based on the most abundantly present species: the peptide acylated with 3-anteisotridecenoic acid.) Found: C, 47.2; H, 6.7; N, 12.4; Ca, 4.4; loss of weight on drying at 110° *in vacuo*, 8.6%.

The amphoteric form of amphomycin was prepared from the Ca salt as described in ref 2. The crude material was purified by countercurrent distribution in a system of 1-butanol–pyridine–AcOH–H_2O (4:2:1:7) through 60 transfers.[15] Analysis of the purified peptide, dried at 60°, indicates an extensive loss of water, probably through ring closure of aspartyl–methylaspartyl residues to aminosuccinimide derivatives.

Anal. Calcd for $C_{58}H_{91}N_{13}O_{20}$: C, 54.0; H, 7.1; N, 14.1. Calcd for $C_{58}H_{85}N_{13}O_{17}$: C, 56.4; H, 6.9; N, 14.7. Calcd for $C_{58}H_{83}N_{13}O_{16}$: C, 57.2; H, 6.9; N, 14.9. Found: C, 56.9; H, 6.9; N, 14.8.

Carboxyl Groups in Amphomycin. A suspension of the Ca salt (85.1 mg) in H_2O (5.0 ml) had a pH of 7.0. On the pH Stat, set at 10.5, rapid (4.5 min) consumption of 0.047 *N* NaOH (1.35 ml) was recorded, corresponding to a neutralization equivalent of 1347. A second titration gave 1353. The sample dissolved during the addition of alkali. To exclude the possibility of ring opening (hydrolysis) during titration, phthalylglycine was titrated under similar conditions. One equivalent of alkali was consumed in 4 min; a second required 17 more min (ring opening).

Titration with alkali indicated no free α-carboxyl groups. An aqueous solution (1 ml) of the amphoter (22.9 mg/ml), when titrated with 0.05 *N* NaOH, showed a distinct break at 1.08 ml (pH 6.5–7), with an average p*K* of 4.4 for the three titrated carboxyl groups. This is the p*K* reported[29] for side-chain carboxyls. Continuation of the titration above pH 7 resulted in the liberation of the amino group, protonated by the fourth carboxyl. Yet, no sharp end point could be observed and no p*K* was calculated for the amine.

Rearrangement, probably ring closure, was noted on storage of the aqueous solution at room temperature. Samples (1 ml) of the above-mentioned solution, titrated 8, 15, 22, 29, and 36 days later, revealed the break at 0.76, 0.63, 0.55, 0.54, and 0.54 ml, respectively. The other half of the solution was stored frozen, and 1-ml aliquots titrated as controls did not change in 36 days.

In order to establish more conclusively whether α- or β-carboxyl groups of the aspartyl (and β-methylaspartyl) residues are free, amphomycin amphoter (230 mg) was dissolved in 5 *N* HCl in MeOH (5 ml). The solution was kept in an ice bath for 1 hr, then at room temperature for 2 hr. After evaporation *in vacuo*, the oily residue was dried over NaOH. The resulting dry foam was dissolved in THF (1 ml), the mixture cooled to 0°, and $LiBH_4$ (75 mg) added in three portions. The mixture was stirred at room temperature for 20 hr and cooled to 0°, and 1 *N* HCl (3 ml) was added slowly, when the excess of the reducing agent decomposed with strong bubbling. After 4 hr, water was added to bring the volume to 10 ml. An aliquot (0.05 ml) was hydrolyzed with 6 *N* HCl (1 ml) at 110° for 16 hr, and then applied to the amino acid analyzer. Only a trace of aspartic acid remained, but the full amounts of proline, glycine, valine, pipecolic acid, and α,β-diaminobutyric acid expected for an amphomycin hydrolysate were found together with the peaks of homoserine and its lactone. Only traces of β-homoserine or its lactone could be detected.[30]

Free Amino Group of Amphomycin. Deamination of the antibiotic was carried out as described by Fujino, *et al.*,[7] for glumamycin. A solution of the amphoter (0.75 g) in 80% acetic acid (8 ml) was cooled in an ice–water bath. Sodium nitrite (75 mg) and 1 *N* HCl (1.2 ml) were added in small portions over a 5-hr period. After 2 days at room temperature, a sample (0.10 ml) was diluted with 6 *N* HCl (1 ml) and hydrolyzed at 110° for 16 hr. The following ratios were found: Asp–MeAsp, 3.9; Thr, 0.3; Pro, 1.0; Gly, 2.0; Val, 1.0; Pip, 1.0; Dab[t], 1.1; Dab[e], 0.2.

After ninhydrin treatment[19] of amphomycin (amphoter), quantitative amino acid analysis gave the following ratios: Asp–MeAsp, 3.7; Pro, 1.1; Gly, 2.0; Val, 0.8; Pip, 1.0; Dab (mainly threo), 1.2. In the untreated portion, the ratios were: Asp–MeAsp, 3.8; Pro, 1.0; Gly, 2.0; Val, 0.7; Pip, *ca.* 1.0; Dab[t], 1.0; Dab[e] 0.8.

Partial Hydrolysis of Amphomycin with 6 *N* Hydrochloric Acid. Amphomycin amphoter (700 mg) was dissolved in 6 *N* HCl (15 ml) and the solution was refluxed for 80 min (*cf.* ref 3). The hydrolysate was diluted with an equal amount of water and extracted with ethyl acetate (3 × 10 ml). The aqueous layer was evaporated *in vacuo*, and the residue (550 mg) was redissolved in 15% aqueous pyridine (5 ml), applied to a column of Dowex 1-X8 (22 × 1.5 cm) in acetate cycle, and eluted with a series of buffers: (1) pH 6.0, pyridine acetate, 60 ml; (2) pH 3.5, pyridine acetate, 120 ml; (3) pH 2.3, 0.2 *N* acetic acid, 180 ml. The eluents were collected in 2-ml fractions. Paper electrophoresis of small samples showed that the neutral and basic components were completely eluted in the first 30 tubes. The acidic peptides were found in tubes 90–180.

(29) C. Tanford, "Physical Chemistry of Macromolecules," Wiley, New York, N. Y., 1967, p 556.

(30) O. O. Blumenfeld and P. M. Gallop, *Biochemistry*, 1, 947 (1962).

Table II. Infrared Spectra of Amphomycin and Related Compounds

Ir band	Lit., cm^{-1} [a]	Calcium amphomycin	Amphomycin amphoter Purified by CCD	Crude	"Amphomycin" with R_f 0.5[b]	Hexapeptide II	Sodium aspartocin[d]
Amide I (trans)	1650, S[c]	1650, S	1660, S	1660, S	1660, S	1650, S	1650, S
Amide I (cis)	1670–1690, S					1671, S	
C=O in succinimide	1780, M 1700, S		1787, M		1790, M		
C=O in COOH	1710, S 1730, S		1720, S	1720, S	1723, S		
C=O in COO$^-$	1610–1550, S 1400, S	1580, S 1400, S					1585, S 1400, S
Amide II (trans)	1550, S	1530, S	1528, S	1530, S	1570, S	1570, S	1535, S
Amide II (cis)	1440–1455, M	1450, M	1436, M	1450, M	1430, M	1430, M	1450, M
Amide III (trans)	1200–1305, M	1240, M	1237, M	1236, M	1250, M	1260, M	1240, M
Amide III (cis)	1305–1345, W–M	1320, M	1330, W–M	1333, W–M	1330, W–M	1335, W–M	1320, W–M
N–H (trans)	3300, S 3180–3145, S	3300, S	3310, S	3320, S	3350, S	3230, S	3300, S
N–H (cis)	3040, M 3080, M	3065, M	3050, M	3050, M	3050, M	3045, M	3050, M

[a] *Cf.* ref 32. [b] Formed on standing in aqueous solution. [c] S = strong; M = medium; W = weak. [d] Nutritional Biochemicals, Cleveland, Ohio.

Separation of the acidic components and final purification were effected by preparative high-voltage electrophoresis on paper in pyridine acetate at pH 6.4 and 33 V/cm. Guide strips were developed with ninhydrin reagent and the peptides were eluted from the paper with 2 *N* acetic acid. Determination of the N-terminal residues of the acidic peptides was carried out by the ninhydrin method.[19] The results are summarized in Table I.

Partial Hydrolysis with 0.25 *N* Acetic Acid. A sample (1.2 g) of amphoter, purified by countercurrent distribution, was dissolved in 0.25 *N* AcOH (150 ml) and refluxed for 1 hr. On subsequent cooling in an ice–water bath, the solution became turbid and gradually a precipitate settled out. The insoluble material gave two spots on tlc, in solvent system B, with R_f values 0.5 and 0.6 corresponding to those of the starting material and FA–Asp (I), respectively. Evaporation of the supernatant solution left a residue (0.53 g) that was distributed in the solvent system 1-butanol–pyridine–AcOH–H_2O (4:2:1:7) through 60 transfers. A 60-tube Craig apparatus, with 10-ml phases, was used. A weight curve revealed a dehydrated form of amphomycin (150 mg, k = 4.2) and a slow-moving band with maximum in tube 10, but with a distribution curve which did not fit the calculated curve. The material in this band was further fractionated by preparative paper electrophoresis at pH 6.4. Several bands were found (corresponding to different degrees of ring closure of the aspartyl–methylaspartyl residues), but all gave the same ratios of components on quantitative amino acid analyses: Asp–MeAsp, 2.9; Pro, 1.0; Gly, 2.0; Val, 0.8; Pip, *ca.* 1.0; Dab^t, 1.0; Dab^e, 0.9.

Samples (1 mg each) of the decapeptide(s) were dansylated[26] in 0.25 *M* $NaHCO_3$ (0.5 ml) with a solution of dansyl chloride (Eastman, 1.25 mg) in acetone (0.5 ml). The pH was adjusted with $NaHCO_3$ to about 10, and the mixture was left to stand at room temperature overnight. After evaporation to dryness with a stream of N_2, the residue was dissolved in 6 *N* HCl and hydrolyzed at 110° for 6 hr. The acid was removed by evaporation and the solid residue extracted with ethyl acetate (2 × 2 ml). Solutions of the extract and of the EtOAc insoluble fraction in 40% aqueous pyridine were examined by tlc. The system benzene–pyridine–AcOH (40:10:1)[31] was used. Comparisons with authentic samples were carried out also by paper electrophoresis (at pH 4.4, 35 V/cm, 2.5 hr). Only the dansyl derivatives of DAB and of *threo*-β-methylaspartic acid were detected in the hydrolysates. (The dansyl derivatives of the diastereoisomers show different mobilities under these conditions.)

A sample (20 mg) of the decapeptide mixture from the slow-moving countercurrent distribution band was dissolved in 0.1 *N* HCl (1.0 ml), and N_2O_3 (generated in a separate flask from $NaNO_2$ and 6 *N* HCl) was bubbled through the solution with the aid of a slow stream of N_2. After 3 hr, spots of the solution on paper gave no more reaction with ninhydrin. After evaporation to dryness, the residue was redissolved in 1 *N* HCl (18 ml); the solution was evaporated on a steam bath with the aid of an N_2 stream. An aliquot of the residue was hydrolyzed with 6 *N* HCl under the usual conditions used for amino acid analysis. The following ratios were determined: Asp–MeAsp, 2.2; Thr, 0.1; Pro, 0.9; Gly, 2.0; Val, 0.9; Pip, 1.2; Dab^t, 0.7; Dab^e, 0.3. High-voltage paper electrophoresis (pH 5.3, pyridine acetate, 1.5 hr, 30 V/cm) carried out on this hydrolysate and on a hydrolysate of the decapeptide before deamination revealed that, on deamination, the β-methylaspartic acid content of the hydrolysate was reduced to a very small value.

A sample of decapeptide (3 mg) was treated with ninhydrin as described above, with the exception that the plate was developed for 24 hr. Hydrolysis and amino acid analysis gave the following ratios: Asp–β-MeAsp, 2.4; Pro, 0.8; Gly, 2.0; Val, 0.8; Pip, *ca.* 1.0; Dab^t, 0.5; Dab^e, 0.3. The untreated control gave the following analysis: Asp–MeAsp, 2.9; Pro, 1.0; Gly, 2.0; Val, 0.7; Pip, *ca.* 0.9; Dab^t, 1.1; Dab^e, 1.0.

Spontaneous Hydrolysis of Amphomycin in Water. An aqueous solution of the amphoter (22.9 mg/ml) was stored at room temperature for about 2 months. On tlc in solvent system B, a ninhydrin positive spot, with R_f 0.1, appeared. After preparative tlc and ninhydrin treatment,[19] the original Asp–MeAsp/Gly ratio of 3.0:2.0 was reduced to 1.9:2.0. The destruction of MeAsp was confirmed by paper electrophoresis.

Reductive Cleavage with Na in Liquid NH_3.[28] A sample of the amphoter (140 mg) was dissolved in liquid NH_3 (50 ml) to which methanol (0.08 ml) had been added. Small pieces of sodium were added until the blue color persisted. After 20 min, addition of methanol and sodium was repeated, and 15 min later the color was discharged with a few drops of methanol. The ammonia was allowed to evaporate, and the residue was dried *in vacuo*. An aliquot of the residue was hydrolyzed for amino acid analysis: Asp–MeAsp, 3.9; Pro, 0.6; Gly, 2.0; Val, 0.4; Pip, 1.0; Dab^t, 0.4; Dab^e, 0.8.

Infrared Spectra. The spectral bands of interest are listed in Table II. A peak around 1790 cm^{-1}, absent in both the calcium salt and crude amphoter but appearing in a sample purified by CCD and in altered amphomycin (R_f 0.5), is strong evidence for succinimide carbonyls. The accompanying peak at 1700 cm^{-1} of succinimide could not be clearly distinguished, probably because it is masked by the strong absorbance of carboxyl carbonyl in the same region.

In all spectra in Table II, peaks were observed in the regions associated with the cis-amide peptide bond. According to Blahá, *et al.*,[32] the cis-amide III band (1330–1335 cm^{-1}) and, to a certain extent, the cis N–H stretching bands (3040, 3080, 3145–3180 cm^{-1}) are especially characteristic in this respect.

Diketopiperazine (DKP) Formation from L-Prolylglycine. Aliquots (1 ml) of a 0.2% solution of the dipeptide (prepared by conventional methods) in 0.25 *N* AcOH were heated in evacuated, sealed ampoules at 110° for different periods of time. The solu-

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tions were then examined on tlc in system A. An authentic sample of the DKP -L-Pro-Gly- was used for comparison. Spots were revealed by ninhydrin and with *tert*-butyl hypochlorite, followed by KI-starch.[33] In a solution that was heated for 45 min, no DKP was detected, while in a solution heated for 26 hr more than half of the dipeptide was converted to the DKP.

Acknowledgments. Amphomycin (Ca salt) was obtained as a gift from H. Lundbeck and Co., Copenhagen-Valby, Denmark. The authors express their gratitude to Dr. L. Szabo for the generous samples. Elemental analyses were carried out by Mr. Joseph Alicino of the Squibb Institute for Medical Research, and amino acid analyses by Mrs. Delores J. Gaut. The assistance of Mr. Anthony Severdia in the ir studies is greatly appreciated. The entire study was supported by a grant from the U. S. Public Health Service (NIH AI-07515).

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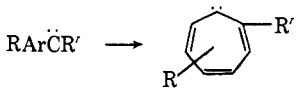
Communications to the Editor

Generation of a Ten-π-Electron Carbocyclic Aromatic Carbene by a Carbene–Carbene Rearrangement

Sir:

To date, carbene–carbene rearrangements of aryl carbenes to aromatic carbenes have been limited to high temperatures (225–900°), the gas phase, and six-π-electron aromatic carbenes (**1**).[1] At this time, we



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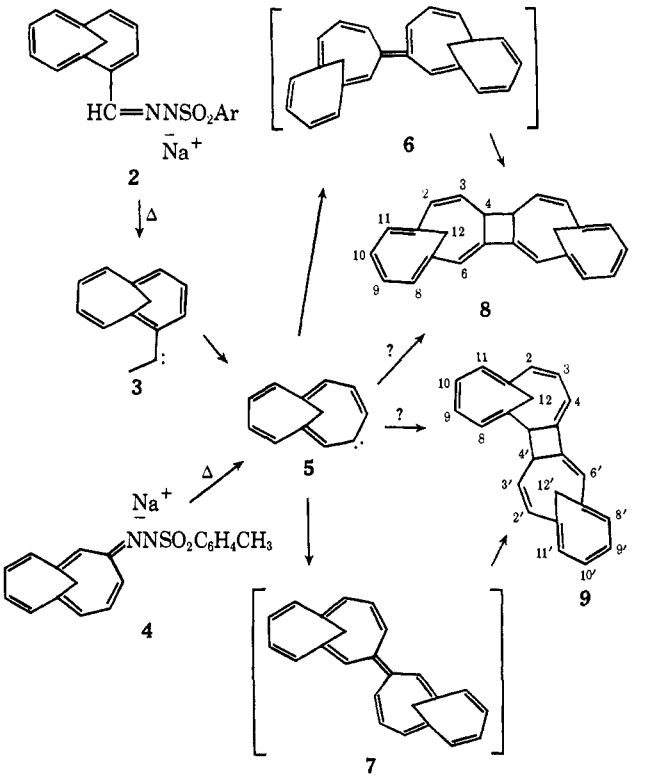
report the isolation and characterization of substantial yields (50–60%) of **8** and **9** from the thermal generation of **3** in diglyme at moderate temperatures (as low as 130°). Taken with the formation of the same products from thermolysis of the sodium salt of the ketone tosylhydrazone **4**, these products constitute strong evidence for the first known rearrangement of an arylcarbene to an aromatic carbene[2] in solution. In addition, the products are also indicative of either an unprecedented 22 π-electron electrocyclic ring closure of the undecafulvalenes **6** and **7** or an atypical aromatic carbene.

The parent methanoannulene was synthesized according to the method of Vogel[3] as modified by Untch.[4] The hydrocarbon was converted to the carboxylic acid[3] and the acid converted to the aldehyde by initial quantitative reduction (lithium aluminum hydride), followed by oxidation (76% yield) of the primary alcohol with freshly prepared MnO_2[5] in acetonitrile. The tosylhydrazone[6] and its sodium salt (**2**) were prepared in the conventional way.

In a typical reaction, the carbene was generated by addition over a 10-min period of 0.5 g of **2** in 15 ml of dry diglyme to 50 ml of rapidly stirred dry diglyme (dry nitrogen swept) maintained at about 135° (minimum of 130°). The reaction mixture was then rapidly cooled to room temperature (to minimize product polymerization), poured into water, and extracted with pentane. Careful column chromatography on silica gel eluting with pentane typically resulted in isolation of two major hydrocarbon products,[7] **8** and **9**, in 50–60% total yield along with a modest amount of annulenyl aldehyde azine.

The sodium salt of 3,8-methano[11]annulenone tosylhydrazone (**4**) was synthesized from the corresponding ketone which was obtained from reduction of 11-chloro-3,8-methano[11]annulenone.[8] Thermolysis of **4** in re-



(1) R. C. Joines, A. B. Turner, and W. M. Jones, *J. Amer. Chem. Soc.*, **91**, 7754 (1969); J. A. Myers, R. C. Joines, and W. M. Jones, *ibid.*, **92**, 4740 (1970); T. Mitsuhashi and W. M. Jones, *ibid.*, **94**, 677 (1972); E. Hedaya and M. E. Kent, *ibid.*, **93**, 3283 (1971); P. Schissel, M. E. Kent, D. J. McAdoo, and E. Hedaya, *ibid.*, **92**, 2147 (1970); G. G. Vander Stouw, A. R. Kraska, and H. Shechter, *ibid.*, **94**, 1655 (1972), and references cited therein; W. J. Baron, M. Jones, Jr., and P. P. Gaspar, *ibid.*, **92**, 4379 (1970).

(2) For a report on the properties of an isomeric ten-π-electron carbocyclic aromatic carbene, see R. A. LaBar and W. M. Jones, *ibid.*, **95**, 2359 (1973).

(3) E. Vogel, *Chem. Soc., Spec. Publ.*, No. **21**, 113 (1967).

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(6) Except where noted, all new compounds gave satisfactory elemental analyses.

(7) Subjection of **8** and **9** to the original reaction conditions resulted in no detectable interconversion but only polymerization to colorless solids.

(8) We thank Professor E. Vogel for generously providing a sample of the chloroannulenone.