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THE STRUCTURAL CHARACTERIZATION OF ENTEROMYCIN CARBOXAMIDE

A NEW STREPTOMYCETE ANTIBIOTIC

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Abstract—A crystalline antibiotic has been isolated from an unknown species of *Streptomyces* and its structure established as N-(O-methyl-*aci*-nitroacetyl)-3-amino acrylamide (I). The relationship between this antibiotic and enteromycin[1] is discussed.

DURING the course of an investigation of the antibiotic activity produced by an unknown species of *Streptomyces*, a new crystalline antibiotic was isolated and identified as N-(O-methyl-*aci*-nitroacetyl)-3-amino acrylamide (I), the amide of the known antibiotic enteromycin.*[1]

Special problems were encountered in isolating and characterizing the antibiotic due to its very poor solubility in most common solvents and its marked instability towards heating. It was found, indeed, to be soluble only in glacial acetic acid, dimethylsulfoxide, dimethylformamide or, to a much smaller extent, in methanol. Enteromycin carboxamide* could be crystallized from these solvents in small white rosettes, m.p. 155° (dec). The antibiotic proved to be extremely unstable and only some 10% of the material being crystallized could be recovered as crystalline antibiotic even when a nitrogen atmosphere was used. A similar loss was encountered upon recrystallization. NMR examination of the crude material, before crystallization, established the presence of enteromycin carboxamide (I) and thus diminished the likelihood that I was formed by some thermal process from an uncharacterized precursor. The residue generally proved to be intractable gums.

Although the NMR spectrum of these gums was very poor, containing a series of very broad maxima indicative of generalized decomposition, the IR and UV spectra were very similar to those of the material obtained after heating the antibiotic in the dry state at 110°. This product (III) will be described later.

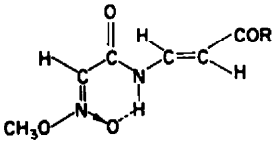
The crystalline antibiotic has an empirical formula of $C_6H_9N_3O_4$ established on the basis of several analyses. Its low solubility, however, and lack of volatility prevented the determination of its mol. wt. by conventional means or by mass spectroscopy, and its poor crystal form eliminated use of the X-ray technique. Basic hydrolysis of the antibiotic liberates ammonia, identified as ammonium chloride by paper chromatography. The amide function thus suggested is also indicated by a band in

* The name enteromycin amide has been used in the Lit. to describe a degradation product of enteromycin. The substance so named is an amidine resulting from the addition of ammonia to the *aci*-nitro ether function. For ease of reference and to avoid confusion with this substance, we have chosen to name our compound enteromycin carboxamide.

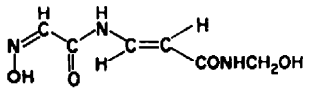
[1] K. Mizuno, *Bull. Chem. Soc. Japan* **34**, 1633 (1961).

the IR spectrum at 1674 cm^{-1} while other bands, at 1110 and 985 cm^{-1} suggest an ether and a *trans* double bond respectively. The UV spectra in neutral and acidic media are essentially identical (λ max 230, 270 (sh) and 300 mμ) but in alkaline solution particularly distinctive changes occur suggesting decomposition to an ultimately stable tautomeric fragment. Maxima are initially observed at 272 and 341 mμ, shifting finally to a single peak at 268 mμ (after 1 hr), which disappears upon acidification (leaving a solution transparent throughout the UV region) and is regenerated by basification. The antibacterial activity was also found to disappear rapidly in alkaline solutions.

At this point a striking similarity was noted between these spectra and those of the known antibiotic enteromycin, the structure of which was established by Mizuno[1] to be N-(O-methyl-*aci*-nitroacetyl)-3-amino acrylic acid (II). Unlike enteromycin,

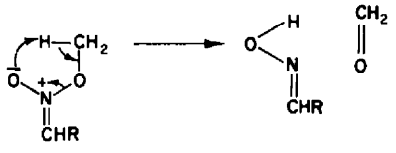


I, R = NH_2
II, R = OH



III

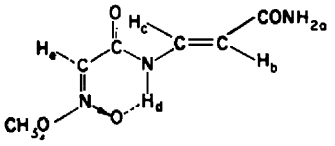
however, our antibiotic was not titratable as an acid and since its molecular formula indicated an additional NH_2 and a lack of one oxygen atom it seemed likely that we had isolated the carboxamide of enteromycin. A key reaction performed by Mizuno in establishing the enteromycin structure was the isolation of glycyl-β-alanine after hydrogenation. It was expected, therefore, that hydrogenation of I under the same conditions would produce glycyl-β-alanyl amide. This was found to be the case. The complex mixture produced was examined by paper chromatography using several solvent systems and compared with an authentic sample of glycyl-β-alanyl amide. The mobilities and ninhydrin color clearly established the major product to be identical to the model compound. Further confirmation of this result was obtained by hydrolysis of the hydrogenation mixture to give glycine and β-alanine, identified by paper chromatographic comparison with authentic samples. These results established the sequence of all the nitrogens and all but one of the carbons in the molecule. The remaining carbon atom, from the spectra of I and by analogy to enteromycin, was thought to occur as an *aci*-nitro-N-(O-methyl)group, an inference consistent with the production of formaldehyde concomitant with loss of the ether peak in the NMR and IR spectra on heating the compound. This reaction, the suggested mechanism of which is shown below,[1] is typical of the *aci*-nitro ether grouping.



Although enteromycin carboxamide is poorly soluble in most suitable solvents, it was possible to measure NMR spectra in dimethyl sulfoxide and trifluoroacetic acid if care was taken to prevent decomposition upon preparing the solutions. Although

not all of the peaks were visible, the gross features agree well with the proposed structure (Table 1).

TABLE 1. NMR (60 mc) SPECTRA OF ENTEROMYCIN CARBOXAMIDE



Protons	Chemical Shift (c/s)	
	D_6-DMSO	TFA
a	390–465 broad	—
b	348 (d, 1) J = 14 cps	382 (d, 1) J = 14 cps
c	obscured	obscured
d	—	—
e	425	440
f	225 (s, 3)	245 (s, 3)
f′	257 (s, 1/2)	250 (s, 2/3)

The observed coupling constant for H_b (14 c/s) is consistent with the IR suggestion of a *trans* double bond and the chemical shift places it α-to a carboxyl derived function. Unfortunately proton c is buried under several protons in dimethylsulfoxide and under the solvent resonance in trifluoroacetic acid. The presence of *syn* and *anti* forms in the antibiotic is suggested by the presence of a satellite singlet near the strong H_f peak (labeled f′ in the chart). Although it is a sharp singlet in a clear region it does not integrate to be a whole proton. In apparent confirmation H_e is not a sharp singlet but bears a shoulder. These minor peaks could result from the slightly different molecular environment about the *aci*-nitro ether function in the two possible double bond orientations in much the same way as has been shown to occur with aldehyde 2,4-dinitrophenylhydrazones.[2]

Thus the spectral and chemical data are in accord with the hypothesis that the new antibiotic is in fact carboxamide of enteromycin. The thermal instability of the two antibiotics is satisfactorily explained by the presence of the *aci*-nitro ether function which characteristically decomposes to formaldehyde and an oxime upon heating. An interesting aspect of the chemistry of enteromycin carboxamide resides in the fact that in contrast to enteromycin itself the liberation of formaldehyde proceeds only in very poor yields. In fact, on some occasions no formaldehyde could be detected, and in this event the residual solid proved to have the same empirical formula as the starting material but lacked O- or N-methyl groups. This finding is confirmed by the progressive disappearance of the strong ether band at 1110 cm^{-1} during the heat treatment. Mild acidic hydrolysis of the heat degradation product liberated acetaldehyde and formaldehyde while enteromycin carboxamide itself liberates only acetaldehyde under these conditions. Both the heat degradation product and enteromycin carboxamide yield glycyl-β-alanyl amide upon hydrogenation in acidic media and the ultraviolet absorption spectrum of the heat degradation product matches that of demethoxyenteromycin (obtained by thermal degradation of enteromycin) indicating that the molecular skeleton is still intact. These observations suggest that an internal

[2] G. T. Karabatos, R. A. Taller, and F. M. Vane, *J. Amer. Chem. Soc.* **85**, 2327 (1963).

rearrangement occurred during the heat treatment which involved the methyl ether function in such a way as to produce a grouping which was readily removed by hydrogenation in acidic media and was also capable of liberating formaldehyde upon mild acid hydrolysis. The simplest explanation for the observed results would be the formation of a methylol function by reaction of the liberated formaldehyde with the primary amide function as is illustrated by formula III. Such adducts between formaldehyde and amines and amides of all kinds are well known and have been isolated from many different systems.[3] Ample precedent exists for such a reaction although the conditions of formation in this case are rather special. The fact that enteromycin itself does not react in the same way would follow as a natural consequence of its being a carboxylic acid and not a carboxamide. The expected poor basicity of the secondary amide grouping in enteromycin carboxamide (actually a vinylogous imide function) diminishes the probability of reaction at that site. Unfortunately the heat degradation product is unstable in all the solvents which have been examined to date and this has so far eliminated the possibility of a diagnostic NMR spectrum.

EXPERIMENTAL

Isolation of enteromycin carboxamide. The fermentation broth (30 l.) was filtered to remove suspended solids and the beer filtrate slurried with 5·5% (w/v) magnesol for 30 min. The mixture was filtered, the filtrate discarded and the magnesol cake slurried with 85% aqueous acetone (30 min, 6 l.). Filtration of this mixture followed by concentration of the filtrate to an aqueous phase precipitated an amorphous brown solid (2·0 g). This material was dissolved in hot glacial acetic acid and from this solution, on cooling, the antibiotic crystallized as small white rosettes, m.p. 155° (dec) λ_{max}^{MeOH} 230, 270 (sh), 300 mμ ($E_{1cm}^{1\%}$ 650, 740, 820). No change was observed in the UV spectrum on acidification; however, with base a marked shift was noted; λ_{max}^{MeOH} 272, 341 mμ ($E_{1cm}^{1\%}$ 680, 860) and after 1 hr, λ_{max}^{NaOH} 268 mμ ($E_{1cm}^{1\%}$ 1080). (Found: C, 38·6; H, 5·0; N, 21·7; O, 35·2; OMe, 7·0. $C_6H_9N_3O_4$ requires: C, 38·5; H, 4·8; N, 22·4; O, 34·2; OMe, 8·0%).

Degradation of enteromycin carboxamide

(a) *By base:* A sample of enteromycin carboxamide (1·0 g) was dissolved in 0·1 N NaOH (20 ml) and the flask connected *via* a glass tube to one containing 10 ml 1·0 N HCl. This apparatus was allowed to stand at room temp for 18 hr. The acid solution was taken to dryness, redissolved in 1 ml water and paper chromatographed on Whatman No. 1 paper using a butanol–acetic acid–water (4:1:5) system. The chromatograms were first sprayed with a 1% solution of 1,2-napthaquinone-4-sulphonic acid sodium salt, dried, and then sprayed with sat $NaHCO_3$ aq (Folin's reagent).[4] The hydrochlorides of methyl and dimethylamine and NH_4Cl were also examined. From the colour and R_f of the spot given by the reaction mixture basic degradation of enteromycin carboxamide produced ammonia.

(b) *By acid.* A sample of crystalline enteromycin carboxamide (50 mg) was dissolved in dimethylsulfoxide (0·5 ml) and the solution diluted with water (5 ml). After addition of some conc. H_2SO_4 (3 drops) the solution was placed in a Conway dish containing excess 2,4-dinitrophenylhydrazine reagent and the dish maintained at 35° in an incubator for 5 days. Examination of the reagent solution by thin layer chromatography on silica gel plates using Prelog's system (CCl_4–ether (4:1))[5] and comparing with various known samples established that acetaldehyde had been released from enteromycin carboxamide.

(c) *By heat.* A sample of crystalline enteromycin carboxamide (100 mg) was heated in the absence of solvent at 110° in a stream of N_2 for 6 hr. The N_2 flow was arranged to carry the volatile degradation products into 2,4-dinitrophenylhydrazine reagent which was subsequently examined by thin layer chromatography using Prelog's system. The presence of formaldehyde 2,4-dinitrophenylhydrazone was indicated. The solid residue remaining melted at 158° (dec) and showed no evidence

[3] J. F. Walker, *Formaldehyde* p. 206. Reinhold, New York (1944).
[4] K. Blau, *Biochem. J.* **80**, 193 (1961).
[5] V. Prelog, A. M. Gold, G. Talbot and A. Zamojski, *Helv. Chim. Acta* **45**, 4 (1962).

of an ether peak in the IR; $\lambda_{max}^{H_2O}$ 222, 280 mμ. (Found: C, 38·8, H, 4·5; N, 23·0; OMe, 0·5. $C_8H_9N_3O_4$ requires C, 38·5; H, 4·8; N, 22·4%).

Hydrolysis of this material under exactly the same conditions as those described for the acid degradation of enteromycin carboxamide yielded formaldehyde and acetaldehyde, identified as their 2,4-dinitrophenylhydrazones by thin layer chromatography on silica gel.

Hydrogenation of enteromycin carboxamide. A crystalline sample of enteromycin carboxamide (400 mg) was dissolved in hot glacial acetic acid and PtO_2 (100 mg) was added. The mixture was stirred in an atm. of H_2 for 18 hr. The total apparent uptake was 100 ml. The catalyst was removed by centrifugation and the solvent by distillation under vacuum. The residue, a brown gum, was dissolved in methanol and the solution allowed to stand at room temp for 48 hr. A small quantity of microcrystalline material was precipitated (Fraction A), but the mother liquor on concentration yielded only gum (Fraction B). The solid and gums were separately dissolved in some methanol to which a drop of HCl was added. These solutions were then examined by paper chromatography using ninhydrin for detection and compared with an authentic sample of glycyl β-alanyl amide hydrochloride[6] using several systems (Table 2). Mixed samples were inseparable. The crude hydrogenate (Fraction B) was dissolved in 25 ml 6 N HCl and the solution placed in a sealed tube and heated at 120° for 12–14 hr. The final solution was examined by paper chromatography using the butanol–acetic acid–water (4:1:5) system and compared with glycine, β-alanine and DL-alanine (Table 3). Ninhydrin was used for detection. The R_f and color indicated that glycine and β-alanine were present.

TABLE 2. PAPER CHROMATOGRAPHY OF ENTEROMYCIN CARBOXAMIDE HYDROGENATION PRODUCTS AND GLYCYL-β-ALANYL AMIDE HYDROCHLORIDE

System	R_f		
	Crude hydrogenate Fraction B	Solid hydrogenate Fraction A	Glycyl-β-alanyl amide Hydrochloride
BuOH–H_2O–HOAc (4:5:1)	0·21 (Br) 0·32 (B) 0·42 (B)	0·20 (Br)	0·23 (Br)
BuOH–Pyridine–H_2O (10:1:5)	0·07 (Br) 0·12 (P) 0·19 (P)	0·07 (Br)	0·07 (Br)
BuOH saturated with 3% NH_4OH solution	0·05 (P) 0·10 (P) 0·19 (Br)	0·20 (Br)	0·19 (Br)
BuOH saturated with 1% *p*-toluenesulphonic acid solution	—	0·4 (Br)	0·4 (Br)
5% Na_3PO_4 solution	—	0·86 (Br)	0·86 (Br)

Colour code: Br = Brown B = Blue P = Purple

TABLE 3. PAPER CHROMATOGRAPHY OF HYDROLYSATE OF ENTEROMYCIN CARBOXAMIDE HYDROGENATION PRODUCT, β-ALANINE, DL-ALANINE AND GLYCINE

Compound	R_f	Color
β-Alanine	0·30	Blue
DL-Alanine	0·28	Purple
Glycine	0·19	Red-brown
Hydrolysate of enteromycin carboxamide Hydrogenation product	0·14	Yellow
	0·19	Red-brown
	0·30	Blue

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[6] Supplied by Cyclo Chemical Corp., Los Angeles, Calif.