FURTHER STUDIES ON ASN-136 AND MONOKETO-ORGANOMYCIN CYSTAURIMYCIN, A BROAD SPECTRUM SUBSTANCE PRODUCED BY PARTIAL ENZYMIC DIGESTION OF MONOKETO-ORGANOMYCIN

GAMAL M. IMAM and HANS KÜNTZEL

Max-Plank-Institut für Experimentelle Medizin, Abteilung Chemie,
Hermann-Rein-Straße 3, 3400- Göttingen, West-Germany (BRD)

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The two antibiotics ASN-136 and monoketo-organomycin (MKOM) showed very close similarities in their UV, IR spectra and elemental analysis to those of tuberactinomycin and yazumycin respectively. Further chemical and enzymic studies revealed the novelty of the two former antibiotics. Partial enzymic hydrolysis of MKOM yielded a hydrolytic product of more potent inhibitory action compared with the parent antibiotic. Having cystine as the N-terminus and taurine as the C-terminus in its molecule, this enzymic degradation product was designated cystaurimycin. Performic acid oxidation of MKOM and of cystaurimycin improved their growth inhibitory effects on the test organisms used.

Isolation and characteristics of ASN-136 and MKOM are already described.[1,2] On acid hydrolysis ASN-136 splits into arginine and three unidentified ninhydrin-positive compounds[1] while MKOM yielded glycine, phenylalanine, cystine, glutamic, citric, fumaric and pyruvic acids in addition to two unidentified substances of positive ninhydrin reaction.[2] However, a comparison of elemental analysis, UV and IR spectra of ASN-136 and those of tuberactinomycin (tuberactin)[3] and of MKOM and those of yazumycin[4] indicated very close similarities. The present antibiotics were, however, differentiated from these antibiotics[3,4] by the absence of lysine and its homologues as well as serine from their molecules.

While gram-negative bacteria are sensitive to tuberactinomycin,[3] they were almost resistant to ASN-136.[1] Yazumycin is mainly active against *Mycobacterium smegmatis*, gram-positive and gram-negative bacteria[4] but MKOM is mainly active against gram-negative ones.

The present paper concerns the further characterization of ASN-136 and MKOM, production of cystaurimycin from MKOM and investigation of the effect of performic acid oxidation on the growth inhibitory effects of the latter two substances.

Materials and Methods

ASN-136 and MKOM were prepared as described,[1,2] purified by repeated precipitation from aqueous solutions by addition of excess ethanol and then stored dry at −5°C till used.

Acid Hydrolysis: 6 N HCl in the presence and absence of 0.1% β-mercaptoethanol for 20 hours at 110°C was used. A sample was withdrawn, diluted with water to 2 N HCl, 2,4-dinitrophenylhydrazine was added to the final concentration of 4 g/liter and cooled overnight. The resulting hydrazones were extracted with ethyl acetate and concentrated (see later). The rest of the hydrolysate was analyzed for its contents of amino and aliphatic acids as described later.

Enzymic Hydrolysis: The method given by HILL and SCHMIDT[5] was followed using papain, leucine aminopeptidase (LAP) and prolidase (Sigma, U.S.A.). The reaction was stopped by addition of few drops of HCl to pH 1.2 followed by lyophilization. The dry hydrolysate was dissolved in 0.1 N HCl and centrifuged. The clear supernatant was applied to Dowex-50 X2 [H^+] column (0.5×3 cm,

Sirva - W. Germany), washed with water and then eluted with 3 M ammonium hydroxide. The alkaline eluate was lyophilized, redissolved in the suitable solvent and analyzed as in case of acid hydrolysates.

The undigested fraction(s) of a given antibiotic was separated from the amino acids using two-dimensional separation technique as described by KATZ *et al.*[6] as well as by ascending paper chromatography using Whatmann No. 1 filter sheets and butanol - acetic acid - water (4: 1: 5, v/v) solvent. The dried chromatograms were sprayed with 0.02% ninhydrin in acetone. Spots other than amino acids were cut out and eluted with 6 N HCl. A sample was directly hydrolyzed with subsequent analysis of the hydrolysate while another sample was dried under reduced pressure, dissolved in phosphate buffer, pH 7, and assayed for its antimicrobial activity before and after performic acid oxidation.[7] The rest of the chromatogram, apart from the stained spots, was found to contain no amino or aliphatic acids after acid and alkaline hydrolysis.

N-Terminal Determination

(1) Using dansyl chloride (Dans-Cl)*: Dansylation of antibiotic was made in presence of 0.3 M triethylamine of pH 10 and an equal volume of ^{3}H-labelled Dans-Cl solution (specific activity 7 Ci/mmol; The Radio-chemical Centre, Amersham, Bucks., U.K.). The reaction mixture was incubated in a water bath at 37°C for 2 hours and dried. The dansyl antibiotic was hydrolyzed for one hour with 6 N HCl containing 0.5% β-mercaptoethanol and dried *in vacuo*. Purification, separation and identification of dansyl amino acids was conducted as given later.

(2) Using LAP: Digestion was made according to the method of HIRS *et al.*[8] using an enzyme substrate ratio 1: 40 (w/w) with subsequent analysis of the hydrolysates as described before.

C-Terminal Determination

(1) By Hydrazinolysis: Anhydrous hydrazine (95% +; Roth - W. Germany), 2 ml, in the presence of 5 mg hydrazine sulphate and 30 mg Amberlite IRC–50 [H^+], was used for each 2.5 mg of antibiotic. The evacuated tubes were then sealed, incubated at 60°C for 18~20 hours and lyophilized over P_2O_5 and H_2SO_4 for removal of excess hydrazine. The liberated free C- terminal amino acids and amino acids hydrazides were eluted from the resin by water followed by centrifugation. Free amino acids were separated from the hydrazides as described by BRAUN and SCHROEDER[9] and analyzed.

(2) By use of carboxypeptidases: This was made according to the method given by AMBLER.[10]

Amino Acid Analysis

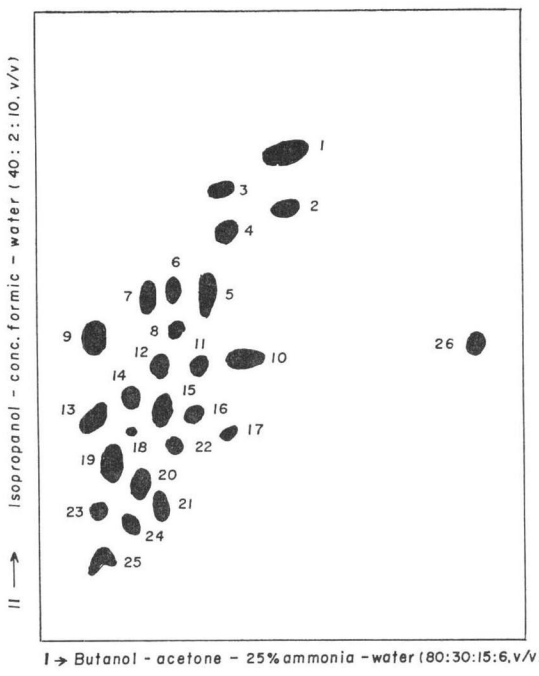
(1) Direct amino acid analysis: Two-dimensional separation of amino acids was performed using cellulose TLC-plates (20×20 cm, 0.25 mm thick, Macherey - Nagel, W. Germany). Amino acids were located by spraying with ninhydrin (0.1%) dissolved in a mixture of collidine - acetic acid - methanol (5: 20: 75, v/v), heated at 90°C for few minutes, kept in a humid dark place for 1 hour and investigated. Sharp separation between the various amino acids (Fig. 1) could be repeatedly achieved.

(2) By dansyl amino acid analysis: A sample of antibiotic hydrolysate was dried *in vacuo*, dissolved in 0.1 M $NaHCO_3$ (pH 8.9) and dried. ^{3}H- labelled Dans-Cl was added, the mixture incubated at 37°C for 1 hour and then dried. Excess Dans-Cl and dansylamine were extracted from the solid material by neutral peroxide-free ether. The remaining material was dissolved in water, acidified to pH 1.2 with formic acid and centrifuged. A mixture of 18 non-labelled dansyl amino acids (5 pmol each) was added to the supernatant and the solution was evaporated. The dry material was then dissolved in 0.01 M triethylamine (pH 10) and loaded onto a small column of Dowex-50 X1 (formate form) equilibrated with the same buffer. After desalting, using the column buffer, dansyl amino acids were eluted with 0.01 M formic acid containing 25% acetone. Dans-OH remained tightly fixed to the resin.

The acid eluate was dried *in vacuo*, redissolved in acetone - formic acid (19: 1, v/v) solution and loaded onto polyamide plates (Schleicher and Schüll, W. Germany). Separation of dansyl amino acids was made using the method of HARTLEY[11] as modified by LEE and SAFILLE[12] and by RAMSHAW *et al.*[13] The dry plates were examined by the UV light and the fluorescent spots were cut out as described by BROWN and PERHAM,[14] placed in scintillation vials containing a small volume of acetone - formic acid solution and left for 30 minutes for elution. Aquasol Scintillation cocktail was added, polyamide material was removed using a long needle and counted for ^{3}H in Packard Tri-Carb Scintillation

* Abbreviations: dansyl=1-dimethylaminonaphthalene-5-sulfonyl; dans-OH=dansylic acid, 1-dimethylaminonaphthalene-5-sulphonic acid.

Fig. 1. Thin-layer chromatography of amino acids on cellulose plates.



1 Isoleucine
2 Phenylalanine
3 Leucine
4 Valine
5 Methionine
6 Tryptophan
7 Alanine
8 Proline
9 Glutamic acid
10 Threonine
11 Tyrosine
12 Glucosamine
13 Aspartic acid
15 Serine
16 Galactosamine
17 Methionine sulfone
18 Methionine sulfoxide
19 Arginine
20 Lysine
21 Histidine
22 Taurine
23 Cysteic acid
24 Ornithine
25 Cystine
26 Ethanolamine

spectrometer.

Analyses of aliphatic and keto acids: These were conducted as described by SHIMI *et al.*[15,16] and by SHIMI and IMAM.[17,18]

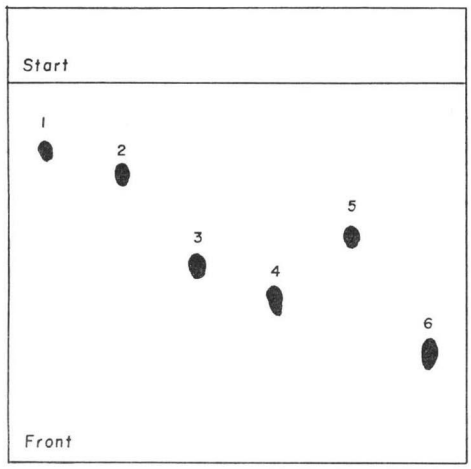
Results and Discussion

A. MKOM

On investigating the acid hydrolytic artifacts of MKOM, the two unidentified compounds,[1] using the present methods of analyses, were identified as ornithine and taurine. Taurine was separated from the antibiotic hydrolysate using Dowex-50 X8 [H^+], 50~100 mesh (Sirva, W. Germany). Taurine was eluted with water and was the only amino compound detected in the eluate as revealed by TLC on cellulose plates as well as on electrophoresis using Whatmann No. 3MM filter sheets, 50 mM acetate buffer (pH 3.8), 90 v/cm, 2 mA/cm for 30 minutes. Only one ninhydrin-positive spot was always obtained when a sample of the water eluate was chromatographed solely or after being mixed with an authentic sample of taurine. Thus the following compounds remain as the components of the MKOM molecule: glycine, cystine, phenylalanine, glutamic acid, ornithine, taurine, pyruvic, citric and fumaric acids. The latter compound was present in relatively low amounts compared with citrate and pyruvate.

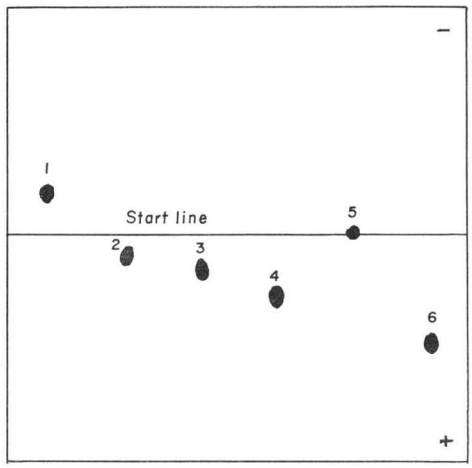
Using LAP for N-terminal determination, glycine and phenylalanine were the only amino acids liberated from the antibiotic molecule with up to 24 hours of incubation. Analysis of dansyl MKOM indicated, however, the presence of glycine as the N-terminal amino acid. While carboxypeptidases A and or B failed to release any amino acid from the antibiotic molecule with up to 18 hours of incubation, taurine was detected as the C-terminal compound by hydrazinolysis. Failure of carboxypeptidases to hydrolyze taurine from the C-terminal position is not unexpected in light of the specificity of these

Fig. 2. Chromatography of MKOM and its enzymic degradation products.



Solvent used: butanol - acetic - water (4: 1: 5, v/v); Whatmann No. 1

Fig. 2a. Electrophoresis of MKOM and its enzymic degradation products.



Buffer used: formic - acetic - water (4: 1: 45, v/v), pH 3.1; Whatmann No. 3MM; 60 v/cm, 30 min.

Where: 1=MKOM, 2=performic acid-oxidized MKOM, 3=MKOM after digestion with LAP, 4=oxidized form of (3), 5=cystaurimycin, 6=performic acid-oxidized cystaurimycin.

enzymes.[19] Digestion of MKOM with papain, LAP and prolidase resulted in the liberation of glycine, phenylalanine and glutamic acid. No other components were detected with up to 24 hours of incubation. Of interest is that the same result could be obtained by using papain alone in an enzyme substrate ratio of 1 : 40 by weight and an incubation period of 2 hours in phosphate buffer of pH 8.5 at 37°C. That none of the aliphatic acids was found in the enzymic hydrolysates would indicate that none of these compounds was linked to either the N- or C-terminal residue. In addition, the absence of O-dansyl tyrosine and ε-dansyl lysine in the hydrolysate of dansyl MKOM confirms the absence of the corresponding amino acids in the antibiotic molecule. Similar results were obtained when the performic acid-oxidized MKOM was used. Acid hydrolysis of the undigested fractions indicated the presence of all of the compounds present in the intact antibiotic molecule indicating that the existence of glycine, phenylalanine and glutamic acid was not only restricted to the N-terminal region of the antibiotic molecule. N- and C-termini analyses, using Dans-Cl and hydrazine respectively, for the enzymic undigested fractions revealed (N- . . . C-termini): glycine . . . taurine, glutamic . . . taurine and cystine . . . taurine for those obtained after digestion with carboxypeptidases; LAP and papain – LAP – prolidase mixture (or papain alone) respectively.

No sharp differences were found in the UV and IR spectra and behaviour towards different chemical tests of the various undigested compounds when these were compared with those exhibited by the intact antibiotic molecule[1] but they showed differences in their chromatographic and electrophoretic behaviour (Fig. 2).

Results of the antimicrobial studies of the various enzymic undigested compounds are given in Table 1 along with MKOM for comparison. These compounds were tested before and after being oxidized with performic acid. The data indicate that the antimicrobial activities of the different tested compounds are different from that of MKOM and that the oxidized forms are of more potent inhibitory

Table 1. Antimicrobial studies on MKOM and its enzymic degradation products

Test organism	M.I.C. (mcg/ml)					
	MKOM		MKOM after digestion with LAP		MKOM after digestion with papain (cystaurimycin)	
	−	+	−	+	−	+
* *Escherichia coli* B-2346	0.1	0.05	3.0	1.0	0.01	0.005
* *Escherichia coli* NCTC 6820	3.0	1.5	30.0	10.0	0.1	0.01
* *Escherichia coli* 0-127, NCTC 9707	5.0	3.5	25.0	12.5	0.1	0.05
* *Klebsiella pneumonia* 0-2, NCTC 5046	1.2	0.6	7.5	5.0	0.1	0.05
* *Salmonella typhi*	100.0	100.0	R	R	30.0	15.0
* *Salmonella typhosa* NRRL-B-210	R	R	R	R	50.0	35.0
* *Proteus mirabilis*	20.0	15.0	80.0	60.0	10.0	6.5
* *Proteus rettgeri* 1163-CDC	R	R	R	R	75.0	50.0
* *Pseudomonas aeruginosa* ATTC 9027	10.0	10.0	50.0	50.0	5.0	5.0
* *Staphylococcus aureus* D6	10.0	10.0	75.0	50.0	5.0	1.5
* *Bacillus subtilis* D161 - staphylomycin R	1.5	1.5	30.0	20.0	0.5	0.1
Bacillus subtilis D161 - chloramphenicol R	20.0	20.0	R	80.0	10.0	3.5
* *Bacillus cereus* NRRL B-569	10.0	10.0	100.0	80.0	7.5	5.0
Bacillus mycoides	100.0	80.0	R	125.0	75.0	30.0
Shigella equis	100.0	100.0	R	R	75.0	50.0
Candida albicans Y-477	R	R	R	R	100.0	80.0
Saccharomyces cerevisiae NRRL Y-567	R	R	R	R	75.0	50.0
Penicillium chrysogenum B-2000	R	R	R	R	50.0	30.0
Aspergillus niger	R	R	R	R	120.0	90.0
Streptomyces AS-400 (MKOM-producing)	R	R	R	R	60.0	25.0
End group:- N-terminus	Glycine		Glutamic acid		Cystine	Cysteic acid
C-terminus	Taurine		Taurine		Taurine	Taurine

+ Performic acid-oxidized, − not oxidized and * pathogenic organisms.
M.I.C.=Minimum inhibitory concentration. R=Resistant.

actions than the corresponding non-oxidized ones. Oxidation of MKOM improved its activity against *Escherichia coli* and *Klebsiella pneumonia* while the rest of the organisms responded more or less similarly to the two forms of MKOM. Removal of glycine and phenylalanine from the N-terminal region of MKOM by LAP yielded a compound of low antimicrobial activity and despite of this its antimicrobial activity was improved after performic acid oxidation though the activities of the two forms remained beyond that of MKOM. Further removal of glutamic acid by papain or by the enzyme mixture used resulted in the production of a degradation product (with N-terminal cystine and C-terminal taurine) which, unexpectedly, was found of potent inhibitory action on the growth of the used test organisms including those which are resistant to MKOM, the parent antibiotic. This enzymic degradation product was designated cystaurimycin. The antimicrobial activity of the latter substance was appreciably increased after performic acid oxidation (Table 1). Of interest is that cystaurimycin as well as its performic acid-oxidized form (having cysteic acid as N-terminus and taurine as C-terminus) could inhibit the growth of *Streptomyces* AS-400, the MKOM-producing organism.

Cystaurimycin as well as its oxidized form could be produced by digestion of MKOM and its performic acid-oxidized form respectively by papain as mentioned before. The conversion was found to be almost quantitative. Pure forms of these compounds could be obtained by applying the enzymic hydrolysates at pH 1.2 to column chromatography using Dowex-50 X8 [H^+], 50~100 mesh with subsequent elution with water. The biologically active compounds are then precipitated from their concen-

trated aqueous solutions by addition of excess acetone or ethanol, cooling overnight at 5°C and separated by centrifugation.

Cystaurimycin and its performic acid-oxidized form showed similar chemical and physical properties to MKOM but they differ from the latter in their N-termini, in their antimicrobial scope of activity (Table 1), in their chromatographic behaviour (Fig. 2) and by having no sharp melting points.

The *in vivo* studies revealed no lethal effects when 50 and 35 mg/kg body weight of cystaurimycin and its oxidized form respectively were injected to Swiss mice intravenously. Higher doses, particularly of the oxidized form, were found to result in the disturbance of the heart beats followed by paralysis and death after 36 hours of injection.

B. ASN–136

Out of the three unidentified components of the antibiotic molecule, only one could be identified as ethanolamine. The latter was found to form the C-terminal residue as indicated by the hydrazine reaction while carboxypeptidases failed to release this compound. Digestion with LAP as well as analysis of the dansyl antibiotic revealed arginine as the N-terminal amino acid residue. Digestion with LAP - papain - prolidase mixture led to the breakdown of the antibiotic molecule into arginine and two ninhydrin-positive unknown compounds of Rf values 0.35 and 0.45 when separated on Whatmann No. 1 filter sheets using butanol - acetic acid - water (4: 1: 5, v/v) as the developing solvent. Acid hydrolysis of the substance located at Rf 0.45 resulted in the production of ethanolamine and an unidentified ninhydrin-positive compound of Rf value 0.12 using the same chromatographic system. No further degradation of the other unknown compound could be achieved. Thus the antibiotic molecule is composed of arginine, two unknown compounds and ethanolamine.

The above results indicate the novelty of the antibiotics under investigation. MKOM and cystaurimycin are characterized by the presence of taurine in their molecules and by the absence of lysine and its homologues. The latter compounds were reported to be present in the molecules of yazumycin,[4] racemomycins A and C[20,21] and streptothricins.[22] ASN–136 is different from tuberactinomycins[3,23,24,25] and viomycin[26] in having no lysine, serine and gaunidino amino acid moieties in its molecule.

The presence of taurine in the molecules of MKOM and cystaurimycin was confirmed by multi-dimensional chromatography.[27] Taurine might have been present originally in the antibiotic molecule as cysteic acid, which might have been decarboxylated, or as cystine followed by oxidation and decarboxylation within the cells of the producing organism. It is unlikely, however, that such chemical changes would have occurred during the isolation of the original materials.

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