METABOLIC PRODUCTS OF MICROORGANISMS 116*
O-[L-NORVALYL-5]-ISOUREA, A NEW ARGININE ANTAGONIST

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From a bacterial culture a new antibiotic, O-[L-norvalyl-5]-isourea, was isolated. The structure was determined by mass spectrometry and confirmed by chemical synthesis. The antibiotic activity is inhibited by L-arginine, L-ornithine and L-citrulline.

Previously arginine antagonists have been found in plants *e.g.* canavanine[1], homoarginine[2], indospicine[3] and in a streptomyces strain L-N^5-(1-iminoethyl)-ornithine[3] (Table 1). The arginine antagonist described in this paper was found in a bacterial strain (Table 3, I).

The antibiotic was isolated from the cultures by centrifugation and subsequent adsorption on charcoal and elution with methanol-water. Further purification was achieved by ion-exchange chromatography on Dowex 50 WX 2 and chromatography on Sephadex G 10 and Biogel P 2.

The active fractions were collected and lyophilized. The colorless material shows a positive reaction with ninhydrin; however, the SAKAGUCHI reaction[5] for guanidine compounds is negative.

The agar diffusion assay shows the antibiotic to be highly active against *Escherichia coli* K 12 and *Bacillus subtilis* Tü 203 on a chemically defined medium. The minimal inhibitory concentration is 20~40 μg/ml on *E. coli* K 12 submersed culture in defined media and 5~10 μg/ml on *B. subtilis* Tü 203. The antibiotic activity can be inhibited by L-arginine, L-ornithine or L-citrulline. The antibiotic is readily cleaved by arginase and the resulting products show no antibiotic activity.

Table 1. Arginine antagonists

Structure	Name	Reference
$H_2N-C(=NH)-NH-O-CH_2-CH_2-CH(NH_2)-COOH$	Canavanine	(1)
$H_2N-C(=NH)-NH-CH_2-CH_2-CH_2-CH_2-CH(NH_2)-COOH$	Homoarginine	(2)
$H_2N-C(=NH)-CH_2-CH_2-CH_2-CH_2-CH(NH_2)-COOH$	Indospicine	(3)
$H_3C-C(=NH)-NH-CH_2-CH_2-CH_2-CH(NH_2)-COOH$	Iminoethyl-ornithine	(4)

Materials and Methods

1. Materials

Bacterium Tü 222 is a

* Metabolic products of microorganisms, 115; G. WINKELMANN and H. ZÄHNER, Eisenaufnahme bei *Neurospora crassa*. I. Zur Spezifität des Transports. Arch. Mikrobiol., in press.

Table 2. Further characterization of *Bacterium* Tü 222 ; growth at 27°C[6,7]

	Reaction		Reaction		Reaction
Adonitol	(−)	Sucrose	+	Indole	(−)
Dulcitol	(−)	Xylose	+	Methyl red	+
Inositol	(×)	Ammonium		VOGES-PROSKAUER	+
D-Mannitol	+	citrate	+	Liquefaction	
Salicin	(+)	Malonate	(−)	of gelatin	(×)
Sorbitol	(−)	Tartrate	(−)	Thioglycollate medium	
Ammonium		Acid form		aerobe zone	+
glucose	+	glucose	+	microaerobe zone	++
Fructose	+	lactose	+	anaerobe zone	+
Arabinose	+	Gas form		Nitrate to nitrite	+
Galactose	+	glucose	(−)	Catalase	+
Maltose	+	lactose	(−)	Oxidase	(−)
Raffinose	(−)	Motility	+	Macrolide antibiotics	(−)
Rhamnose	+	H_2S production	(−)		
Trehalose	+	NH_4^+ production	(−)		

Symbols : + positive reaction. (+) positive reaction after 3 or more days. (×) weak positive reaction after 3 or more days. (−) no reaction

1.25~3.4 μ gram-negative, rod-like, fermentative, flagellated bacterium (lateral bundles consisting of 3~4 flagellates about four times as long as the bacterium). Colonies on agar media show a white color, which later becomes yellow.

2. Fermentation

The Tü 222 strain was incubated in a 10-liter fermentor on defined medium (9.1 liters end volume, 27°C, aerated at 8 liters per minute and agitated at 400 rpm).

Composition of medium : 2 g NH_4NO_3, 1 g KH_2PO_4, 2 g K_2HPO_4, 2 g $Na_2HPO_4 \cdot 12\,H_2O$, 0.2 g $MgSO_4 \cdot 7\,H_2O$, 0.2 g KCl, trace elements according to WAGNER *et al.*[8], 5 g glucose (separately sterilized) in one liter of deionized H_2O.

This broth (8.3 liters) were inoculated with 800 ml of an 18-hour culture (500 ml flask, 1 buffle, 100 ml broth, shaking at 120 rpm, 27°C) and harvested after 4 hours.

3. Isolation

The fermentation broth was centrifuged without additives. The liquid phase was stirred for 20 minutes with 0.5 % charcoal. The charcoal was removed by filtration and discarded. The filtrate was stirred for another 20 minutes with 3.5 % charcoal. The active material, which was adsorbed on the charcoal, was eluted with two portions of methanol - water (7 : 3 vol. corresponding to 20 % of the initial volume of the culture centrifugate). The eluate was dried *in vacuo*.

A solution of 6.8 g solid (from 6.75 liters of culture centrifugate=70.4 g of dry material) in 300 ml of distilled water was chromatographed on an ion-exchange column (Dowex 50 WX 2, 50~100 mesh, H^+ form. Column : 60×2.4 cm). After washing with 400 ml of distilled water the column was eluted with 0.5 N NaCl solution (fractions of 20 ml). Fractions 94~164 were combined and concentrated under reduced pressure. The high NaCl content was reduced by repeated extractions with 98 % methanol. The dried extract was dissolved in 5 ml of water and applied to a Sephadex G 10 column (65×1.8 cm, 2.5 ml fractions). Fractions 31~33 were combined and lyophilized to yield 117 mg of a white solid that still contained an unknown amount of NaCl. The material was finally applied to a Biogel P 2 column (90×1.5 cm, elution with 0.01 M acetic acid, fractions of 3 ml). Fractions 26~30 contained the active compound. Yield : 80 mg of a colorless, hygroscopic material.

Thin-layer chromatography was performed on silica gel and cellulose plates (Merck, Darmstadt). A violet colored spot was obtained after spraying with ninhydrin. (Silica gel : *n*-butanol - acetic acid - water, 3 : 1 : 1, Rf 0.2; cellulose : ethanol - acetic acid - water 4 : 1 : 1, Rf 0.45.)

4. Gas Chromatography-Mass Spectrometry

The GC-MS investigations were performed on an LKB 9000 Gas Chromatograph-Mass Spectrometer with a 1.5 m glass column, packed with 3 % OV 17 on Chromosorb WAW (100~120 mesh), or 10 % Dexsil 300 on Supelcoport (80~100 mesh). Mass spectra were taken at 70 eV, 250°C ion source temperature, 3.5 kV accelerating voltage.

5. Gas Chromatography on Optically Active Stationary Phase

Determination of the configuration of amino acids was done on a 150 m stainless steel capillary, coated with N-trifluoroacetyl-L-phenylalanyl-L-leucyl-O-cyclohexylester[9], using the trifluoroacetyl-isopropylester-derivative of the amino acid.

6. Hydrolysis and Formation of Derivatives

Hundred μg samples of the active material were hydrolysed in 0.5 ml hydrochloric acid (6 N, 20 hours at 100°C or 1 N, 3 hours at 80°C). After removing the hydrochloric acid in a stream of dry nitrogen, the hydrolysis products were converted to volatile derivatives. Another sample was treated with 100 μl of 1 % aqueous KOH at 100°C for 10 minutes. The solution was neutralized with 6 N HCl. Trimethylsilyl derivatives were formed by addition of 100 μl bis-trimethylsilyl-trifluoroacetamide (Regis Chemical Comp.) and heating at 100°C for 1 hour in a vial with a Teflon-lined screw cap. This solution was used directly for the GC investigation. Trifluoroacetyl derivatives were formed by addition of 100 μl of CH_2Cl_2 and 25 μl of trifluoroacetic anhydride to the dried hydrolysate and keeping the samples at room temperature for 1 hour. In a second step esterification was achieved by adding an excess of diazomethane in ether to the acylated sample in methanol.

7. Enzymatic Hydrolysis of the Antibiotic with Arginase

The assay was performed as described by SCANNELL[4] with minor modifications. To 1 mg of arginase from calf liver (Boehringer) in 0.5 ml 0.1 M $MnSO_4$, pH 7, 10 mg of the arginine antagonist in 5 ml of 0.1 M $MnSO_4$, pH 9, was added after 4 hours of preincubation at 37°C. Incubation was continued for 24 hours, the $Mn(OH)_2$ precipitate was centrifuged and the liquid was lyophilized.

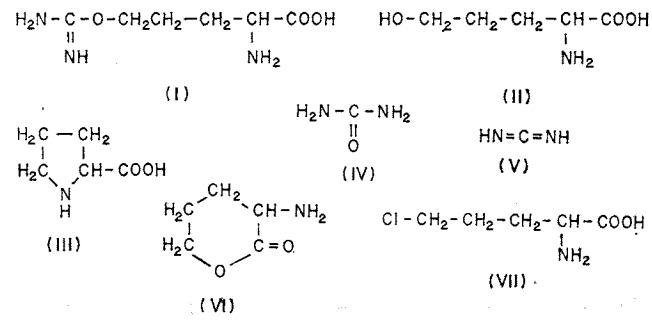
8. Synthesis of L-δ-Hydroxy-norvaline (II, Table 3)

One g of L-ornithine and 1.5 g of $CuSO_4 \cdot 5\,H_2O$ were dissolved in 3 ml of H_2O. The pH was adjusted to 5.5 with 1 N NaOH. The complex was heated at 100°C for a few minutes, cooled to room temperature, 420 mg of $NaNO_2$ in 2 ml of H_2O were added and the reaction mixture was kept at room temperature for 16 hours. The Cu^{2+} was precipitated by passing H_2S gas through the solution. Filtration and lyophilization of the filtrate yielded 1.2 g of a colorless solid. Thin-layer chromatography showed two major ninhydrin positive spots, one of which was identical with that of ornithine. The excess of ornithine could be removed by chromatographic separation on DEAE-Sephadex (100 × 1.7 cm; elution with 0.1 M CH_3COOH). Fractions of 6 ml were collected, the δ-hydroxy-norvaline was eluted in fractions 27~31. (Yield: 22 mg.)

9. Synthesis of O-[L-Norvalyl-5]-isourea (I, Table 3)

The synthesis of (I) was attempted by direct reaction of carbodiimide with δ-hydroxy-norvaline in acidic solution[10~12]. Carbodiimide was prepared from thiourea by reaction with yellow mercury (II) oxide[13]. The reaction of carbodiimide with δ-hydroxy-norvaline was investigated under various conditions. Best results were obtained by

Table 3. Structures of identified compounds



reacting equal amounts of carbodiimide and δ-hydroxy-norvaline in a solution of anhydrous hydrochloric acid (0.1 M) in acetic acid (50 mg of δ-hydroxy-norvaline, 50 mg of carbodiimide in 1 ml solvent were kept at room temperature for 10 hours.)

The reaction mixture was purified by chromatography on Sephadex A 25 (column: 100×1.7 cm, elution with 0.1 M acetic acid, elution volume: 60~75 ml).

The crude product, obtained after lyophilization, was further purified by preparative thin-layer chromatography (silica gel, Merck; solvent system: ethanol-acetic acid-water, 4:1:1). The yield was about 1 mg of a hygroscopic colorless compound.

Results and Discussion

Infrared and ^1H NMR spectra of purified samples of the antibiotic did not give specific information about the structure. The UV spectrum showed only end absorption.

The compound was not sufficiently volatile for mass spectrometry and trimethylsilylation apparently resulted in decomposition. GC-MS analysis of the reaction mixture showed three peaks in the gas chromatogram. A similar chromatogram and identical mass spectra were obtained after mild acid hydrolysis (1 N HCl, 3 hours, 80°C) and silylation (Fig. 1). The three peaks were identified by their mass spectra as trimethylsilyl derivatives of proline (III), urea (IV) (Fig. 2, Table 3) and δ-hydroxynorvaline (II) (Fig. 3, Table 3) by comparison with the mass spectra of synthetic samples.

A second sample was hydrolysed in 1% KOH (10 minutes, 100°C). In addition to the derivatives of proline, urea and δ-hydroxy-norvaline, carbodiimide

Fig. 1. Gas chromatogram of I after treatment with (1) 3 N HCl and (2) BSTFA

1.5 m glass column, 3% OV 17 on Chromosorb WAW, 80~150°C. Temperature program: 6°C/minute. Carrier gas: Helium, 25 ml/minute. Peak 1 = proline, peak 2 = urea, peak 3 = δ-hydroxy-norvaline (TMS 2) peak 4 = δ-hydroxy-norvaline (TMS 3).

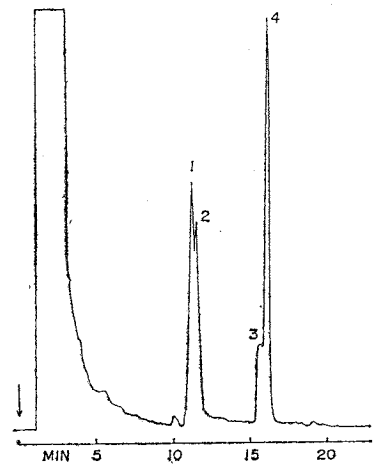


Fig. 2. Mass spectrum of peak 2 in Fig. 1.

LKB 9,000, 70 eV, GC-MS

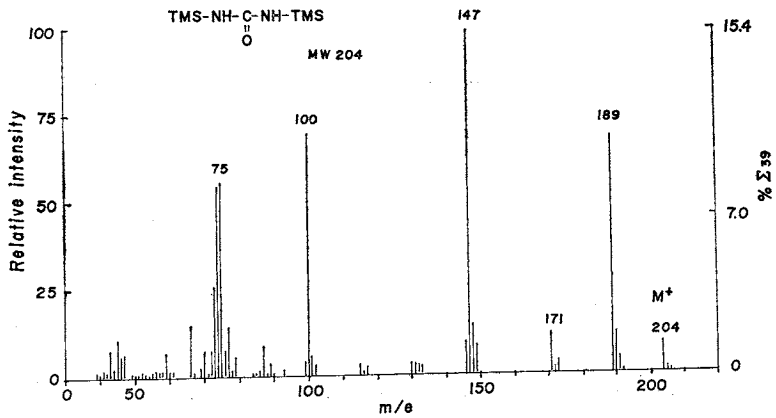
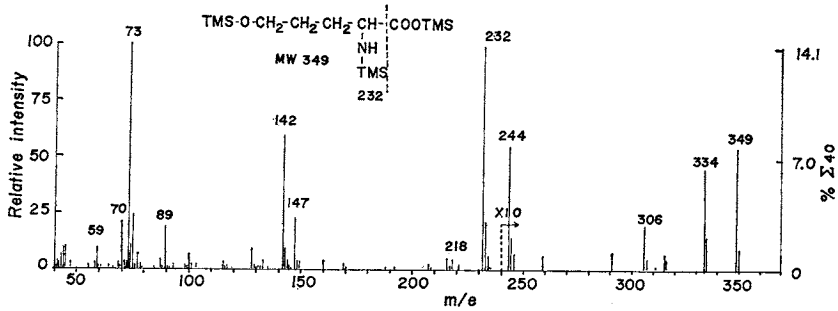


Fig. 3. Mass spectrum of peak 4 in Fig. 1.
LKB 9,000, 70 eV, GC-MS



(V) (Figs. 4 and 5) could be detected as its trimethylsilyl derivative.

Acid hydrolysis (6 N HCl, 18 hours, 100°C), trifluoroacetylation and methylation of a sample yielded the gas chromatogram shown in Fig. 6. The mass spectra also gave evidence of proline and δ-hydroxy-norvaline. The two major peaks were identified as the lactone of δ-hydroxynorvaline (VI) and δ-chloro-norvaline (VII) (Table 3). Under the above conditions urea is not derivatised.

Gas chromatography of the trifluoroacetyl-isopropyl ester of δ-hydroxy-norvaline on a capillary column, coated with an optically active stationary phase, conclusively showed it to have the L-configuation.

L-δ-Hydroxy-norvaline was also found after treatment of a sample of the arginine antagonist with the enzyme arginase. By

Fig. 4. Gas chromatogram of I after treatment with (1) 1 % KOH and (2) BSTFA

1.5 m glass column, 10 % Dexsil 300 on Supelcoport 80~100 mesh, 100~160°C, Temperature program : 6°C/minute, Carrier gas : 25 ml He/min. Peak 1=carbodiimide, peak 2=urea, peak 3=proline, peak 4,5 not identified, peak 6=δ-hydroxy-norvaline

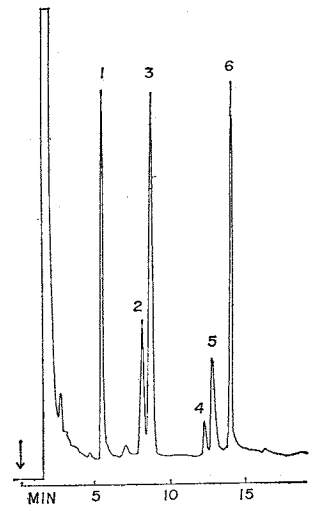
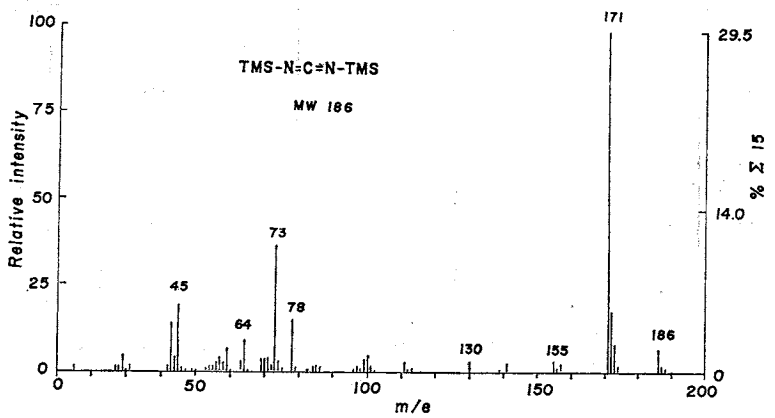


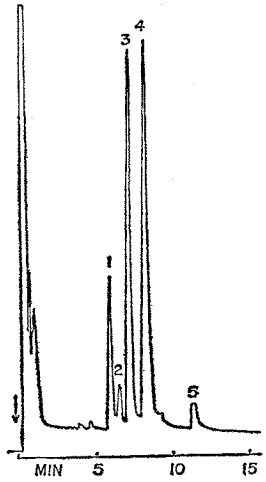
Fig. 5. Mass spectrum of peak 1 in Fig. 4.
LKB 9,000, 70 eV, GC-MS



comparing this result with the reaction of arginase with arginine, which yields ornithine, and in accordance with the products found after hydrolysis, the structure which is shown in Table 3 for the arginine antagonist (I) was assigned.

Fig. 6. Gas chromatogram of I after treatment with (1) 6 N HCl, (2) TFA_2O/CH_2Cl_2 and (3) CH_2N_2

Same conditions as in Fig. 1 Peak 1= δ-hydroxy-norvaline, peak 2=proline, peak 3= δ-chloro-norvaline, peak 4= lactone (VI)



It is known[10,11], that O-alkylated isourea compounds are easily hydrolyzed under acidic conditions, yielding urea and the corresponding hydroxy compounds. In hydrochloric acid isourea derivatives can be converted into urea and the corresponding alkyl chloride[10,11]. The observation of δ-chloro-norvaline after hydrolysis in 6 N hydrochloric acid agrees with this reaction. The presence of proline and of the lactone in the hydrolyzate can be easily explained by ring formation of δ-hydroxy-norvaline.

The comparison of natural and synthetic I shows identical Rf values on cellulose and silica gel thin-layer plates in two different solvent systems (see 3). Synthetic I yields the same products after acid hydrolysis and GC-MS investigation of trimethylsilyl derivatives. It further exhibits identical antibiotic activity as the natural arginine antagonist.

Acknowledgements

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