ENDURACIDIN, A NEW ANTIBIOTIC. II
ISOLATION AND CHARACTERIZATION*

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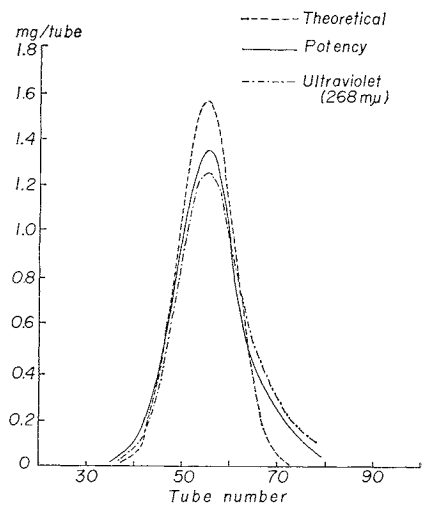
Enduracidin is a basic compound with ultraviolet absorption maxima at 230 and 263 mμ in 90 % methanol and is a colorless crystalline powder decomposing at 225~240°C. It is positive to DRAGENDORFF, ninhydrin, BIURET and BARTON'S[1] reagents but negative to MOLISCH reagent. Hydrolysis of enduracidin with 6N HCl yields, aspartic acid, threonine (and/or allo-threonine), serine, glycine, alanine, ornithine, citrulline, α-amino-4-hydroxyphenylacetic acid, α-amino-3,5-dichloro-4-hydroxyphenylacetic acid and two unidentified basic amino acids.

Characterization of Enduracidin

Enduracidin was obtained from the culture broth[2] by separation of the mycelium and extraction with wet methanol or acetone therefrom. The isolation procedure is summarized in Chart 1. Pure enduracidin was subjected to 150 transfers in a counter-current distribution apparatus using system acetic acid, *n*-butanol and water, 1 : 4 : 5. The distribution was analyzed by determination of biological activity against *Sarcina lutea* and ultraviolet absorption at 268 mμ. These analyses indicated good agreement with the theoretical curve (K=0.59) (Fig. 1).

Fig. 1. Counter-current distribution curve of enduracidin
Solvent : *n*-BuOH - AcOH - H_2O(4:1:5)



The ultraviolet spectrum of enduracidin exhibits two maxima at 230 mμ ($E^{1\%}_{1cm}$=220±15) and 263 mμ ($E^{1\%}_{1cm}$=150±15) in 90 % methanol, two maxima at 230 mμ ($E^{1\%}_{1cm}$=195±10) and 273 mμ ($E^{1\%}_{1cm}$=112±10) in N/10 aqueous hydrochloric acid, and a maximum at 253 mμ ($E^{1\%}_{1cm}$=335±10) with shoulder at about 287 mμ in N/10 aqueous sodium hydroxide (Fig. 2). Its infrared spectrum exhibits characteristic absorptions at 5.7 μ (-CO-OR), 6.0 μ (-CO-NH-), 11.8~12.2 μ (aromatic ring) (Fig. 3).

Enduracidin, obtained by these procedures, is a colorless powder melting at 225~240°C with decomposition and gives $[\alpha]_D^{23}$+85±10° (c 0.5 in dimethylformamide).

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Fig. 2. Ultraviolet spectra of enduracidin

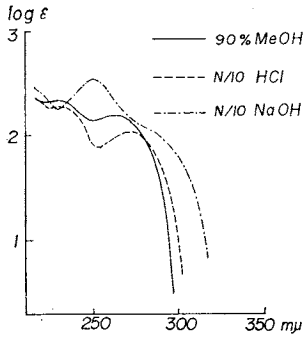
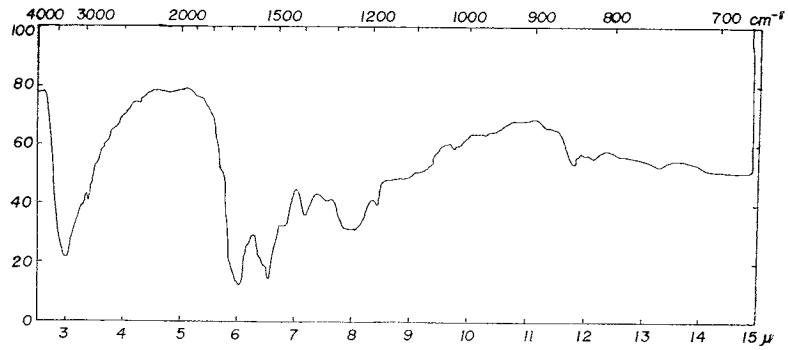


Fig. 3. Infrared spectrum of enduracidin



Analysis: C 53.2± 0.5, H 6.54±0.3, N 14.45±0.5, Cl 2.9±0.3 %

It is easily soluble in dil. hydrochloric acid, pyridine, dimethylformamide; soluble in methanol, aqueous alcohols and aqueous ketones and is insoluble in water, ethylalcohol, *n*-butanol, acetone, ethylacetate, diethylether, chloroform, benzene, hexane and petroleum ether.

Enduracidin is stable in the solid state and is also relatively stable in aqueous systems between pH 3.5 and 7.5 (no loss for more than three days at 40°C). It is less stable at a pH less than 3.0 and unstable in more basic solutions than pH 9.0.

Two solvent systems are useful in the paper chromatographic analysis of enduracidin. *n*-Butanol – acetic acid – water (4 : 1 : 5) gives an Rf-value of 0.45±0.1 and *n*-butanol – pyridine – water (4 : 3 : 7) shows an Rf-value of 0.80±0.1 (Whatman No. 1) (Tables 1, 2).

Table 1. Physical and chemical properties of enduracidin

Form and color	White powder
Melting point	205~225°C(soft), 225~240°C(dec.)
Analysis	C 53.2±0.5, H 6.54±0.3, H 14.45±0.5, Cl 2.9±0.3 %
Minimum molecular weight	2,200~2,600
Optical activity	$[\alpha]_D^{23}+85°\pm10°$ (c : 0.5, in DMF)
Solubility easy soluble	pyridine, DMF, dil, HCl
soluble	MeOH, aq. alcohols, aq. acetone
sparingly soluble	EtOH, BuOH, acetone
insoluble	Et_2O, EtOAc, $CHCl_3$, hexane, C_6H_6, *etc.*
Stability	aq. soln. <pH 3 loses activity (40°, 72 hrs.) pH 3.5~7.5 stable <pH 9 loses activity (40°, 24 hrs.)
Color reaction positive	ninhydrin, Dragendorft, biuret, B.P.B. -$HgCl_2$, 1% $FeCl_3$·1% $K_3Fe(CN)_6$
negative	Molisch, Fehling, *etc.*
Paper chromatography (Whatman No. 1)	*n*-BuOH-AcOH-H_2O (4:1:5) Rf 0.45±0.1
	n-BuOH-pyridine-H_2O (4:3:7) Rf 0.80±0.1

On the basis of microbiological[2,3] and physicochemical properties, enduracidin is a new member of the basic, non-ionic chlorine containing, peptide family of antibiotics, which includes actinoidin[4], M-411[5] and vancomycin[6].

Enduracidin was hydrolyzed with 6 N hydrochloric acid for 6 and 48 hours at 110°C. From the hydrolyzates, a water soluble amino acid fraction was separated by solvent extraction and a part was further converted into 2, 4-dinitrophenyl derivatives. Mixtures of amino acids and those of the dinitrophenylated amino acids were chromatographed on filter paper in two way runs (Figs. 4, 5, 6). Of the common amino acids, aspartic acid, serine,

Table 2. Biological properties of enduracidin

(1) Antimicrobial spectrum (HIGASHIDE *et al.*)

Microorganisms	MIC (mcg/ml)
S. aureus FDA 209P	0.1
S. aureus OM & EM-R	0.2
S. aureus CTC-R	0.1
S. aureus CP-R	0.1
S. aureus CHM-R	0.1
S. aureus MM-R	0.1
S. aureus GM-R	0.2
S. aureus NV-R	0.1
B. subtilis PCI 219	0.1
B. cereus IFO 3466	2.0
B. brevis IFO 3331	2.0
Sar. lutea IFO 3232	0.2
Micro. flavus IFO 3242	0.5
X. oryzae	2.0
E. coli IFO 3044	>100
P. vulgaris IFO 3045	>100
Myco. avium IFO 3153	5.0
Myco. avium SM-R	5.0
Myco. avium DM-R	5.0
Myco. smegmatis IFO 3083	2.0
Myco. phlei IFO 3158	5.0
Myco. sp. ATCC 607	5.0
Pir. oryzae	>100
Asp. niger IFO 4066	>100
Pen. chrysogenum IFO 4626	>100
C. albicans IFO 0583	>100
Sacch. cerevisiae	>100

(2) Acute toxicity in mice (YOKOTANI *et al.*)

Route	LD_{50}
Intraperitoneal	888 mg/kg
Subcutaneous	>2,500 mg/kg
Intramuscular	>2,500 mg/kg

Fig. 4. Paper chromatogram of amino acids obtained by 6-hour hydrolysis of enduracidin (6N HCl, 110°C)

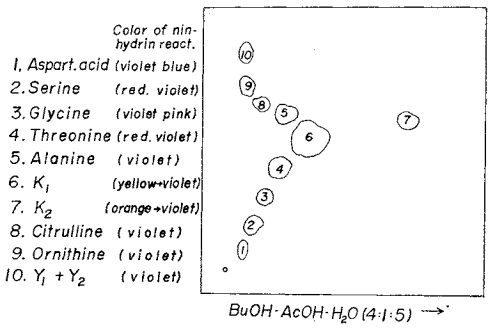


Fig. 5. Paper chromatogram of amino acids obtained by 48-hour hydrolysis of enduracidin (6N HCl, 110°C)

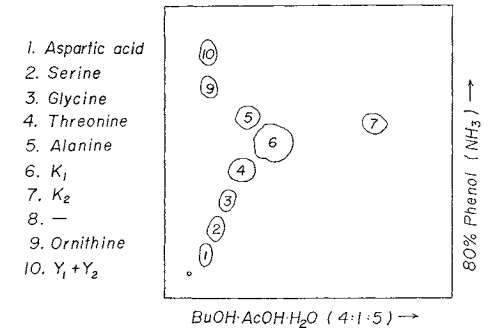


Fig. 6. Paper chromatogram of DNP-amino acids obtained by 48-hour hydrolysis of enduracidin (6N HCl, 110°C)

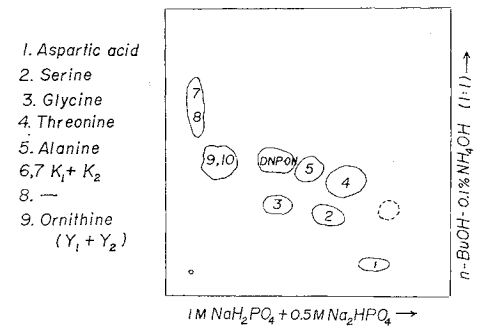


Fig. 7. Amino acid analysis of enduracidin (6N HCl, 110°C, 6 hours)
(Beckman model 120B accelerated system)

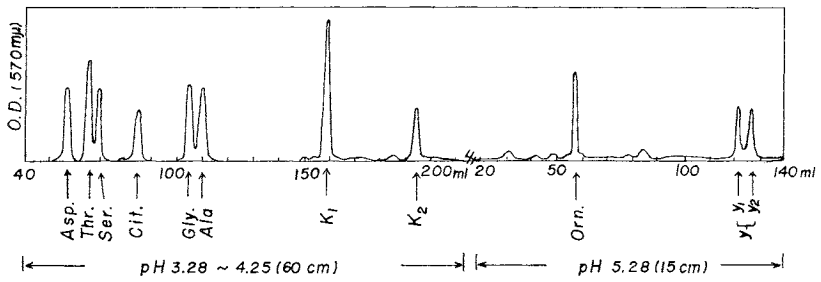


Chart 1. Separation and purification of enduracidin

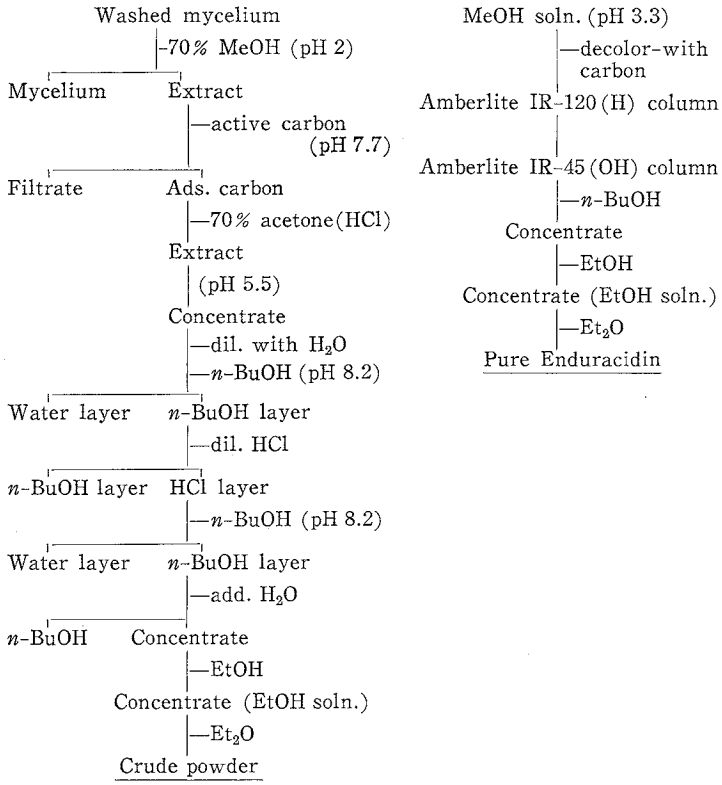


Chart 2. Isolation of K_1, K_2

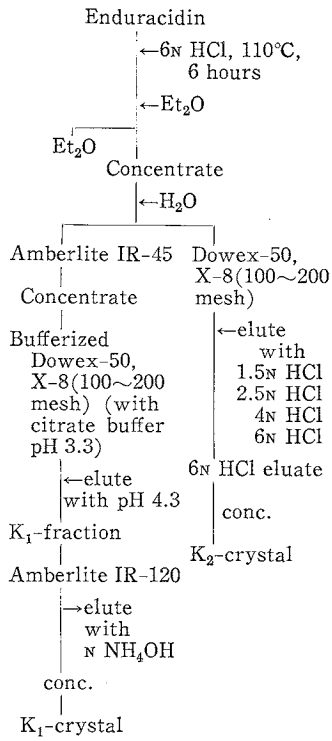


Table 3. Physical and chemical properties of K_1

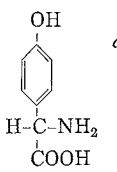
Crystal form and color	colorless needle
Melting point	214°C (dec.) (prism 218°C dec.)
Molecular weight	167 (mass)
Analysis found	C 57.34, H 5.54, N 8.25%
calculated for $C_8H_9NO_3$	C 57.45, H 5.39, N 8.38%
Color reaction, positive	ninydrin yellow→violet SAKAGUCHI blue-violet 1% $FeCl_3$·1% $K_3Fe(CN)_6$
Paper chromatography (Whatman No. 1)	n-BuOH - AcOH - H_2O (4 : 1 : 5) Rf 0.27
Treatment of K_1 with Ac_2O·H_2O	K_1-monoacetate
EtOH·HCl	K_1-ethylester
Chemical structure of K_1	 α-Amino-4-hydroxy-phenylacetic acid

Table 4. Physical and chemical properties of K_2

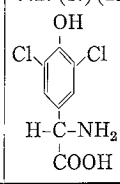
Crystal form and color	colorless needle
Melting point	210°C (prism 212°C) (dec.)
Molecular weight	236 (mass.)
Analysis found	C 40.56, H 3.04, N 5.86, Cl 29.95%
calculated for $C_8H_7NO_3Cl_2$	C 40.65, H 2.97, N 5.93, Cl 30.05%
Optical activity	$[\alpha]_D$+87.6° (C : 3, in N HCl)
Color reaction, positive	ninhydrin, orange→violet, SAKAGUCHI blue—violet 1% $FeCl_3$·1% $K_3Fe(CN)_6$
Paper chromatography (Whatman No. 1)	n-BuOH·AcOH·H_2O (4 : 1 : 5) Rf 0.52
Treatment of K_2 with EtOH·HCl	K_2-ethylester ($C_8H_6NO_2Cl_2$·OC_2H_5)
Nuclear magnetic resonance (in DMSO·D_2O)	1.13 ppm(t.)(3H), 4.02 (q.)(2H), 4.39(s.)(H), 7.27(s.)(2H)
Chemical structure of K_2	 α-amino-3,5-dichloro-4-hydroxy-phenylacetic acid

Fig. 8. Ultraviolet spectra of K_1

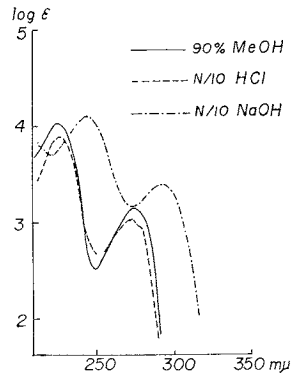


Fig. 9. Infrared spectra of K_1

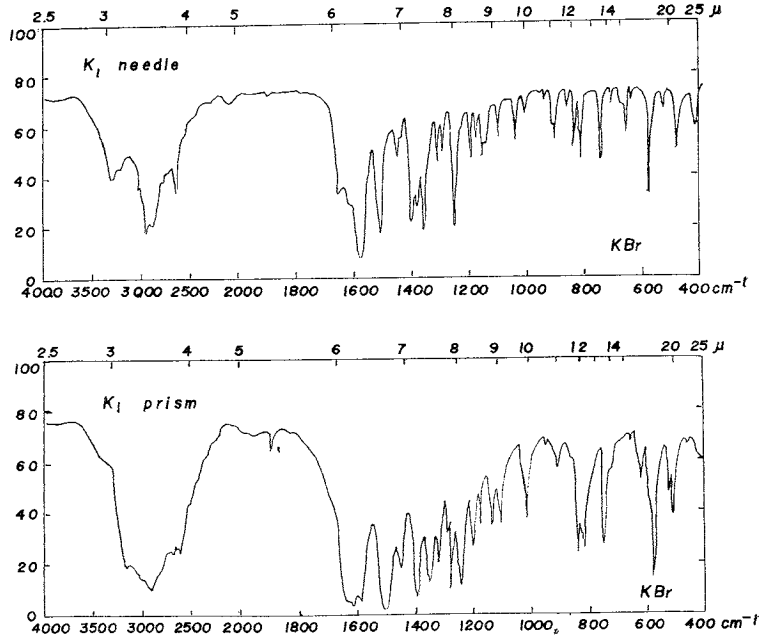


Fig. 10. Ultraviolet spectra of K_2

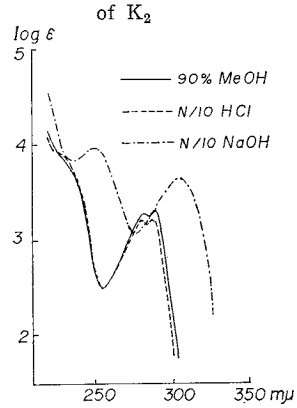
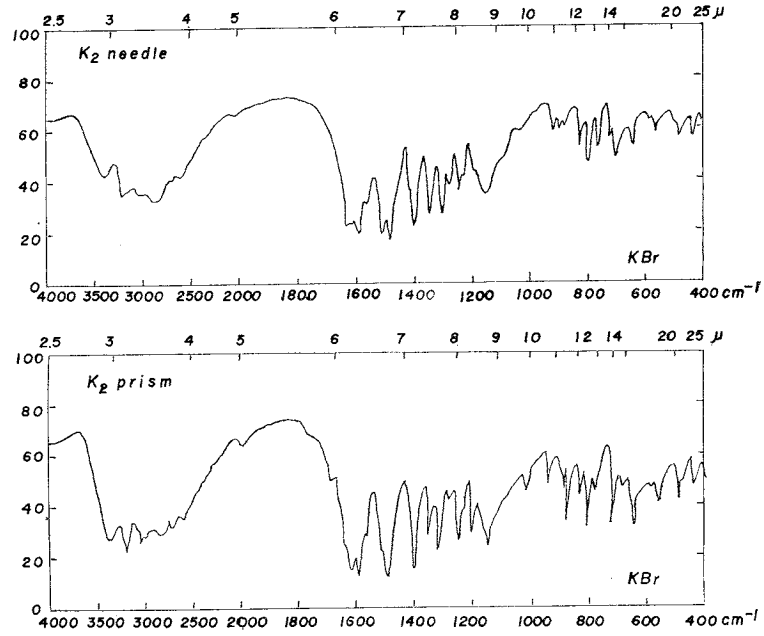


Fig. 11. Infrared spectra of K_2



glycine, threonine (and/or allothreonine), and alanine were detected. Besides these, two unusual components (K_1 and K_2) were detected by ultraviolet light (2,537 Å). These components turned yellow to violet and orange to violet, respectively, with ninhydrin reagent. Spot No. 8 in Fig. 4, which was detected in the 6-hour hydrolyzate but not detected in the 48-hour hydrolyzate, was presumed to be citrulline. Spots No. 9, and No. 10 which showed Rf values near zero in the system *n*-BuOH : AcOH :

H_2O (4 : 1 : 5) and Rf values of 0.8 and 0.9 in the 80 % phenol (NH_3) system were presumed to be ornithin and unidentified basic amino acids (Y_1 and Y_2, as shown in Fig. 7).

The two unusual amino acids, K_1 and K_2, gave a positive BARTON[1] test and negative EHRLICH test. They showed an unconventional color reaction to ninhydrin reagent and also showed a blue color with SAKAGUCHI reagent. The isolation of the two amino acids, K_1 and K_2, is illustrated in Chart 2. Their physicochemical properties are shown in Tables 3 and 4. The ultraviolet spectra of K_1 and K_2 obtained in various solvents are shown in Figs. 8 and 10. Their infrared absorption spectra (Figs. 9 and 11) showed a characteristic aromatic moiety. The NMR spectrum of K_1 N-mono acetate was useful for the structural elucidation of K_1.

K_1 monoacetate		
(in DMSO)	6.64 ppm (d, J=4.2) 7.09 ppm (d, J=4.2)	AB type four aromatic protons
	9.25 ppm	one aromatic hydroxyl
	8.30 ppm (d, J=4)	one -NH(Ac)
	5.09 ppm (d, J=4)	one -CH-
(treated with D_2O)	6.64 ppm (d, J=4.2) 7.09 ppm (d, J=4.2)	AB type four aromatic protons
	5.09 ppm (S)	one -CH-

The structure of α-amino-4-hydroxyphenylacetic acid for K_1 was deduced from the above data and was confirmed by synthesis[7]. The NMR spectrum of K_2 was the key to the elucidation of structure.

K_2		
(in DMSO)	7.5 ppm (S)	two aromatic protons
	8.95 ppm	one aromatic hydroxyl
	5.33 ppm	two amino protons, one $-NH_2$
	4.99 ppm	one methine proton
(treated with D_2O)	7.5 ppm (S)	two aromatic protons
	4.99 ppm (S)	one methine proton

The above data and the similarity of the color reaction to that of K_1 suggested the structure α-amino-3,5-dichloro-4-hydroxyphenylacetic acid for K_2. This structure was confirmed by synthesis[8] and X-ray[9] analysis of K_2.

Experimental

Isolation of Enduracidin

A 4,500 liter portion of fermentation broth of *Streptomyces fungicidicus* No. B-5477 was filtered with 135 kg of Hyflo Super Cel (Johns-Manville, New York, N. Y.) to give 1,100 kg of wet mycelium. The water washed mycelium was extracted under stirring with 4 volumes of 70 % aqueous methanol (adjusted to pH 2.0 with conc. hydrochloric acid) for 2 hours and filtered. The wet methanol extract (5,000 liters ; 500 mcg/ml) was adjusted with 2.5 N aqueous sodium hydroxide to pH 6.0 ; a flocculent precipitate appeared and was removed by filtration. Addition of aqueous sodium hydroxyde was continued until the pH of the extract became 7.5 to 8.0. The active substance in the extract was adsorbed on 142 kg of a mixture of activated carbon (Shirasagi A, Takeda Chemical Industries, Ltd., Osaka, Japan) and Hyflo Super Cel (John-Marnville, New York, N. Y.) (1 : 1). After 1-hour agitation by batch process the adsorbent was removed by filtration.

After washing with water, the adsorbent was treated with 14,000 liters of acidic 70 % aqueous methanol containing 2 % (v/v) of N hydrochloric acid under stirring for 2 hours. This procedure was repeated once more and both eluates were combined and adjusted with sodium hydroxide to pH 5.5 and then concentrated to 800 liters of aqueous solution (2,130 mcg/ml=1.7 kg activity). This aqueous concentrate was diluted with 4,000 liters of water and adjusted with 4 N aqueous sodium hydroxide to pH 8.2. The activity in the aqueous solution was transferred to 4,500 liters (1,500 liters×3) of *n*-butanol. After washing with 1,600 liters of water the activity in *n*-butanol was transferred to 1,600 liters×3 of N/200 hydrochloric acid and back to 1,500 liters×3 of *n*-butanol at pH 8.2. The *n*-butanol solution was washed with 1,500 liters×2 of water and concentrated azeotropically with water below 50°C under reduced pressure to 5,000 ml. After addition of 10,000 ml of ethanol, the concentrate was concentrated again to 5,000 ml to remove water and then placed in 10 volumes of diethyl ether to give one kg of amorphous precipitate. This precipitate was recovered by filtration (one kg 900 mcg/mg).

One kg of precipitate (crude antibiotic) was dissolved in 500 liters of 70 % aqueous methanol and left standing overnight. A small volume of inactive precipitate appeared, and was removed with 200 g of activated carbon (Shirasagi A) by filtration. The filtrate was diluted with 1,500 liters of 70 % aqueous methanol and this methanolic solution was stirred for one hour with 800 g of activated carbon (Shirasagi A) at pH 3.3, and filtered. The filtrate was successively passed through columns (height/diameter=7.5) of 20 liters of Amberlite IR-120 (H) (S. V. 1.0) and of 20 liters of Amberlite IR-45 (OH). Concentration of the effluent under reduced pressure reduced the content of methanol to 35 %; then distillation was continued azeotropically with *n*-butanol to 3 liters of concentrate, which was then poured into 10 volumes of diethyl ether to give 700 g of pure colorless crystalline powder (970~1,030 mcg/mg).

Acid hydrolysis of enduracidin for amino acid analysis and paper partition chromatography

Ten milligrams of enduracidin in one ml of 6 N HCl was heated at 110°C for 6 or 48 hours in a sealed tube. The acid hydrolyzates were dehydrated in a vacuum desiccator over NaOH and $CaCl_2$ and used for amino acids analysis and paper partition chromatography.

α-Amino-4-hydroxyphenylacetic acid (Compound K_1)

Five grams of enduracidin in 400 ml of 6 N HCl was refluxed for 6 hours. After being extracted with diethyl ether the resultant aqueous hydrolyzate was concentrated azeotropically with *n*-butanol *in vacuo* to dryness then diluted with 300 ml of water and passed through a column of 400 ml of Amberlite IR-45 (OH) to remove the excess HCl. The effluent was concentrated to give *ca.* 3.5 g of residue and used for paper partition chromatographic or ion exchange chromatographic separation of compound K_1.

i) Paper partition chromatographic method: One gram of the above concentrate was dissolved in 15 ml of 50 % aqueous methanol and applied to the origin line, 7 cm distant from the end of square (40 cm×40 cm) Tōyō filter paper No. 526. Twelve sheets of the filter paper were required for complete utilization of the above solution. The papers were developed with a solvent system of AcOH-butanol-H_2O (1 : 4 : 5) and zones, having Rf value of 0.27±0.05 and showing yellow color with ninhydrin reagent, were cut off and extracted with 70 % aqueous methanol. The extract was passed through a column of Amberlite IR-120 (H). Elution of the column with 1 N NH_4OH and concentration of the eluate yielded crystals of compound K_1. Compound K_1 exhibits in two crystalline modifications; a needle form (optically active substance, $[\alpha]_D^{23}+335°\pm5°$ (c 1, 0.1 N HCl)) and a plate form (racemate) and recrystallization from hot water gave 200 mg of colorless needles, m. p. 214°C (dec.) and from the mother liquor, colorless prisms, m. p. 218°C (dec.), were obtained.

Anal. Found: C 57.34, H 5.54, N 8.25 %
Calcd. for $C_8H_5NO_3$: C 57.45. H 5.39, N 8.38 %

ii) Ion exchange chromatographic method: Acid hydrolyzate obtained from 5 g of enduracidin was diluted with 150 ml of 0.2 M citrate buffer (adjusted to pH 3.3) and passed through 300 ml (2.2×78 cm) of citrate buffer (pH 3.3) pretreated Dowex 50×8 (100~200 mesh), at S.V. 0.2. The column was then eluted with buffer solution at pH 4.3, and 10 ml aliquots of the eluate were tested with ninhydrin reagent. Ninhydrin positive (yellow color) fractions were passed through a column of Amberlite IR-120 (H) and the column was eluted with aqueous ammonia. Concentration of the ammoniacal eluate gave 500 mg of crystalline (needles) compound K_1.

Ethyl α-amino-4-hydroxyphenylacetate (Ethyl ester of compound K_1)

Fifty milligrams of compound K_1 in 5 ml of 18 % ethanolic hydrogen chloride was heated for 1 hour and left overnight. The reaction mixture was extracted with a mixture of ethyl acetate and water (1 : 1) and the ethyl acetate extract was washed with aqueous sodium hydrogen carbonate solution and water and then dried with sodium sulfate. Concentration of the ethyl acetate solution yielded needles, m.p. 148~152°C.

Anal. Found: C 61.38, H 6.79, N 7.06 %

Calcd. for $C_{10}H_{13}NO_3$: C 61.52, H 6.71, N 7.18 %

α-Monoacetylamino-4-hydroxyphenylacetic acid (N-monoacetate of compound K_1)

To 70 milligrams of compound K_1 in 4 ml of distilled water was added 1 ml of acetic anhydride dropwise. The mixture was held at 60°C for 3 hours under agitation. The reaction mixture was concentrated and washed with petroleum ether and extracted with ethyl acetate. The ethyl acetate solution was washed with water and concentrated to give needles, m. p. 155°C.

Anal. Found: C 57.35, H 5.35, N 6.50 %

Calcd. for $C_{10}H_{11}NO_4$: C 57.41, H 5.30, N 6.70 %

α-Amio-3,5-dichlor-4-hydroxylphenylacetic acid (Compound K_2)

i) Paper partition chromatographic method: Acid hydrolyzate of enduracidin was chromatographed on the filter paper by the same method as in the case of compound K_1. The zone having a Rf value of 0.52±0.05 and showing orange color with ninhydrin reagent was cut off and extracted with acidic (HCl) 70 % aqueous methanol. The extract was passed through a column of Amberlite IR-120 (H). Elution of the column with 1 N NH_4OH and concentration of the eluate gave needles of compound K_2.

ii) Ion exchange chromatographic method: Acid hydrolyzate obtained from 5 g of enduracidin was passed through a column (2.2×78 cm) of Dowex-50×8 (H) (100~200 mesh). The column was then eluted with 1.5 N, 2.5 N, 4 N and 6 N aqueous hydrochloric acid in order. The eluate with 6 N hydrochloric acid was concentrated to give needles of compound K_2. Compound K_2 also exists in two crystalline modification; a needle form (optically active substance $[\alpha]_D^{21}+87.6°\pm8°$ (c 3, 1 N HCl)) and pillar form (racemate). The latter was suitable for X-ray analysis. Recrystallization from water gives pure needles of compound K_2, m. p. 208~210°C (dec.). From the mother liquor, another pillar form, m. p. 211~212°C (dec.), of compound K_2 was obtained.

Anal. Found: C 40.56, H 3.04, N 5.86, Cl 29.95 %

Calcd. for $C_8H_7NO_3Cl_2$: C 40.65, H 2.97, N 5.93, Cl 30.05 %

Ethyl α-amino-3,5-dichlor-4-hydroxyphenylacetate (Ethyl ester of compound K_2)

One hundred milligrams of compound K_2 was dissolved in 50 ml of 18 % ethanolic hydrogen chloride and refluxed for 1 hour and then left overnight. The reaction mixture was treated in the same manner as compound K_1. Recrystallization from ethyl acetate gave needles of compound K_2 ethyl ester, m. p. 180~182°C (dec.).

Anal. Found: C 45.75, H 4.16, N 5.05, Cl 26.71 %

Calcd. for $C_8H_7NO_3Cl_2 \cdot C_2H_4$: C 45.50, H 4.20, N 5.30, Cl 26.86 %.

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