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Shigenobu Okuda,*1 Yūya Nakayama,*2 and Kyosuke Tsuda*1 : Studies on Microbial Products. I. Helvolic acid and Related Compounds. I. 7-Desacetoxyhelvolic Acid and Helvolinic Acid.

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Cephalosporin P_1 (I),[1] fusidic acid (II)[2] and helvolic acid (III)[3] have hitherto been reported as the interesting antibiotics with unique protolanostane type framework. This paper deals with the isolation of additional homologues, 7-desacetoxyhelvolic acid (IV) and helvolinic acid (V), from a culture broth of *Cephalosporium caerulens* besides helvolic acid (III).

A) 7-Desacetoxyhelvolic Acid

Ethyl acetate extract of the broth filtrate was evaporated and the residue was triturated with methanol to remove a greater part of helvolic acid (III). The helvolic

culture filtrate (pH 7~8)
water layer— adjusted to pH 2, extracted with ethyl acetate
ethyl acetate extract
concd. in reduced pressure
dried product
insoluble portion (helvolic acid)— extracted with MeOH
MeOH extract
concd. in reduced pressure
dried product
filtrate— warmed in 0.1*N* NaOH at 50°, adjusted to pH 2, then filtered
precipitate
dissolved in $CHCl_3$, silica gel chromatography, eluted with $CHCl_3$–MeOH
IV fraction
recrystallized from MeOH
IV crystal

Fig. 1. Separation of 7-Desacetoxyhelvolic Acid (IV) from Culture Broth

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acid deficient methanol solution was evaporated and the residue was treated in 0.1*N* sodium hydroxide solution at 50° for 15 min., whereupon the remaining helvolic acid (Ⅲ) was hydrolyzed into helvolinic acid (Ⅴ). After acidification, the resulting precipitate was collected by filtration, dissolved in chloroform and purified through silica gel chromatography to afford white fine needles (Ⅳ), m.p. 214.5~215°, $[\alpha]_D^{27}$ −51.7°, a yield of 30 mg./1000 ml.

This material is easily soluble in ethyl acetate, methanol, ethanol, acetone, chloroform, while it is insoluble in petroleum ether. This compound is also soluble in 0.1*N* sodium hydroxide and the resulting solution gave a recovery of original compound in a quantitative yield after heating at 50° for 15 min. followed by acidification. On the other hand, helvolic acid (Ⅲ) is converted into helvolinic acid (Ⅴ) under these conditions.

A treatment of this acid with diazomethane afforded methyl ester (Ⅵ) m.p. 178~179°, $[\alpha]_D^{33}$ −54°. The analytical data of the acid (Ⅳ) and methyl ester (Ⅵ), were in good agreement with the molecular formulas, $C_{31}H_{42}O_6$ and $C_{32}H_{44}O_6$, respectively. Infrared (IR) spectrum of Ⅳ by potassium bromide disk method exhibits absorptions (ν, cm^{-1}) at 1240 (-OAc), 1250 (>C=O), 1670 ($\alpha\beta$-unsaturated ketone), 1700 (>C=O, $\alpha\beta$-unsaturated acid) and 1730 (-OAc). In nuclear magnetic resonance (NMR) spectrum of Ⅵ, the signals corresponding to C_7-H and C_7-acetoxyl methyl were not observed, compared with that of helvolic acid methyl (Ⅶ) (cf. Table I). UV spectrum of Ⅳ showed the absorption maximum corresponding to $\alpha\beta$-unsaturated ketone at 232.5 mμ (cf. Table Ⅱ).

Table I. Comparison of Nuclera Magnetic Resonance Spectrum

	C_1, C_2-H	C_7-H	C_{16}-H	C_{24}-H	-C-OCH_3 (O)	C_7-OAc	C_{16}-OAc	C_{26}, C_{27}-CH_3	C_4-CH_3	-C(O)-CH_3
Helvolic acid methyl (Ⅶ)	2.70 4.17 doublet J=11.4 c.p.s.	4.79 singlet	4.17	4.89	6.37	7.90	8.05	8.30 8.38	8.72 doublet	8.55 8.82 9.08
7-Desacetoxy-helvolic acid methyl (Ⅵ)	2.70 4.16 doublet J=11.0 c.p.s.	—	4.22 doublet J=8.0 c.p.s.	4.94	6.38	—	8.06	8.31 8.39	8.84 doublet	8.68 8.89 9.10

Assignment of Protons: τ value

Table Ⅱ. Comparison of Ultraviolet Spectrum

	Absorption λ_{max}^{EtOH} mμ	Absorption intensity log ε
Helvolic acid (Ⅲ)	231.0	4.24
7-Desacetoxyhelvolic acid (Ⅳ)	232.5	4.20

Since this compound is produced by a helvolic acid producing strain, the facts above mentioned strongly suggested the absence of the C_7-acetoxyl grouping in Ⅳ. Then the interrelation between Ⅲ and Ⅳ was attempted, according to the scheme in Chart 1.

Catalytic hydrogenation of Ⅳ with palladium-charcoal in methanol solution afforded tetrahydro derivatives (Ⅸ), m.p. 219°, $[\alpha]_D^{25.5}$ −13.2°. This compound is completely identical with 7-desacetoxytetrahydrohelvolic acid (Ⅸ), prepared from Ⅲ *via* zinc acetic acid treatment followed by catalytic hydrogenation.

Consequently the structure of the above newly isolated acid, m.p. 214.5~215°, is represented by Ⅳ.

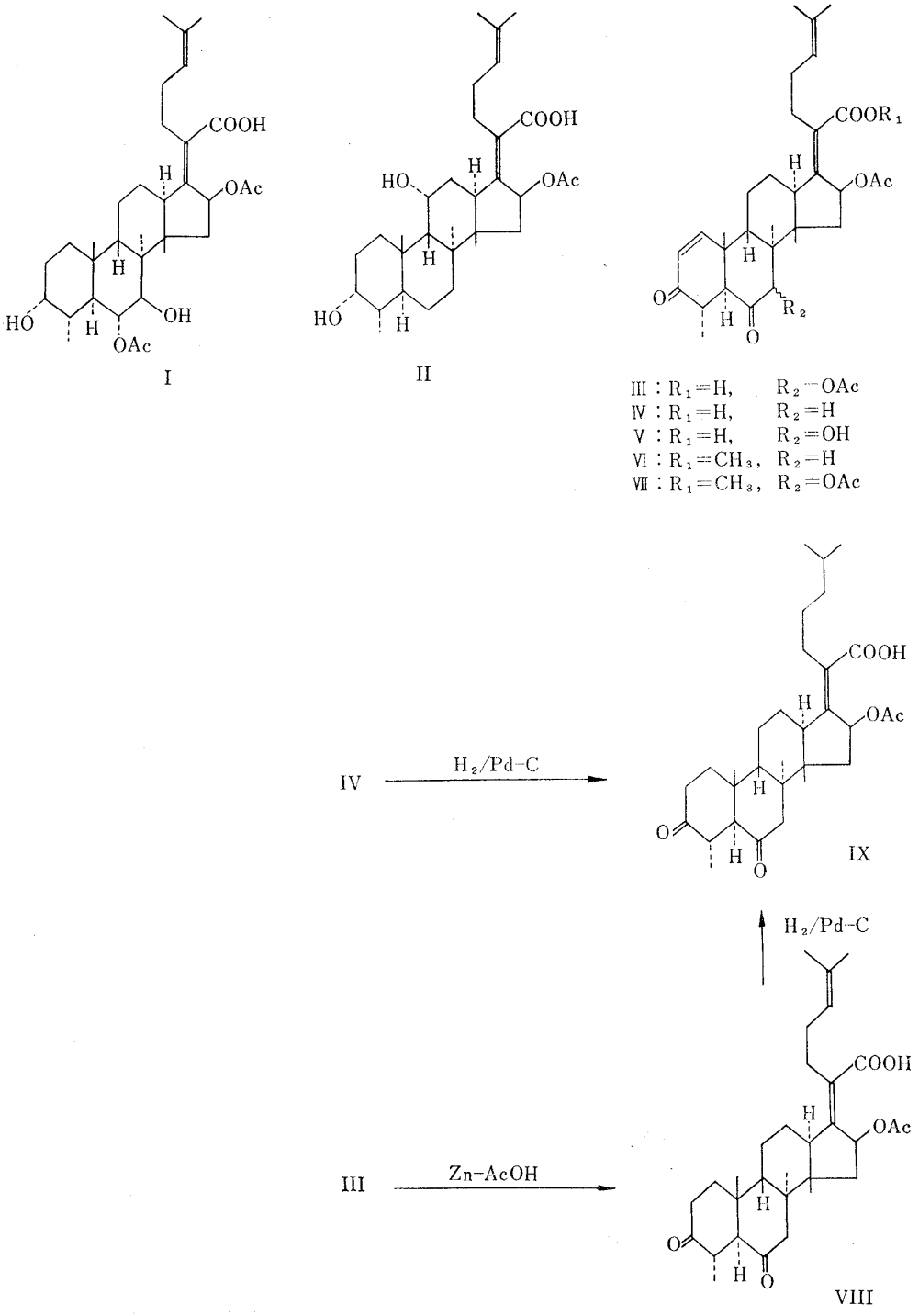

Chart 1.

B) Helvolinic Acid

Cram and his coworkers[3b] reported that mild hydrolysis of III with 0.1*N* sodium hydroxide solution gave rise to the removal of one of the two acetyls yielding helvolinic acid (V), which regenerated III by acetylation.

After cautious separation of metabolites from *Cephalosporium caerulens* especially preventing hydrolysis of acetoxy group was carried out, the methanol soluble fraction (a mixture of III, IV and V) was purified through silica gel chromatography (cf. Fig. 2). White fine needles, m.p. 202.5~203°, $[\alpha]_D^{24}$ −88.9°, thus obtained, was identical with an authentic specimen of helvolinic acid (V).

culture filtrate (pH 7~8)
water phase— adjusted to pH 2, extracted with ethyl acetate
ethyl acetate extract
concd. in reduced pressure
dried product
insoluble portion (helvolic acid)— extracted with MeOH
MeOH extract
concd. in reduced pressure
dried product
petroleum ether solution— washed with petroleum ether
residue
dissolved in $CHCl_3$, silica gel chromatography, eluted with $CHCl_3$-MeOH
V fraction
recrystallized from aqueous MeOH
V crystal

Fig. 2. Separation of Helvolinic Acid (V) from Culture Broth

The fact that IV and V were isolated from a culture broth of *Cephalosporium caerulens* indicates some possibilities that these compounds might serve as precursors of helvolic acid (III).

C) Antibiotic Activity of IV

7-Desacetoxyhelvolic acid exhibits a similar antibiotic spectrum as in the case of helvolic acid (III) or helvolinic acid (V) but the activity against *Staphylococcus aureus* of this compound is around one half compared with III (cf. Table III).

Table III. Biological Activities of IV and III

Test strain \ Sample No.	IV	III
Staphylococcus aureus 209P	12.5	6.25
	25.0	12.5
Staphylococcus 76 (Penicillin resistant)	6.25	1.56
	12.5	6.25
Bacillus anthracis	12.5	6.25
	25.0	25.0
Salmonella entertidis 1891	>100	>100
	>100	>100
Salmonella typhi (Yomo)	>100	>100
	>100	>100
Salcina lutea 9341	3.12	0.8
	25.0	12.5
Escherichia coli	>100	>100
	>100	>100
Proteus vulgaris X-28	>100	>100
	>100	>100

MIC value : γ/ml.
Method : agar streak in Buillon agar
Lower : 48 hr., Upper : 24 hr.

Experimental

Production and Isolation of 7-Desacetoxyhelvolic Acid (IV)

The fermentation medium contatined 5% glucose, 2% cornsteep liquor, 0.2% NaCl, 0.3% KCl, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.01% $ZnSO_4$, 0.001% $MnSO_4$, 0.0001% $CuSO_4$, 0.4% $CaCO_3$. The strain (*Cephalosporium caerulens*) on an agar slant was inoculated into the medium.

After the fermentation at 27° for 7 days in shaking flasks, the mycelium were removed from the culture broth by filtration. The filtrate 5.51 L. thus obtained was adjusted to pH 2 and extracted twice with 1.2 L. each of ethyl acetate. The ethyl acetate extracts were combined and concentrated to dryness under reduced pressure. The syrupy residue was added with 50 ml. of MeOH and then the resulting precipitates were filtered off. After the filtrate was evaporated under reduced pressure, the residue was dissolved in 250 ml. of 0.1*N* NaOH and allowed to stand at 50° for 10 min. The reaction mixture was acidified to pH 2 and the resulting solids were collected by filtration.

This dried product (3.6 g.) was dissolved in 36 ml. of $CHCl_3$ and chromatographed in a column packed with 100 g. of silica gel. The column was washed with $CHCl_3$ and the elution was carried out with $CHCl_3$ containing 1% MeOH. Ⅳ and Ⅴ were eluted in this order. The fractions containing Ⅳ were dried *in vacuo* and the residue obtained was crystallized from MeOH to give crystls of Ⅳ, 80 mg. White needles, m.p. 214.5~215°, $[\alpha]_D^{27}$ −51.7°(c=0.5, $CHCl_3$). *Anal.* Calcd. for $C_{31}H_{42}O_6$: C, 72.91; H, 8.29. Found : C, 72.66; H, 8.27.

IV-Methyl Ester (VI)

Ⅳ (50 mg.) was esterified with diazomethane in $CHCl_3$. The resulting solution was evaporated to dryness and the residue was recrystallized from aqueous acetone to give crystals of Ⅵ 30 mg. White needles, m.p. 178~179°, $[\alpha]_D^{33}$ −54.0° (c=0.5, $CHCl_3$). *Anal.* Calcd. for $C_{32}H_{44}O_6$: C, 73.25; H, 8.45. Found : C, 73.08; H, 8.43.

7-Desacetoxytetrahydrohelvolic Acid (IX)

A) Onto a refluxing solution of 1 g. of Ⅲ in 150 ml. of AcOH–H_2O (1:1), 100 g. of Zn-dust were added with vigorous stirring. Heating and stirring were continued for 15 min. UV absorption due to α,β-unsaturated ketone completely disappeared within 10 min. under these conditions. After cooling, Zn-dust was removed by filtration and the filtrate was concentrated until $Zn(OAc)_2$ was precipitated. The precipitated $Zn(OAc)_2$ was filtered and washed with acetone. The filtrate and washing were combined and concentrated again until another $Zn(OAc)_2$ was precipitated. After several similar treatments, the final concentrated solution was added with H_2O and the precipitated solid was extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with H_2O, $NaHCO_3 \cdot H_2O$, and H_2O, and then evaporated *in vacuo* after drying over Na_2SO_4. Recrystallization of the residue from acetone–H_2O afforded 600 mg. of prisms (Ⅷ), m.p. 202~203°. *Anal.* Calcd. for $C_{31}H_{44}O_6$: C, 72.62; H, 8.65. Found : C, 72.60; H, 8.67. $[\alpha]_D^{18}$ +3.6°(c=0.56, EtOH).

The solution of 600 mg. of Ⅷ, obtained above, in 30 ml. of MeOH was shaken with 30 mg. of 10% Pd-C under atmosphere of hydrogen until 1.2 equivalent moleclues of hydrogen were absorbed. A usual work up gave rise to crystalline material which was purified *via* recrystallization from MeOH to aflord needles, m.p. 219°. *Anal.* Calcd. for $C_{31}H_{46}O_6$: C, 72.34; H, 9.01. Found : C, 72.38; H, 8.94.

B) 50 mg. of Ⅳ was dissolved in 10 ml. MeOH and hydrogenated at an atmospheric pressure in the presence of 5 mg. of 5% Pd-C. After 2 hr., 2 moles of hydrogen were absorbed, and then the catalyst was filtered off. Evaporation of the filtrate gave crude material, which was recrystallized from MeOH affording 41 mg. of crystalline Ⅸ. White needles m.p. 219°, $[\alpha]_D^{25.5}$ −13.2°(c=0.76, $CHCl_3$). *Anal.* Calcd. for $C_{31}H_{46}O_6$: C, 72.34; H, 9.01. Found : C, 72.51; H, 9.28. The IR spectrum (Nujol) was completely identical with the standard sample prepared as described above. The mixed melting point showed no depression.

Production and Isolation of Helvolinic Acid (V)

The fermentation was carried out similary to the case of the production of Ⅳ. The broth filtrate 1.5 L. was adjusted to pH 2, which was extracted twice with 300 ml. each of ethyl acetate. The ethyl acetate extract thus obtained was evaporated under reduced pressure and the residue was washed by petroleum ether. The resulting solid was collected by filtration and dried.

The dried product (1.3 g.) was dissolved in 14 ml. of $CHCl_3$ and chromatographed on a column of silica gel (40 g.). After the column was washed with 160 ml. of $CHCl_3$, it was eluted with $CHCl_3$ containing 2% of MeOH. Evaporation of the fraction containing Ⅴ gave crude powder (200 mg.), which was recrystallized from H_2O-MeOH affording 21 mg. of crystalline Ⅴ. White needles, m.p. 202.5~203°, $[\alpha]_D^{24}$ −88.9° (c=1, $CHCl_3$). *Anal.* Calcd. for $C_{31}H_{42}O_7$: C, 70.09; H, 8.04. Found : C, 70.05; H, 8.01.

This sample of Ⅴ showed the same IR spectrum as the standard sample prepared from helvolic acid by the method of Cram, *et al.*[3b] and the mixed melting point showed no depression.

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Summary

Two new antibiotics, 7-desacetoxyhelvolic acid and helvolinic acid were isolated from the culture broth of *Cephalosporium caerulens*. Characterization and structural studies of these substances are described.

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