ARGLECIN, A NEW MICROBIAL METABOLITE
ISOLATION AND CHEMICAL STRUCTURE[1]

KUNIAKI TATSUTA, TSUTOMU TSUCHIYA,
TETSUYA SOMENO and SUMIO UMEZAWA

Department of Applied Chemistry, Faculty of Engineering,
Keio University, Koganei-shi, Tokyo, Japan

HAMAO UMEZAWA and HIROSHI NAGANAWA

Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

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A new streptomycete metabolite named arglecin was isolated from the culture filtrates of the strains KA57-AG3, KB59-M1 and the other 11 strains by the screening of compounds with positive WOOD and diacetyl reactions. The structure was determined as 5-amino-2-(3-guanidinopropyl)-3,4-dihydro-4-isopropylpyridin-3-one.

As the results of our screening of culture filtrates of microorganisms for chemical color reactions, dienomycins[2] and sphydrofuran[3] have already been found as new metabolites of streptomycetes. This screening method appears to be useful in finding the significant metabolites which may reveal biosynthetic sequences in microorganisms. This paper describes the isolation and the chemical structure of a new metabolite named arglecin which was found by the screening of compounds of positive WOOD[4] and diacetyl reactions.

Screening Method

The screening method was essentially the same as that described in the previous paper[2] except for the additional use of diacetyl color reaction. The metabolites in culture filtrates were checked by thin-layer chromatography on the plates coated with microcrystalline cellulose powder with the solvent system: *n*-butanol - acetic acid - water (12 : 3 : 5), and the spots were detected by WOOD reagent as described in a previous paper[2]. Among about 3,000 strains tested, the culture filtrates of approximately 17 strains of streptomycetes showed positive color reactions (blue or purple) for WOOD reagent, the color reactions being proved not to originate from the nutrients. The thin-layer plate was also examined by the diacetyl reagent which is known to be sensitive to a variety of biologically important substances having such molecular moieties as guanidine (for example, arginine and streptomycin) and amidine (creatine). The detection method by the diacetyl reagent was as follows: the developed plate was heated to 100°C and sprayed with an equal volumes of 1% α-naphthol in 8% aqueous sodium hydroxide and 0.1% diacetyl in water, which was mixed just before use. Purple red color appeared immediately in most cases. The culture filtrates of

the 13 strains among the 17 strains described above showed the strong color spot at Rf 0.8 both for WOOD (blue) and diacetyl (purple red) reagents, and the laboratory numbers KA55-C4, KA57-AG3, KA74-C6, KB59-M1, KB84-AG2, KB93-M2, KC8-AG8, KC46-AG2, KC52-AG3, KC64-C3, KC87-AG2, KD15-C10 and KD19-AG1 were given to the strains.

Characters of the Arglecin-producing Strains*

The strain KA57-AG3 can be summarized as follows: no whorls; open or compact spirals; surface of spore is smooth; pale yellowish brown to dark brown growth; pale pink to pale brown aerial mycelium; chromogenic type which produces melanoid pigment; strong proteolytic activity; strong hydrolysis of starch. Among known species, the strain KA57-AG3 was most closely related to *Actinomyces toxytricini*[5,6] PREOBRAZHENSKAYA and SVESHNIKOVA. Therefore, the strain KA57-AG3 was designated *Streptomyces toxytricini*. The characters of the strain KB59-M1 can be summarized as follows: it forms neither whorls nor typical spirals, but sometimes extending aerial hyphae forming hooks; surface of spore is smooth; growth is pale yellow to pale yellowish brown and to pale brown on various media; aerial mycelium is pinkish white to light brownish gray and to reddish gray on various media; soluble pigment is none on synthetic media; chromogenic type which produces melanoid pigment; it has strong proteolytic activity on milk and strong hydrolytic activity on starch. Among known species, the strain KB59-M1 was most closely related to *Streptomyces lavendulae*[7,8] (WAKSMAN and CURTIS) WAKSMAN and HENRICI.

Production and Isolation

For laboratory production, 125 ml of a medium consisting of glycerol 2.5 %, yeast extract 1.0 %, meat extract 0.5 %, peptone 0.5 %, $CaCO_3$ 0.3 %, NaCl 0.2 %, $MgSO_4 \cdot 7H_2O$ 0.05 % and K_2HPO_4 0.05 % (pH 7.0) was inoculated with the strain KA57-AG3 grown on KRAINSKY's glucose asparagine agar slant and shaken-cultured at 27°C for 48 hours. Each inoculum (about 3 ml) was transferred to 125 ml of the same medium in a 500-ml SAKAGUCHI-flask and cultured for 60~70 hours at 27°C on a reciprocating shaker (8 cm amplitude, 130 strokes per minute). The broth (pH 8.0) was filtered and the filtrate (6.5 liters) was extracted with *n*-butanol (4 liters and 2 liters). The combined extracts were evaporated to give a solid (8.3 g), which was chromatographed on a column of alumina (Woelm acid form, 120 g, 4.7×7.0 cm) with methanol. The eluate positive for WOOD reagent was collected and evaporated to dryness. The resultant residue (3.9 g) was dissolved in a small volume of water and charged on a column of Amberlite CG-50 resin (H-form, 400 ml, 4.2×37 cm). After the resin column was washed with water, it was treated with 0.1 N hydrochloric acid as the developing solvent and the WOOD-positive fractions (550 ml) were collected. These were evaporated to a crystalline substance, which was recrystallized from ethanol-ethyl acetate to afford colorless needles of dihydrochloride of arglecin; yield 590 mg: m.p. 176~177°C, $[\alpha]_D^{20}$ 0° (*c* 2.5, water).

* This part was performed by Dr. MASA HAMADA of Institute of Microbial Chemistry.

Arglecin was also obtained from the other 12 strains by the same procedure described above. The strain KB59-M1 produced leupeptins[9] in addition to arglecin.

Chemical and Physical Properties

Arglecin is a basic substance and positive to WOOD, diacetyl and SAKAGUCHI reactions but negative to ninhydrin and EHRLICH reactions. It can also be detected by ultraviolet light.

Arglecin dihydrochloride is soluble in water, methanol, pyridine and dimethylsulfoxide, and insoluble in benzene, chloroform, ether and ethyl acetate.

An aqueous solution of arglecin is stable under acidic and neutral conditions and slightly unstable under basic condition.

Arglecin showed a single spot at Rf 0.6 on a thin-layer chromatogram of silica gel with a solvent system: *n*-butanol - ethanol - water (5 : 1 : 2). On electrometric titration in water, the values of pka 9.1 and >11 were obtained. On high voltage paper electrophoresis (3,500 V/42 cm, 90 mA/20 cm, 15 min.; Toyo Roshi paper No. 51) using a buffer solution of formic acid - acetic acid - water (1 : 3 : 36 by volume), arglecin moved 9~10 cm toward the cathode from the origin and showed a single spot of $Rf_{alanine}$ 0.82.

Elemental analysis of arglecin dihydrochloride revealed the following compositions:

Found: C 44.64, H 7.32, N 21.42, O 5.12, Cl 21.50 %,

Calcd. for $C_{12}H_{21}N_5O \cdot 2HCl$: C 44.45, H 7.15, N 21.60, O 4.93, Cl 21.87 %.

The mass spectrum showed the following prominent peaks: m/e 251, 209, 192, 179, 177, 150, 149, 123, 86, 73. The peak at m/e 251 corresponds to $C_{12}H_{21}N_5O^+$ and this indicates that the most probable empirical formula of arglecin dihydrochloride will be $C_{12}H_{21}N_5O \cdot 2HCl$.

The ultraviolet spectrum of arglecin (Fig. 1) exhibited maxima at the following wave length (ε): 322 (9,600) and 226 mμ (8,000) in water; 321 (9,100) and 232 mμ (8,200) in 0.1 N aqueous sodium hydroxide and 338 (9,400) and 226 mμ (8,700) in 0.1 N hydrochloric acid.

Fig. 1. Ultraviolet absorption spectrum of arglecin.

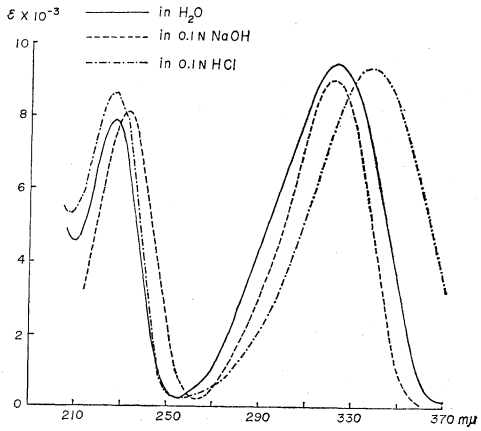


Fig. 2. Infrared spectrum of arglecin dihydrochloride in KBr disk.

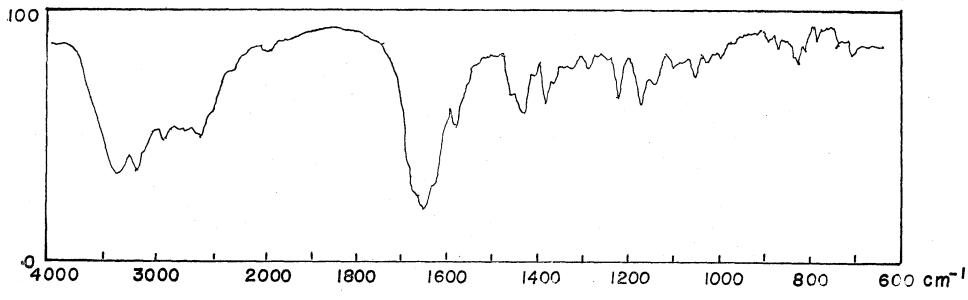
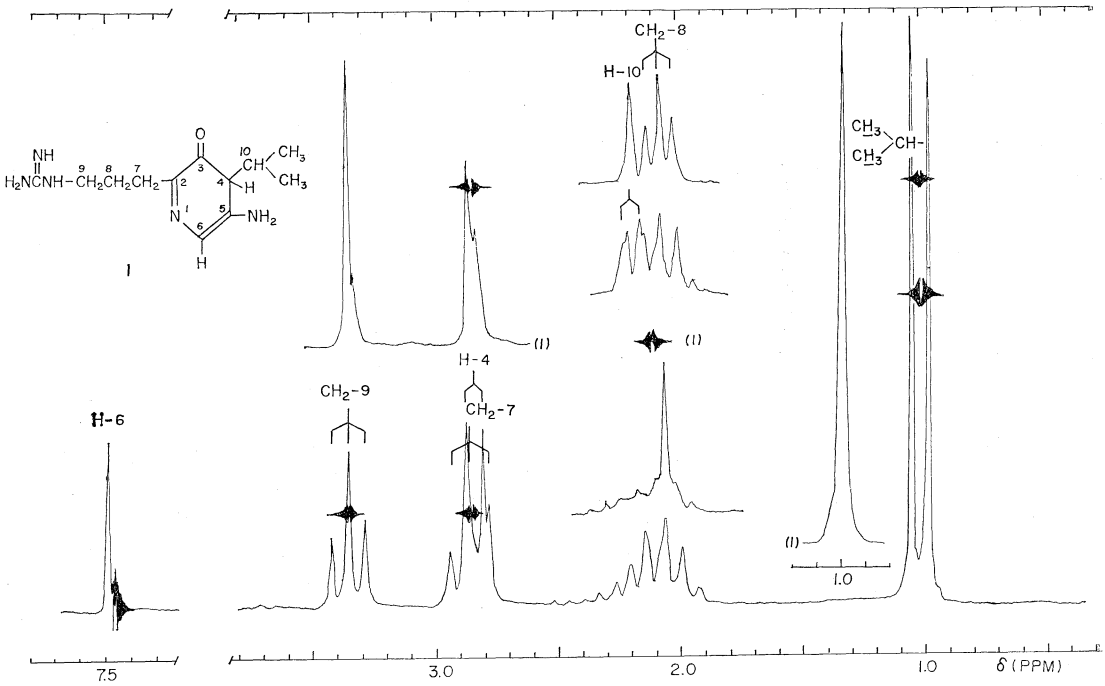


Fig. 3. NMR spectrum of arglecin dihydrochloride in D_2O at 100 MHz.



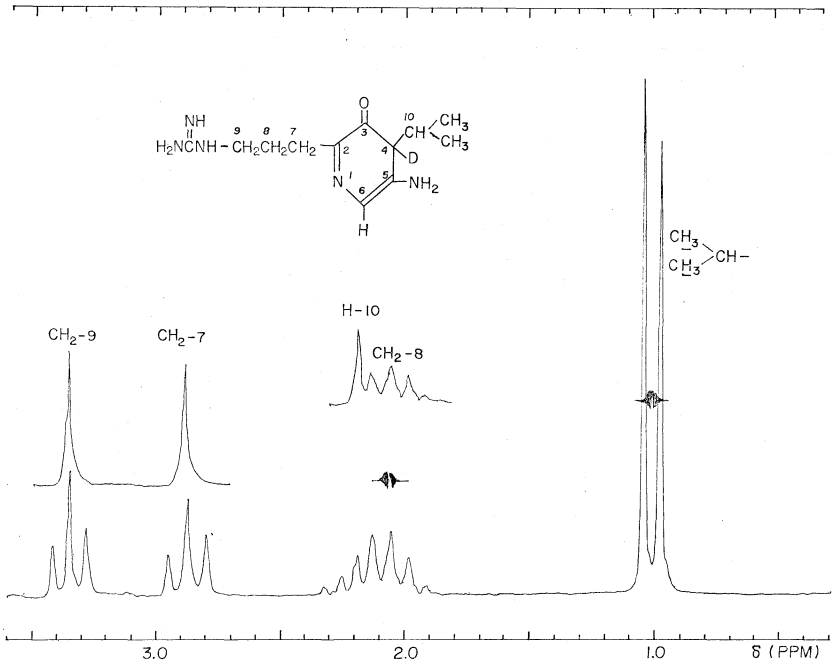
The infrared spectrum (Fig. 2) of arglecin dihydrochloride showed absorptions at 3360, 3200 (NH); 2980~2660 (CH); 2040; 1695, ~1655, 1635, 1590 cm^{-1} (C=O, C=C and guanidinium).

The NMR spectrum (100 MHz) of arglecin dihydrochloride in deuterium oxide was shown in Fig. 3. The signals were divided into five parts, that is, a 6-proton doublet at δ 1.01, 3-proton multiplet at δ 1.9~2.3, 3-proton multiplet at δ 2.8~3.0, 2-proton triplet at δ 3.36 and 1-proton singlet at δ 7.50.

The Structure of Arglecin

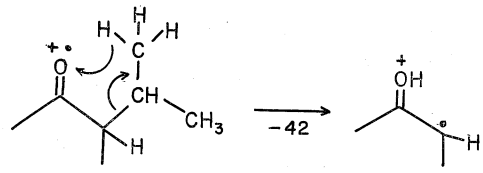
In the first place, the NMR spectrum of arglecin (I) was subjected to detailed analysis (see Fig. 3). Irradiation at δ 2.11 caused a 6-proton doublet at δ 1.01 to collapse to a singlet. Irradiation at δ 1.01 caused the unresolved multiplet at δ 1.9~2.3 to collapse to a simpler pattern including a 1-proton doublet at δ 2.18. Simultaneous irradiation at δ 1.01 and 2.85 caused the multiplet (δ 1.9~2.3) to collapse to a 2-proton triplet at δ 2.06 and a 1-proton singlet at δ 2.18. These observations suggest the presence of a sequence of $-\overset{|}{C}H-CH-(CH_3)_2$. Irradiation at δ 2.11 caused, in addition to the collapsing of the 6-proton doublet at δ 1.01 [$(C\underline{H}_3)_2CH$], a 1-proton doublet at δ 2.84 (this proton is tentatively named as H-4) and two methylene triplets at δ 2.87 and 3.36 [tentatively named as $CH_2(7)$ and $CH_2(9)$, respectively] to change into singlets respectively. Simultaneous irradiation at δ ~2.85 [CH(4) and $CH_2(7)$] and δ 3.36 [$CH_2(9)$] caused the multiplet at δ 1.9~2.3 to change into a 2-proton singlet [δ 2.06; tentatively named as $CH_2(8)$] and a 1-proton multiplet [δ 2.18, $(CH_3)_2C\underline{H}$]. These results clearly show the presence of a sequence of a trimethylene group $-CH_2(7)-CH_2(8)-$

Fig. 4. NMR spectrum of arglecin dihydrochloride deuterated with 1 N deuterochloric acid, in D_2O at 100 MHz.



$CH_2(9)$- and -$\overset{1}{C}H(4)$-$CH(CH_3)_2$. Relating coupling constants are shown in Table 1.

When the solution of the dihydrochloride of **I** in deuterium oxide was allowed to stand at 5°C for a week or the solution of **I** in 1 N deuterochloric acid in deuterium oxide was refluxed for 1 hour, the doublet of the methine proton at δ 2.84 (H-4) of **I** disappeared leaving the triplet at δ 2.87 (H-7) intact and without disturbance of any other signals as shown in Fig. 4. Above change, however, was not observed in 1 N sodium deuteroxide in deuterium oxide. When the above acidic solutions were refluxed in 1 N hydrochloric acid in water for 1 hour, the methine doublet of **I** was again recovered. These phenomena suggested the presence of a carbonyl group situated adjacent to the methine [CH(4)], to which an isopropyl group is attached, and the absence of the other protons situated adjacent to the carbonyl group than H-4. Above conclusion was further confirmed by the presence of a strong peak at m/e 209 in the mass spectrum of **I**. The fragment was interpreted as follows :



From the above data, the presence of the following groups as $-CH_2-CH_2-CH_2-$, -CO-$\overset{1}{C}H$-$CH(CH_3)_2$ and -CH= (an olefinic proton; named as H-6) were concluded.

Table 1. NMR data (δ values)

Compound	I	II	III	IV	V
Solvent	D_2O	D_2O	$CDCl_3$	D_2O	D_2O
H-2				4.04 t (~6)	
H-4	2.84 d (7)*	2.85 d (7.5)	2.67 d (7.5)	~1.8	~1.8
H-5				3.87 m	
H-6	7.50 s	7.49 s	7.28 s	3.30 q (8, 13)	3.30 s
H-6′				3.59 q (4.5, 13)	
CH_2-7	2.87 t (7.5)	2.89 t (7.5)	2.58 t (7)	~1.8	~1.8
CH_2-8	2.06 m (~7)	~2.1	~2.0	~1.7	~1.7
CH_2-9	3.36 t (6.5)	3.19 t (~7)	3.37 q (~6)	3.21 t (6)	3.21 t (6)
$\underline{CH_3}$ / $\underline{CH_3}$ >CH-	1.01 d (7)	0.99 d (7)	0.96 d (7)	0.98 d (6) d (6)	0.98 d (6) d (6)
CH_3 / CH_3 >$\underline{CH}$-	2.18 m	~2.2	~2.0	~1.8	~1.8
$C\underline{H_3}CONH$-			2.00 s	1.99 s	1.99 s
-NH-			~6.5		

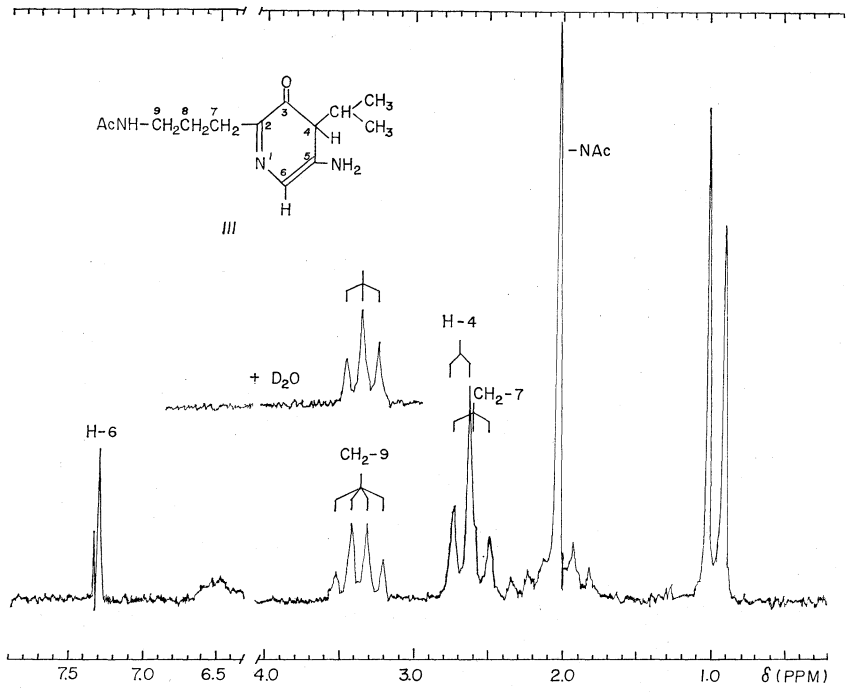
* Coupling constants (Hz) are shown in parentheses.

On the other hand, the diacetyl and SAKAGUCHI-positive characters of I suggest, together with its pka values (9.1 and >11), the presence of a guanidino and an amino groups. When arglecin was treated with barium hydroxide and followed by neutralization with hydrochloric acid, a diacetyl and SAKAGUCHI-negative derivative (II) was obtained as a dihydrochloride-monohydrate; m. p. 232~234°C. Its pka values were 8.9 and 10.6 (in water). The ultraviolet spectrum of II was quite similar to that of I, suggesting the chromophore to be unchanged. In the mass spectrum of II, two prominent peaks (m/e 209 and 167) were discerned. The former was assigned to the molecular ion $C_{11}H_{19}N_3O^+$ and the latter (M^+-42) was assigned to the fragment originated from the same fragmentation as described in I. There exists no significant change between the NMR spectra of I and II except for the chemical shift of H-9 at δ 3.19. From the above results and its ninhydrin-positive character, this compound (II) was elucidated to have the similar structure as I except for the amino group derived from the guanidino group of I.

Treatment of II with acetic anhydride in pyridine gave a ninhydrin-negative mono-N-acetylated compound (III: m.p. 180~181°C; pka 9.4) in a good yield. Another amino group remained unacetylated, and this inertness may be ascribable to the presence of a bulky group [-$CH(CH_3)_2$?] situated near to the amino group. The mass spectrum of III showed the molecular ion at m/e 251 and again the fragment of M^+-42 at m/e 209. The NMR spectrum of III (Fig. 5) showed a 2-proton quartet (J ~6 Hz) at δ 3.37, which collapsed to a triplet upon deuteration with deuterium oxide. This indicates that the methylene (δ 3.37) bears an acetamido group and therefore, in I, the guanidino group is attached to an end of the trimethylene group possibly at C-9.

From the above-mentioned results, the presence of the following moieties were concluded: $H_2NC(=NH)NHCH_2CH_2CH_2$-, -$COCHCH(CH_3)_2$, -CH=, -NH_2 and probably =N-. Since the chemical formula of I ($C_{12}H_{21}N_5O$) has five unsaturation sites, I was

Fig. 5. NMR spectrum of III in $CDCl_3$ with (upper) and without (lower) D_2O at 60 MHz.



predicted to have a ring system having two double bonds in it, to which a guanidinopropyl, an isopropyl, an amino and an oxo groups are attached. The final problem is, therefore, to decide the positions of the above-described groups on the ring, and the species of the ring.

Hygrogenation of **III** with hydrogen and platinum oxide readily gave a tetrahydro derivative (**IV**) as a monohydrochloride in 78 % yield; m.p. 201~202°C. On the other hand, treatment of **III** with deuterium and platinum oxide gave a trideuterated derivative (**V**) as a monohydrochloride in 71 % yield; m.p. 200~201°C. The mass spectra of **IV** and **V** showed the molecular ions at m/e 255 and m/e 258 respectively, which were in accordance with the formulae ($C_{13}H_{25}N_3O_2$ and $C_{13}H_{22}D_3N_3O_2$ respectively) estimated from the elemental analyses of **IV** and **V** respectively. On a silica gel thin-layer chromatogram with a solvent system: ethyl acetate - methanol (2 : 1), **IV** and **V** showed an identical mobility (Rf 0.3). The IR spectra of **IV** and **V** furnished the similar pattern each other: 1700 (C=O), 1650 (amide I) and 1550 cm^{-1} (amide II), suggesting that each of them retained a carbonyl group. By comparison of the molecular weights and formulae of **IV** and **V**, the occurrence of one active hydrogen was concluded. That is, on the above treatment of **III** with deuterium and platinum oxide, a tetradeuterated derivative will first be produced and then it will be changed to the trideuterated derivative during the purification procedure. This change will reasonably be interpreted by assuming the following transformations: $\rangle C{=}N- \longrightarrow \rangle CD-ND- \longrightarrow \rangle CD-NH-$ and $\rangle C{=}C\langle \longrightarrow \rangle CD-CD\langle$. This was confirmed by the NMR spectra of **IV** and **V**. In the NMR spectra of **IV** (Fig. 6), three protons, which were not discerned

Fig. 6. NMR spectrum of IV in D_2O at 100 MHz.

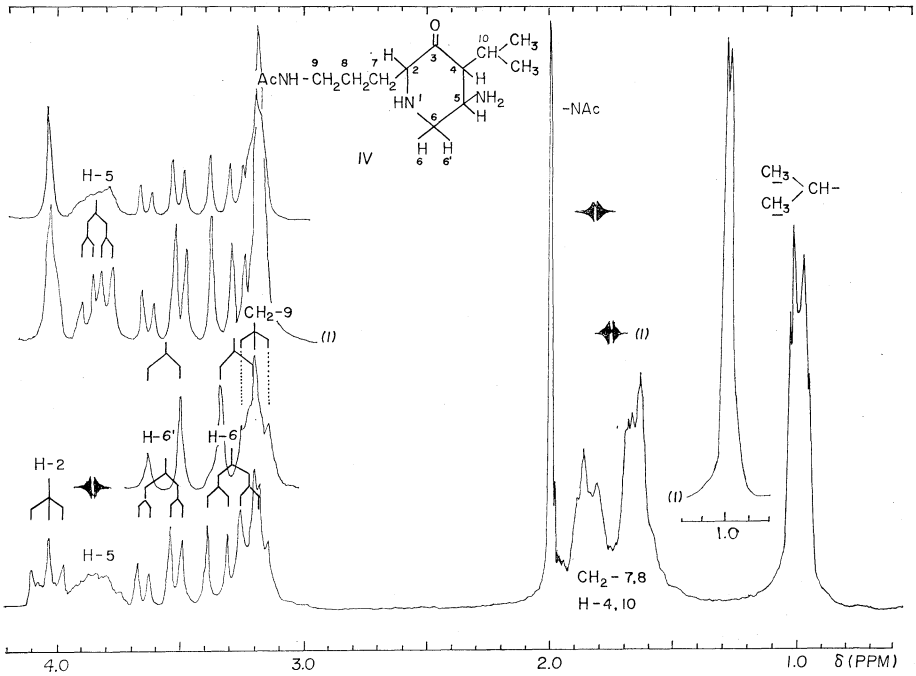
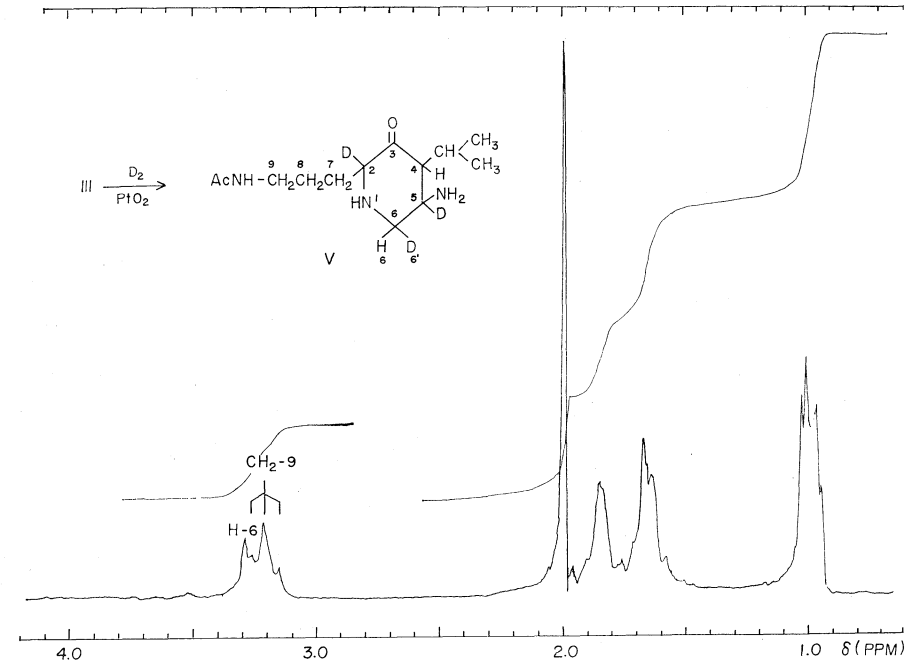


Fig. 7. NMR spectrum of V in D_2O at 100 MHz.



in that of **V** (Fig. 7) and therefore considered to be produced on the hydrogenation of the double bonds of **III**, appeared at δ 4.04 (an ill-defined triplet, tentatively named as H-2), 3.87 (multiplet, tentatively named as H-5) and 3.59 (quartet, tentatively named as H-6'). The triplet at δ 4.04 (H-2) collapsed, on irradiation at $\delta \sim 1.8$, to a sharp

singlet proving that H-2 does not couple with H-5, H-6′ and H-6 (about the latest, we will describe below).

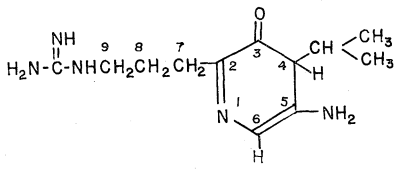
The fact that H-2 appeared as a triplet requires two vicinally situated hydrogens and this is satisfied, in the present case, only by situating a methylene group to the position. Moreover, the observation of the ill-defined triplet of H-2 will be interpreted by assuming the presence of a virtually long-range coupling[10] and this will be satisfied by situating the acetamidopropyl side chain in the vicinal position of H-2. Since H-2 should be born by hydrogenation of $-\overset{|}{C}=N-$ to $-\overset{|}{C}H(2)-NH-$ (this will be understood from the subsequent description), the following reaction was confirmed.

$$AcNHCH_2CH_2CH_2-\overset{|}{C}=N- \text{ (in III)} \longrightarrow AcNHCH_2CH_2CH_2-\overset{|}{C}H(2)-ND- \text{ (in IV in } D_2O).$$

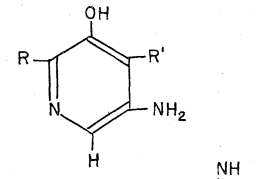
In the NMR spectrum of **IV**, another quartet (δ 3.30, tentatively named as H-6) was discerned and this was collapsed to a singlet at the same shift-value (δ 3.30) in **V**. Thus, it can be concluded that this proton in **V** corresponds to the proton at δ ~7.4 [-CH(6)=] in **I**, **II** or **III**. Irradiation of H-5 (δ 3.87) caused the octet due to H-6 and H-6′ to collapse to an AB quartet (J 13 Hz) indicating the presence of the grouping $-CH(6)H(6')-\overset{|}{C}H(5)-$ in **IV** and $-CDH(6)-\overset{|}{C}D-$ in **V** and the absence of the other hydrogens adjacent to the methylene protons [-CH(6)H(6′)-]. Irradiation at δ 1.75 caused the multiplet at δ 3.87 (H-5) to collapse to a clear quartet (J 4.5 and 8.0 Hz), indicating the coupling of H-5 with a proton at δ~1.75 (tentatively named as H-a). From the shift-value of H-5 (δ 3.87) and aforementioned results, H-5 should be attached to the carbon bearing the amino group. Thus, the presence of the group ($=\overset{|}{C}-NH_2$) is indicated in **I**, **II** or **III**. Therefore, the following reaction was confirmed: $-CH(6)=C(NH_2)-\overset{|}{C}Ha-$ (in III) ⟶ $-CH(6)H(6')-CH(5)(NH_2)-\overset{|}{C}Ha-$ (in IV).

Since, as mentioned above, H-2 in **IV** did not couple with H-5, H-6′ and H-6, and the methylene [CH(6)H(6′)] coupled only with H-5, the two parts $AcNHCH_2CH_2CH_2-\overset{|}{C}H(2)-NH-$ and $-CH_2(6, 6')-CH(5)(NH_2)-\overset{|}{C}Ha-$ should be combined as $AcNHCH_2CH_2CH_2-$

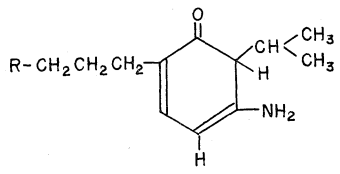
Chart 1



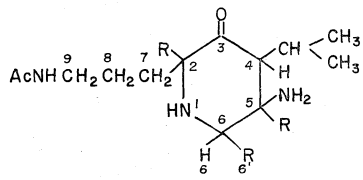
I. Arglecin



I′. R= $-CH_2CH_2CH_2NHC(=NH)NH_2$
R′= $-CH(CH_3)_2$



II. R= $-NH_2$
III. R= $-NHCOCH_3$



IV. R = -H
V. R = -D

$\overset{|}{C}H(2)$-NH-$CH_2(6, 6')$-$CH(5)(NH_2)$-$\overset{|}{C}Ha$-. The way of attachment of the above chain to the other part -$COCH(4)$-$CH(CH_3)_2$ was solved by the fact that H-2 was coupled only with a methylene group as described before. Thus, H-a should be equal to H-4.

From the aforementioned results, the structure of arglecin is concluded to be 5-amino-2-(3-guanidinopropyl)-3,4-dihydro-4-isopropylpyridin-3-one (I). The structure is reasonable from the biosynthetic viewpoint because it can be constructed from arginine and leucine.

On the structural viewpoint, it is characteristic that arglecin exists largely in the form of I and not in the form of I' (see Chart 1). This will be the reflection that structure I acquires more resonance energy than I' does. The optical inactivity of arglecin, however, suggests that I and I' will exist in an equilibrium in an appropriate medium although the molar intensity is much inclined to I. The speed of the equilibration will be accelerated by acid as shown by the exchange of H-4 with deuterium. The high pka value (9.1) of the amino group is thought to be due to the difficult conversion to I'.

Experimental

Thin-layer chromatography was performed by the use of silica gel ("Silica-Rider" purchased from Daiichi Pure Chemicals Co.) or microcrystalline cellulose powder ("Avicel" purchased from Funakoshi Yakuhin Co.) and the chromatograms were visualized by spraying with Wood, diacetyl, ninhydrin (0.5 % ninhydrin in pyridine) or sulfuric acid. The NMR spectra were recorded with Varian-A-60D and HA-100D spectrometers. Tetramethylsilane (for the solution of deuterochloroform) and sodium 4,4'-dimethyl-4-silapentane-1-sulfonate (for the solution of deuterium oxide) were used as the internal standards. All data concerning the NMR spectra were described in Table 1.

Treatment of arglecin with barium hydroxide (Synthesis of II). Arglecin dihydrochloride (2.0 g) in 1 N aqueous barium hydroxide solution (50 ml) was refluxed for 2 days. The solution was cooled, saturated with carbon dioxide and centrifuged. The supernatant layer was evaporated to give a solid which was dissolved in a small volume of ethanol, acidified to pH 2 with diluted hydrochloric acid and the solution was evaporated to dryness. The resulting solid, which showed virtually a single spot (Rf 0.55) on a thin-layer chromatogram with cellulose powder [developed by *n*-butanol - ethanol - water (5 : 1 : 2) with coloration by ninhydrin], was chromatographed on a cellulose powder column ("Avicel" 140 g, 3.7×37 cm) with the same solvent system. The ninhydrin-positive fraction containing II (230~350 ml) was evaporated to give colorless needles of the dihydrochloride-monohydrate of II, which was recrystallized from ethanol - ethyl acetate; yield 1.7 g (92%); m.p. 232~234°C, $[\alpha]_D^{20}$ 0° (*c* 1.0, water).

Found: C 43.81, H 7.88, N 14.27, Cl 23.38. Calcd. for $C_{11}H_{19}N_3O \cdot 2HCl \cdot H_2O$: C 44.00, H 7.72, N 14.00, Cl 23.62 %.

Mass spectrum (m/e): 209 (molecular ion), 179, 167, 166, 150, 149, 137, 123.

IR spectrum (KBr disk): ~3400 (NH); ~2920 (CH); 2460, 2360 (ammonium); 2040, 1690, 1675, 1650, 1635, 1600 (C=O, C=C and C=N); 1510 (NH) cm^{-1}.

UV spectrum: λ_{max} (ε): $\lambda_{max}^{H_2O}$ 321 (12,700), 226 mμ (10,900); $\lambda_{max}^{0.1N\ NaOH}$ 321 (12,600), 232 mμ (11,400); $\lambda_{max}^{0.1N\ HCl}$ 335 (13,500), 227 mμ (13,100).

Acetylation of II (Synthesis of III). To an ice-cold solution of II dihydrochloride-monohydrate (1.2 g) in pyridine (20 ml), acetic anhydride (2.7 ml) was added and the solution was allowed to stand overnight. After addition of a small volume of ethanol, the solution was evaporated to the residue, which showed, on thin-layer chromatography with silica

gel (ethyl acetate – methanol 8 : 1), two spots of Rf 0.6 (trace) and Rf 0.3 (major). Repeated recrystallization of the residue from ethyl acetate – methanol gave colorless needles (Rf 0.3) of the mono-N-acetylated derivative **III**; yield 850 mg (87 %); m.p. 180~181°C.

A small amount of the Rf 0.6-substance was isolated as a colorless syrup, however, the syrup was gradually crystallized with concomitant change to the Rf 0.3-substance during the storage in a desiccator or recrystallization from ethyl acetate – methanol.

Found: C 62.36, H 8.39, N 16.73. Calcd. for $C_{13}H_{21}N_3O_2$: C 62.12, H 8.42, N 16.73 %.

Mass spectrum (m/e): 251 (molecular ion), 209, 179, 150, 149, 137, 123.

IR spectrum (KBr disk): 3280 (NH); 2920~2800 (CH); 1690 (C=O); 1650 (amide I); 1560 (amide II); 1530, 1460, 1365 cm^{-1}.

UV spectrum: λ_{max} (ε): $\lambda_{max}^{H_2O}$ 324 (11,300), 227 mμ (9,300); $\lambda_{max}^{0.1N\ NaOH}$ 322 (10,400), 232 mμ (9,700); $\lambda_{max}^{0.1N\ HCl}$ 342 (12,000), 227 mμ (10,700).

Reduction of **III** with hydrogen (Synthesis of **IV**). A solution of **III** (421 mg) in a mixture of methanol (6 ml), water (1.5 ml) and 1 N hydrochloric acid (0.4 ml) was hydrogenated with platinum oxide (420 mg) and hydrogen at 40°C under 3.5 atm pressure overnight. The solution was filtered and the filtrate was evaporated to give a solid, which gave a single spot (Rf 0.3) on a silica gel thin-layer chromatogram (ethyl acetate – methanol, 2 : 1). The solid was chromatographed on a silica gel column (Mallincklodt 84 g, 2.6 × 31 cm) with the same solvent system and the fraction containing **IV** (440~670 ml) was evaporated to give crystals. Recrystallization from ethanol – ethyl acetate gave colorless needles of the hydrochloride of **IV**; yield 310 mg (78 %); m.p. 201~202°C.

Found: C 53.58, H 9.21, N 14.27, Cl 12.43. Calcd. for $C_{13}H_{25}N_3O_2 \cdot HCl$: C 53.50, H 8.98, N 14.40, Cl 12.15 %.

Mass spectrum (m/e): 255 (molecular ion), 212, 198, 170, 155.

IR spectrum (KBr disk): 3300~3280 (NH); 2950~2760 (CH); 1700 (C=O); 1660 (amide I); 1550 (amide II); 1465, 1375, 1310, 1240 cm^{-1}.

Reduction of **III** with deuterium (Synthesis of **V**). A sample of **III** (290 mg) was treated similarly as above except for the replacements of hydrogen and the solvents with deuterium and deuterated solvents respectively. The monohydrochloride of the trideuterated product **V** was obtained as colorless needles; yield 230 mg (71 %); m.p. 200~201°C.

Found: C 53.18, N 14.45. Calcd. for $C_{13}H_{22}D_3N_3O_2 \cdot HCl$: C 52.95, N 14.25 %.

Mass spectrum (m/e): 258 (molecular ion), 215, 201, 173, 158.

IR spectrum (KBr disk): 3300~3280 (NH); 2950~2750 (CH); 1700 (C=O); 1660 (amide I); 1550 (amide II); 1475, 1455, 1375, 1300, 1160 cm^{-1}.

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