ISOLATION AND CHEMICAL STRUCTURE OF AKLANONIC ACID, AN EARLY INTERMEDIATE IN THE BIOSYNTHESIS OF ANTHRACYCLINES

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The fermentation, isolation and structure elucidation of aklanonic acid are described. The compound was isolated from fermentations of *Streptomyces* strain ZIMET 43,717. Aklanonic acid is a yellow-orange crystalline substance, melting at 203~204°C (dec), having the molecular formula $C_{21}H_{16}O_8$, and possessing UV maxima at 258, 282 (sh) and 438 nm ($CHCl_3$). In dimethyl sulfoxide or pyridine aklanonic acid is unstable and a new compound (aklanone) is formed as a conversion product. The elucidation of the structures has shown that aklanonic acid and aklanone are derivatives of 1,8-dihydroxyanthraquinone.

During our antibiotic screening program, a *Streptomyces* strain (designated as *Streptomyces* sp. ZIMET 43,717) was found to produce a new anthracycline-related pigment. The compound, which has been named aklanonic acid, is not active as an antibiotic. However, aklanonic acid is of particular interest because we have found that this compound was biotransformed to structurally different anthracyclinones in feeding experiments with several pigment-negative mutants[1,6]. Its role in the biosynthetic pathway of anthracyclines is now under study. The present paper is concerned with the physico-chemical properties of aklanonic acid and the results of the structural elucidation. The biological characteristics of the producing strain, which is unable to form aerial mycelia and spores, will be the subject of a separate communication.

Results

Isolation and Physico-chemical Properties

Aklanonic acid was obtained mainly from the mycelium by extraction with acetone. After purification by column chromatography and recrystallization from ethyl alcohol the compound was obtained as pure yellow-orange needles with a melting point of 203~204°C (dec). Aklanonic acid is only slightly soluble in water, moderately soluble in ethyl alcohol, methanol, acetone and chloroform, and soluble in dimethyl sulfoxide and a solution of sodium bicarbonate in water. In concentrated sulfuric acid and sodium hydroxide the compound dissolves with a red color. When dissolved in dimethyl sulfoxide or pyridine aklanonic acid was rapidly converted to a new compound which we have named aklanone. The UV and visible spectrum of aklanonic acid in chloroform contains maxima at 258, 282 (sh), and 438 nm (ε 23,300, 19,300, and 10,300). The IR spectrum in potassium bromide had characteristic absorptions at 1622, 1670 and 1700 cm^{-1}.

Aklanonic acid has the molecular formula $C_{21}H_{16}O_8$. Attempts to derive the exact molecular formula based upon accurate molecular weight measurements by mass spectrometry and microanaly-

Fig. 1. Mass spectrum of aklanonic acid methyl ester.

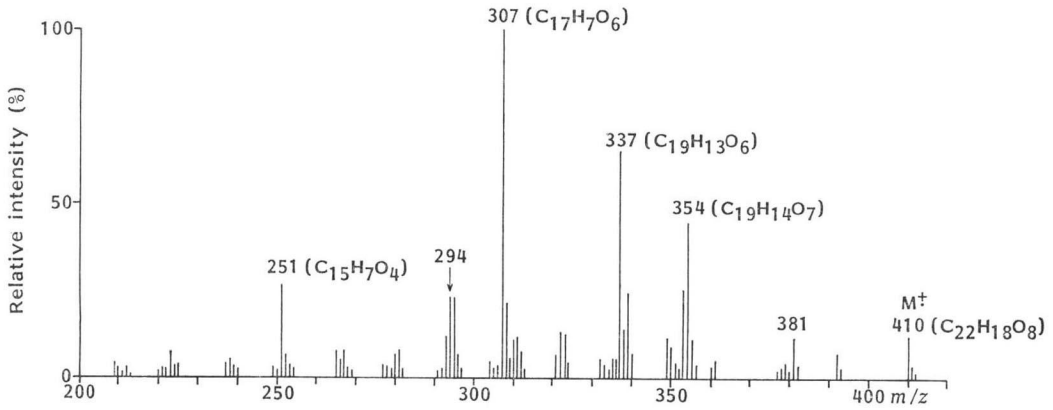
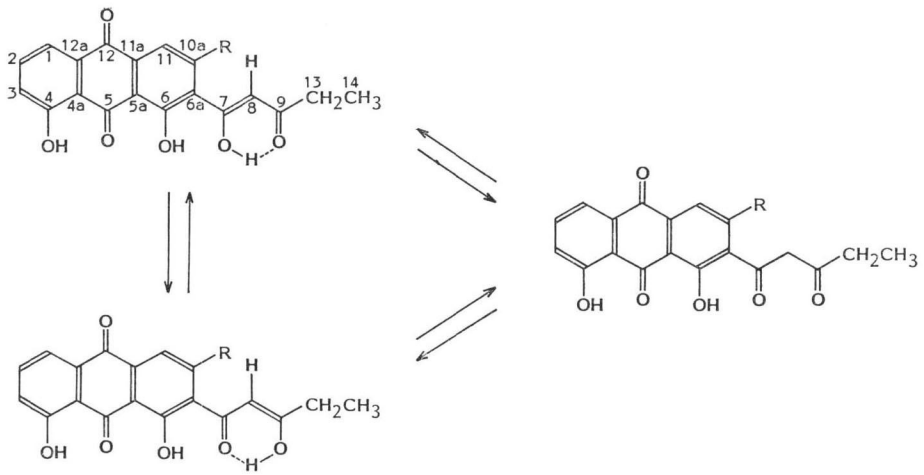


Fig. 2. Structures of aklanonic acid, aklanonic acid methyl ester and aklanone. (Numbering corresponds to that used for anthracyclines).



1 Aklanonic acid: $R=\overset{10}{C}H_2\overset{15}{C}OOH$

2 Aklanonic acid methyl ester: $R=\overset{10}{C}H_2\overset{15}{C}O\overset{16}{O}CH_3$

3 Aklanone: $R=\overset{10}{C}H_3$

tical data were unsuccessful. The mass spectrum of aklanonic acid is completely identical with that of aklanone. Therefore, the molecular formula of aklanonic acid has been deduced from the NMR spectra and from the analytical data of its methyl ester (Fig. 1).

Structure Elucidation

Structural elucidation studies of aklanonic acid have indicated that the compound has formula **1** (Fig. 2). This structure readily accommodates the similarity of aklanonic acid's UV spectrum to that of 1,8-dihydroxyanthraquinone (λ_{max} in chloroform 255, 285, and 435 nm; ε 18,100, 10,300, and 10,100) and the observation of one chelated (1622 cm^{-1}) and one non-chelated quinone carbonyl (1670 cm^{-1}) in the IR spectrum. Furthermore, the presence of an IR band at 1700 cm^{-1} was consistent with a carboxyl group, which was confirmed by conversion of aklanonic acid to its methyl ester, giving rise

Table 1. ^{1}H NMR data of aklanonic acid (**1**), aklanonic acid methyl ester (**2**) and aklanone (**3**) ($CDCl_3$, 200 MHz).

Assignment of protons	Chemical shift δ, ppm (J, Hz)		
	1	**2**	**3**
H-1	7.84 dd (A)	7.83 dd (A)	7.82 dd (A)
H-2	7.71 t (B)	7.69 t (B)	7.68 t (B)
H-3	7.32 dd (C) (J_{AB}=8; J_{AC}=1.7; J_{BC}=8)	7.31 dd (C) (J_{AB}=8; J_{AC}=1.7; J_{BC}=8)	7.29 dd (C) (J_{AB}=8; J_{AC}=1.7; J_{BC}=8)
H-11	7.83 s	7.75 s	7.68 s[a]
4-OH[b]	11.89 s	11.90 s	11.98 s
6-OH[b]	12.70 s	12.54 s	12.43 s
7/9-OH		15.40 s, br	15.47 s, br
H-8	6.04 s	5.91 s	5.80 s
H-10 (CH_2)	3.86 s	3.84 s	—
H-10 (CH_3)	—	—	2.46 s[a]
CH_2 (C_2H_5)	2.47 q (A_2)	2.44 q (A_2)	2.46 q (A_2)
CH_3 (C_2H_5)	1.21 t (X_3) (J_{AX}=7.5)	1.19 t (X_3) (J_{AX}=7.5)	1.19 t (X_3) (J_{AX}=7.5)
OCH_3	—	3.71 s	—

Abbreviations: s Singlet, d doublet, dd doublet of doublets, t triplet, q quartet, br broad signal.

[a] Aklanone shows an unresolved long-range coupling between the H-11 and H-10 (CH_3) protons. This was confirmed by double resonance experiments.

[b] The signals at δ=11.89, 11.90 and 11.98 ppm in the spectra of compounds 1, 2 and 3 were assigned to the OH group at C-4 because of the relatively constant chemical shifts. This is due to the greater distance to the substituents at C-6a and C-10a as compared to the OH group at C-6 which is more influenced. This is in good accordance with the assignments of the corresponding signals in the spectra of aklavinone, aklavinone I and aklavinone II mede by KROHN[8].

to an IR carbonyl band at 1740 cm^{-1} and a singlet (3 protons) with a chemical shift of δ 3.71 ppm in the ^{1}H NMR spectrum. The NMR spectra of aklanonic acid, its methyl ester and aklanone are completely assigned (Tables 1 and 2). It is evident from the spectra that positions 1~4 of the anthraquinone nucleus are occupied by 3 coupled protons and one hydroxyl. One additional proton appearing in the ^{1}H NMR spectrum of aklanonic acid as a singlet with a characteristic chemical shift of δ 7.83 ppm (7.68 ppm in the spectrum of aklanone) must arise from an aromatic proton located at position 11 (*peri* to the anthraquinone carbonyl*).

The structures of the two side chains followed from consideration of the NMR spectra of **1** and its methyl ester (for assignments of the signals see Tables 1 and 2) with supplementary evidence from mass spectra. They occupy the last two free positions, designated 6a and 10a*. The exact location followed from the determination of the structure of aklanone, which is a conversion product of aklanonic acid. The structure of **3** is nearly identical with that of **1**. This is evident from comparison of their NMR spectra. Most of the signals present in the spectra of aklanonic acid are very similar to the corresponding resonances in the spectra of aklanone. The only difference is that the CH_2COOH group in **1** is replaced by a CH_3 group in **3**, giving rise to the ^{1}H NMR signal at δ 2.46 ppm and a ^{13}C resonance at δ 20.70 ppm instead of the CH_2 singlet at δ 3.86 and ^{13}C NMR resonances at 39.44 and 171.10 ppm. Double resonance experiments confirmed an unresolved long-range coupling between the proton at C-11 and the CH_3 protons with coupling constants indicating they are *ortho* to each other

* Numbering corresponds to that used for the anthracycline antibiotics.

Table 2. ^{13}C NMR data[a] in $CDCl_3$ of aklanonic acid methyl ester (**2**) and aklanone (**3**).

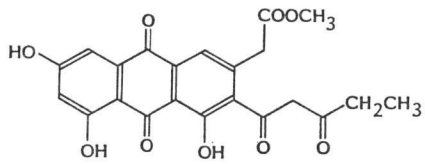
	Chemical shift (ppm)	
	2	**3**
1	120.31 (d)	120.14 (d)
2	137.55 (d)	137.29 (d)
3	124.99 (d)	124.85 (d)
4	160.19 (s)	159.88 (s)
5	192.67 (s)	192.53 (s)
6	162.76 (s)	162.65 (s)
7[b]	183.55 (s)	184.13 (s)
8[b]	102.61 (d)	102.10 (d)
9[b]	195.94 (s)	196.41 (s)
10 (CH_2)	39.44 (t)	—
10 (CH_3)	—	20.70 (q)
11	122.28 (d)	121.93 (d)
12	181.04 (s)	181.42 (s)
13	31.70 (t)	31.81 (t)
14	9.52 (q)	9.47 (q)
15	171.10 (s)	—
16	52.31 (q)	—
4a, 5a	115.20 (s), 115.81 (s)	115.87 (s), 114.13 (s)
6a, 11a, 12a	133.69 (s), 133.48 (s), 132.38 (s)	133.52 (s), 133.31 (s), 132.12 (s)
10a	142.72 (s)	147.23 (s)

^{13}C NMR FT spectra were recorded on a Bruker WP 200 spectrometer at 50.327 MHz. Ppm downfield from TMS. Multiplicities are indicated in parenthesis.

[a] The chemical shifts coinsided with the data reported for the corresponding molecule parts of 1,8-dihydroxy-3-methoxycarbonylmethyl-2-(1-oxopropyl)-9,10-anthraquinone[9] and aklavinone[10].

[b] Assignments were based on data reported for acetylacetone[11] taking into account the effects of the different neighboring groups.

Fig. 3. Structure of compound **58B**[5].



(J=0.6~0.8 Hz). As mentioned before, this proton has been located on C-11 because of its characteristic chemical shift. This is also consistent with a downfield shift of 0.40 ppm which was observed after acetylation of the 6-OH. Consequently, the five-carbon side chain must occupy the position C-6a in both aklanone and aklanonic acid. The formation of **3** from **1** seemed to be unusual; however, a similar reaction has been described by PEARLMAN *et al.*[7].

In Fig. 1 the chemical structures of aklanonic acid, its methyl ester, and aklanone are depicted in three tautomeric forms. However, as is well known from simple 1,3-diketones such as acetylacetone and benzoylacetone, the keto-enol equilibrium lies far to the side of the enol-form. This is also evident for our compounds from the singlets assigned to the olefinic proton at C-8, as well as the resonances of the enolic hydroxyls which in all spectra were found to have the intensity of about 1 proton. (The OH signal in the spectrum of **1** was not observed because of the inter- and intramolecular proton exchange due to the presence of the carboxyl group). Our results are in contrast to findings of TOBE *et al.*[5] who preferred the diketo-form for compound **58B** (Fig. 3), a substance closely related to aklanonic acid methyl ester. In the spectra of all three compounds, aklanone, aklanonic acid, and aklanonic acid methyl ester, the signal which must be assigned to the 8-CH_2 of the keto-form had only very low intensity.

The chemical structure of aklanonic acid as depicted in Fig. 1 is in harmony with our findings that the compound is accepted by several *Streptomyces* strains as a precursor for the biosynthesis of anthracyclines[1].

Discussion

Aklanonic acid is an interesting new anthraquinone derivative isolated from fermentations of *Streptomyces* strain ZIMET 43,717. According to its chemical structure aklanonic acid is related to

the group of anthracyclinones. Similar compounds have been isolated by JIZBA *et al.*[4] and TOBE *et al.*[5]. Aklanonic acid differs from these compounds on the basis of its physico-chemical properties and the results of the structural elucidation. All of these anthraquinone derivatives have been isolated from mutants of anthracycline-producing *Streptomyces* strains. VANĚK *et al.*[3] and TOBE *et al.*[5] suggested that some of these compounds may be intermediates at an early stage in the biosynthesis of anthracyclines. Recently we have shown that, in fact, aklanonic acid can serve as a precursor for different anthracyclinones[1]. More recent experiments with ^{14}C-labeled aklanonic acid confirmed these results and will be published elsewhere[6]. It is most likely that aklanonic acid can not be further transformed by its producing organism and accumulates in the mycelium because of lack of the enzymes involved in the biosynthetic pathway of complete anthracyclines.

Experimental

Isolation of Aklanonic Acid (1)

Streptomyces strain ZIMET 43,717 was used for fermentation. The culture was grown to sporulation for 10 to 14 days at 28°C on agar consisting of sucrose 0.3%, dextrin 1.5%, urea 0.01%, yeast extract 0.1%, Bacto Peptone (Difco) 0.5%, NaCl 0.05%, KH_2PO_4 0.05%, $FeSO_4$ 0.001%, agar 2.0%; pH 6.5~7.0. The following seed medium was used to produce a vegetative inoculum: glucose 1.5%, soybean meal 1.5%, NaCl 0.5%, $CaCO_3$ 0.1%. This medium was inoculated with a spore suspension and then incubated for 48 hours at 28°C on a rotary shaker. The vegetative mycelium of the seed culture was used to inoculate the fermentation medium consisting of glucose 2.0%, soybean meal 2.0%, NaCl 0.5%, $CaCO_3$ 0.3%. Culture flask fermentations were usually conducted at 28°C for 72 to 96 hours on a rotary shaker at 180 rpm.

The red mycelium from the harvested broth (5.6 liters) was extracted with two liters of acetone. Then the red extract was concentrated to 1/4 of its volume *in vacuo*. After addition of 500 ml of $CHCl_3$ and 500 ml of 0.01 N HCl the red color turned to brown. The $CHCl_3$ layer containing the aklanonic acid was washed with H_2O and dried with sodium sulfate. Concentration of the extract to a small volume afforded 811 mg of orange-yellow needles. Analytically pure **1** was prepared by column chromatography on potassium dihydrogen phosphate buffered silica gel[2] by use of the $CHCl_3$ - MeOH (100: 10) system. Compound **1** was eluted as the main yellow band. Recrystallization from EtOH afforded the substance as pure yellow-orange needles.

Aklanonic Acid Methyl Ester (2)

Treatment of **1** in $CHCl_3$ - MeOH (90: 10) with a solution of diazomethane in ether for 2 minutes at 0°C resulted in the formation of **2**. The mixture was concentrated *in vacuo* to give the crude product which was purified by column chromatography on phosphate buffered silica gel, system $CHCl_3$ - MeOH (90: 10), and recrystallization from EtOH to give yellow needles; mp 143~144°C; IR ν_{max} (KBr) cm^{-1} 1740, 1677, 1625; MS *m/z* 410.1010 (M^+, $C_{22}H_{18}O_8$), 392 ($C_{22}H_{16}O_7$), 381 ($C_{20}H_{13}O_8$), 379 ($C_{21}H_{15}O_7$), 354 ($C_{19}H_{14}O_7$), 353 ($C_{19}H_{13}O_7$), 349 ($C_{19}H_9O_7$), 339 ($C_{18}H_{11}O_7$), 337 ($C_{19}H_{13}O_6$), 323 ($C_{18}H_{11}O_6$), 322 ($C_{18}H_{10}O_6$), 307 ($C_{17}H_7O_6$), 295 ($C_{16}H_7O_6$), 294 ($C_{17}H_{10}O_5$), 281 ($C_{16}H_9O_5$), 251 ($C_{15}H_7O_4$).

Anal Calcd for $C_{22}H_{18}O_8$: C 64.39, H 4.39.
Found: C 63.91, H 4.40.

Conversion of 1 to Aklanone (3)

Compound **1** (120 mg) was dissolved in 0.5 ml of DMSO. The mixture was allowed to stand at room temperature for 2 days. Then the precipitated crystals of **3** (87 mg) were filtered off and washed with a small amount of EtOH. The compound was purified by column chromatography on phosphate buffered silica gel, system $CHCl_3$ - MeOH (100: 10) and recrystallization from EtOH; yellow needles, mp 180~181°C; MS *m/z* 352.0951 (M^+, $C_{20}H_{16}O_6$); IR ν_{max} (KBr) cm^{-1} 1680, 1620, 1592. The UV visible spectrum is closely related to that of **1**.

Aklanone Triacetate (4)

To a solution of **3** (67 mg) in 4 ml of pyridine 2 ml of acetic anhydride was added. The mixture

was kept for 20 hours at room temperature, then 150 ml of H_2O was added. The precipitated acetate was filtered off, rinsed with H_2O and dried. Recrystallization from EtOH gave the triacetate as yellow needles; mp 125~126°C; MS *m/z* 478 (M^+, $C_{26}H_{22}O_9$), 436.1178 ($M-CH_2CO$), 418 ($C_{24}H_{18}O_7$), 404 ($C_{23}H_{16}O_7$), 394 ($C_{22}H_{18}O_7$), 377 ($C_{22}H_{17}O_6$), 376 ($C_{22}H_{16}O_6$), 362 ($C_{21}H_{14}O_6$), 352 ($C_{20}H_{16}O_6$), 347 ($C_{20}H_{11}O_6$), 337 ($C_{19}H_{13}O_6$), 334 ($C_{20}H_{14}O_5$), 323 ($C_{18}H_{11}O_6$), 320 ($C_{19}H_{12}O_5$), 295 ($C_{17}H_{11}O_5$), 281 ($C_{16}H_9O_5$).

Anal Calcd for $C_{26}H_{22}O_9$: C 65.27, H 4.60.
Found: C 65.43, H 4.68.

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