AB3217-A, A NOVEL ANTI-MITE SUBSTANCE PRODUCED BY A STRAIN OF *Streptomyces platensis*

KENJI KANBE, YAYOI MIMURA, TSUYOSHI TAMAMURA, SACHIYO YATAGAI, YASUNORI SATO, ATSUSHI TAKAHASHI and KIYOSHI SATO

Central Research Laboratories, Hokko Chemical Industry Co., Ltd., 2165 Shimotoda, Atsugi-shi, Kanagawa 243, Japan

HIROSHI NAGANAWA, HIKARU NAKAMURA and TOMIO TAKEUCHI

Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

YOICHI IITAKA

Laboratory of Biophysics, Faculty of Pharmaceutical Science, Teikyo University, Sagamiko-machi, Tsukui-gun, Kanagawa 199-01, Japan

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AB3217-A, a novel anti-mite substance, was isolated from the fermentation broth of a streptomycete strain. The strain was isolated from a soil collected at Kita-azumi, Nagano Prefecture, Japan, and identified as *Streptomyces platensis* AB3217.

AB3217-A was purified by Amberlite IR120B, Diaion HP-20 and CM-Sephadex C-25 column chromatographies. The molecular formula was determined as $C_{17}H_{23}NO_7$ by elemental analysis, MS and ^{13}C NMR spectroscopy. The structure of AB3217-A was determined to be (1*R*,3*S*,4*S*,-7*R*,8*R*,11*R*,12*S*,13*R*)-4,12,13-trihydroxy-8-(4-methoxyphenyl)-6-aza-2,9,14-trioxatricyclo-[9.2.1.$0^{3,7}$]tetradecane by spectroscopic analysis and X-ray crystallographic analysis. The molecule of AB3217-A has unique structure that deacetylanisomycin and β-D-xylofuranose linked through glycosidic bond and ether bond resulting in the formation of nine-membered ring.

AB3217-A showed marked activity against the two spotted spider mite, *Tetranychus urticae*.

In the course of screening for new anti-mite substances from soil microorganisms, we found that a streptomycete strain AB3217, which was isolated from a soil collected at Kita-azumi, Nagano Prefecture, Japan, produced a new substance, AB3217-A with a unique nine-membered ring structure (Fig. 1).

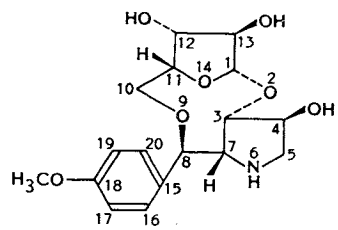
We describe here the taxonomy of the producing organism together with purification, physicochemical properties, structure determination and biological properties of AB3217-A.

Materials and Methods

Taxonomical Examination

Morphological, cultural and physiological properties of strain AB3217 were examined according to the methods described by SHIRLING and GOTTLIEB[1]. Detailed observation of mycelial and spore morphologies was performed with the use of a light microscope (Olympus Model BHS-123N) and scanning electron microscope (Hitachi S-430). Chemical analyses of the cell wall were performed by the method of LECHEVALIER and LECHEVALIER[2].

Fig. 1. Absolute structure of AB3217-A.



Fermentation

A slant culture of strain AB3217 on yeast extract-malt extract agar was inoculated into a 500-ml Sakaguchi flask containing 125 ml of a medium consisting of dextrin 20 g, galactose 20 g, corn-steep liquor 5 g, Bacto-soytone 10 g, $(NH_4)_2SO_4$ 2 g and $CaCO_3$ 2 g in 1 liter of deionized water (pH 7.4 before autoclaving) and incubated at 27°C for 72 hours on a reciprocal shaker (135 rpm). For the production of AB3217-A, 2 ml of the culture was transferred to a 500-ml Sakaguchi flask containing 125 ml of the fresh medium and incubated for 120 hours in the same way as above.

Analytical Procedures

TLC: AB3217-A content was monitored during purification by silica gel TLC (Kieselgel 60 F_{254}, Art. No. 5715, Merck) developed with EtOAc-MeOH (1:1). Spots were detected on TLC with the vanillin-H_2SO_4 reagent. Rf value of AB3217-A was 0.5.

Physico-chemical Characteristics: Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. IR and UV spectra were recorded with a Hitachi 285 spectrophotometer and a Hitachi 557 spectrophotometer, respectively. The ^1H and ^{13}C NMR spectra were measured with a Jeol GX400 spectrometer. The MS was recorded with a Hitachi M-80H mass spectrometer.

Biological Procedures

The anti-mite effect of AB3217-A against the two spotted spider mite (*Tetranychus urticae*) was examined by a pot test in a green house. Twenty adult mites were released on leaves of kidney bean in the first stage. One day after the release, leaves were thoroughly sprayed with the diluted AB3217-A preparation. The numbers of adult mites surviving on the leaves were determined 14 days after treatment. The protection coefficient (%) was calculated from the following formula: $100 \times (1-(\text{number of the mites on treated leaves})/(\text{number of the mites on non-treated leaves}))$. Dicofol, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol 40% emulsified solution, was used as a control agent.

The MIC of AB3217-A was tested according to the agar dilution method with 2-fold dilution. Bacteria were incubated in nutrient agar or Noken agar depending on the organism. Yeasts were incubated in Sabouraud agar and molds were incubated in potato-sucrose agar.

Table 1. Taxonomic characteristics of strain AB3217.

Morphological characteristics:	
Spore chain	Spore chain > 10, spirales
Spore	Oval or crescent
Cultural characteristics:	
Aerial mycelium	Gray color, hygroscopic
Soluble pigment	Yellow or not formed
Chemical characteristics:	
Cell wall type	I
Whole cell sugar pattern	None diagnostic sugars
Physiological properties:	
Positive	Gelatin liquefaction, starch hydrolysis
Negative	Milk coagulation, milk peptonization, nitrate reduction, melanin formation
Carbon utilization:	
Positive	D-Fructose, D-glucose, inositol, D-mannitol, raffinose, sucrose, L-arabinose, D-xylose
Negative	Rhamnose

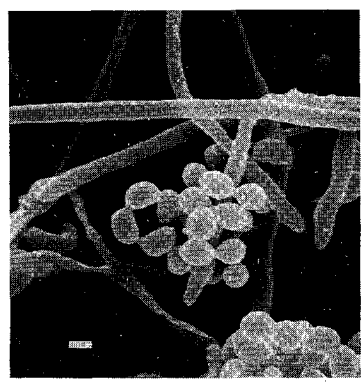
Results

Taxonomic Features of Strain AB3217

Cultural and physiological features of strain AB3217 are summarized in Table 1. Aerial mass

Fig. 2. Scanning electron micrograph of spore chain of strain AB3217 (on yeast extract-malt extract agar, 27°C, 7 days).

Bar: 0.5 μm.



color of the strain was white to light gray or brownish gray to dark gray depending on the medium. Distinct hygroscopic mycelia were observed on ISP No. 2 and ISP No. 4 media. Mature spores occurred in chains of more than 10 spores forming open spirals. The spore was oval to crescent in shape with smooth surface and $0.5 \sim 0.6 \times 0.6 \sim 0.8\,\mu m$ in size (Fig. 2). The reverse side color of colonies was usually pale yellowish brown. Soluble pigment was formed in only two media, ISP No. 2 and ISP No. 7. Melanoid pigment was not formed. Nitrate reduction was negative. The whole cell acid hydrolysate contained LL-diaminopimelic acid and no diagnostic sugars.

From these results, strain AB3217 was considered to belong to the genus *Streptomyces* and related to the "hygroscopic" *Streptomyces* species[3]. Compared with the type strains held in our institute and the published description of *Streptomyces* species, *Streptomyces platensis* IFO 12901 was closest to strain AB3217, the only difference being in the formation of soluble pigments (*S. platensis* is not known to form soluble pigment in any agar media). Therefore, strain AB3217 was classified as a strain of *Streptomyces platensis* and designated as *S. platensis* AB3217. The strain AB3217 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-11095.

Isolation

The culture broth (10 liters) was filtered by using Hyflo Super-Cel (John-Manville Co., U.S.A.). After removing calcium ion from the filtrate by adding oxalic acid, the filtrate was adjusted to pH 7 with 5 N NaOH and then charged on a column of Amberlite IR120B (H^+ form, 2.5 liters). The column was washed with water (7.5 liters) and the active principle was eluted with 2% aq ammonia (7.5 liters). The eluate was concentrated to 100 ml. The concentrate was charged on a column of Diaion HP-20 (2 liters). The column was washed with 30% aq MeOH and the active principle was eluted with absolute MeOH (6 liters). The eluate was concentrated to dryness *in vacuo*. The crude solid was dissolved in 5 ml of water. The solution was chromatographed on a column of CM-Sephadex C-25 (200 ml, triethylamine form) by elution with 0.05 M triethylamine carbonate buffer (pH 7.5) - MeOH (1 : 1). The active eluate was concentrated to dryness, yielding 370 mg of pale yellow powder.

Pure AB3217-A was obtained by crystallization from MeOH to give 160 mg of colorless needles.

Physico-chemical Properties

AB3217-A was obtained as colorless needles: MP 241°C, $[\alpha]_D^{24}$ −52.5° (*c* 1.0, water). It was soluble in water and lower alcohol, and sparingly soluble or insoluble in EtOAc, $CHCl_3$ or *n*-hexane. AB3217-A gave a positive vanillin - H_2SO_4 color reaction. UV $\lambda_{max}^{H_2O}$ nm (ε) 226 (11,400), 272 (1,100), 278 (900); $\lambda_{max}^{0.05\ N\ HCl}$ nm (ε) 227 (11,300), 272 (1,100), 279 (900); $\lambda_{max}^{0.05\ N\ NaOH}$ nm (ε) 226 (10,900), 272 (1,100), 279 (900). IR spectrum is shown in Fig. 3. The molecular formula of the compound was established as $C_{17}H_{23}NO_7$ (MW 353) by elemental analyses, HREI-MS and ^{13}C NMR spectrum, *Anal* calcd for $C_{17}H_{23}NO_7$: C 57.78, H 6.56, N 3.96; found C 57.39, H 6.44, N 4.13; HREI-MS (M^+) *m/z* 353.1469 (calcd 353.1474).

Structure Determination

The UV spectrum of AB3217-A was very similar to that of anisomycin. The ^1H NMR and ^{13}C NMR spectral data of AB3217-A are summarized in Tables 2 and 3. The multiplicity of carbon signals were determined by the distortionless enhancement by polarization transfer (DEPT) experiment. In the ^1H NMR spectrum of AB3217-A, two doublet at δ 6.84 and δ 7.20 and a methoxyl proton singlet at δ 3.72

Fig. 3. IR spectrum of AB3217-A (KBr).

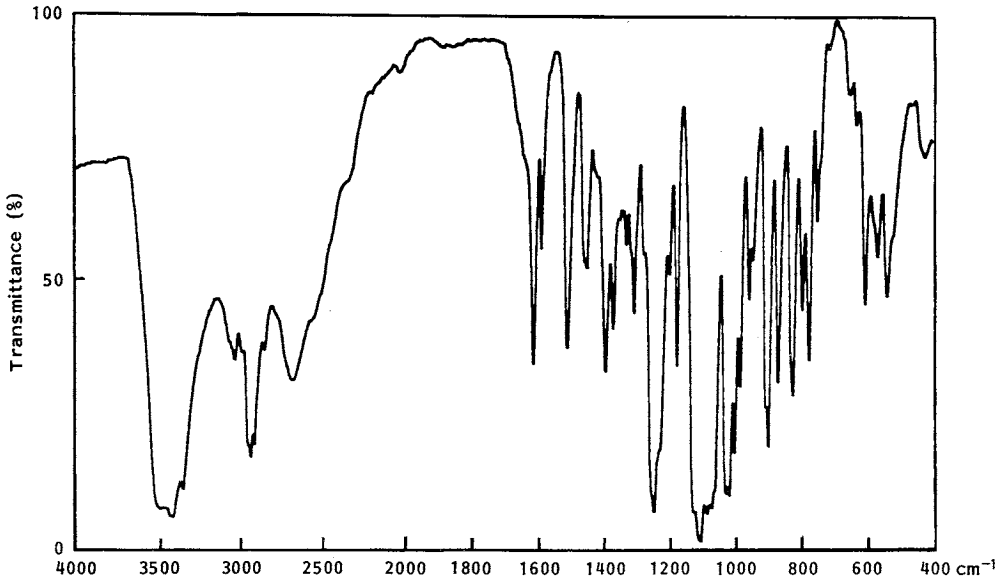


Table 2. ^{1}H NMR data for AB3217-A (400 MHz, DMSO-d_6).

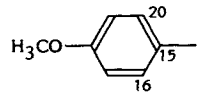
Position	δ (ppm)	J (Hz)
1-H	4.89 s*	
3-H	4.09 m	3.6, 6.4
4-H	3.94 m	
4-OH	4.85 d	4.6
5-H_a	2.41 dd	4.0, 11.0
5-H_b	2.84 dd	5.7, 11.0
6-NH	1.43 bs	
7-H	3.41 m	6.4, 9.6
8-H	4.40 d	9.6
10-H_a	3.68 m	
10-H_b	3.70 m	
11-H	4.18 m	
12-H	3.99 m	
12-OH	5.32 d	4.8
13-H	3.99 m	
13-OH	5.36 d	4.8
16-H	7.20 d	8.6
17-H	6.84 d	8.6
18-OCH_3	3.72 s	
19-H	6.84 d	8.6
20-H	7.20 d	8.6

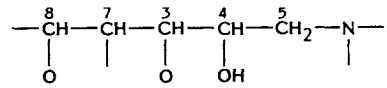
* Multiplicity.

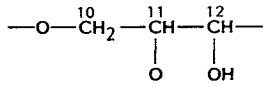
Table 3. ^{13}C NMR chemical shift assignments (ppm) for AB3217-A and protons to which a long range connectivity is observed in the HMBC experiment in DMSO-d_6.

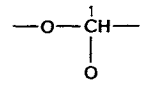
Assignment	^{13}C (ppm)	Long range connectivity in HMBC (^{1}H)
1	107.8	3-H, 11-H, 13-OH
3	82.3	4-H, 5-H_a, 5-H_b, 7-H, 4-OH
4	75.3	4-H, 4-OH
5	51.5	3-H, 4-OH
7	64.4	4-H, 5-H_a, 5-H_b, 8-H
8	82.7	7-H, 16-H, 20-H
10	68.4	8-H
11	78.0	1-H, 12-OH
12	82.1	11-H, 12-OH, 13-OH
13	78.9	12-OH, 13-OH
15	134.9	8-H, 17-H, 19-H
16	128.5	8-H, 20-H
17	113.2	19-H
18	158.2	16-H, 17-H, 19-H, 20-H, 18-OCH_3
19	113.2	17-H
20	128.5	8-H, 16-H
OCH_3	54.9	

of AB3217-A were easily recognizable to be due to the anisyl moiety suggested by UV spectrum. Furthermore, an anomeric proton at δ 4.89 and three hydroxyl protons at δ 4.85, 5.32 and 5.36 were observed. The ^{1}H-^{1}H shift correlation spectroscopy (COSY) and ^{1}H-^{13}C heteronuclear shift COSY experiments indicated the presence of following fragments:









The ^{1}H-^{13}C long range connectivities of AB3217-A were determined by the ^{1}H-detected heteronuclear multiple-bond ^{1}H-^{13}C correlation spectroscopy (HMBC) experiment. As methylene protons (5-H_a, δ 2.41 and 5-H_b, δ 2.84) were coupled to C-7 (δ 64.4), the presence of a pyrrolidine ring was suggested. An oxygen-bearing methine proton (8-H) at δ 4.40 was coupled to an aromatic quaternary carbon (C-15, δ 134.9) and aromatic carbons (C-16, C-20, δ 128.5).

A proton (δ 5.32) of the hydroxyl group attached to C-12 was coupled to C-12 (δ 82.1), C-13 (δ 78.9) and C-11 (δ 78.0). A hydroxyl proton (δ 5.36) at C-13 was coupled to C-13, C-12 and C-1 (δ 107.8). An anomeric proton (1-H, δ 4.89) was coupled to C-11 (δ 78.0). These data suggested the presence of pentose-like moiety. 3-H (δ 4.09) and 8-H (δ 4.40) were coupled to C-1 and C-10, respectively.

All results of HMBC experiments of AB3217-A are shown in Table 3. Thus, the planar structure of AB3217-A was proposed as 4,12,13-trihydroxy-8-(4-methoxyphenyl)-6-aza-2,9,14-trioxatricyclo-[9.2.1.$0^{3,7}$]tetradecane.

The planar structure of AB3217-A was supported by X-ray crystallographic analysis of the free base. The crystal structure was determined by direct methods and refined by the method of block-diagonal-matrix least-squares (R=0.04 including 23H atoms).

To determine the absolute structure of AB3217-A, X-ray crystallographic analysis of the HBr salt was performed. The HBr salt of AB3217-A was prepared by mixing 0.01 mol aq solution of AB3217-A with 0.01 mol aq solution of HBr. The crystals of HBr salt of AB3217-A was grown in MeOH - EtOAc solution. A small crystal with approximate dimensions $0.05 \times 0.15 \times 0.60$ mm was chosen for X-ray work. The crystal data and intensity data were collected by graphite monochromated CuK_α radiation. The crystal data are as follows: $C_{17}H_{23}NO_7$ HBr, FW 434. Orthorhombic, space group $P2_12_12_1$. Cell dimensions, $a=13.059(8)$, $b=23.054(13)$, $c=6.094(4)$ Å, $U=1835$ Å^3. $Z=4$, $D_x=1.571$ g cm^{-1}, μ for CuK_α= 34.1 cm^{-1}.

Intensities of 3,979 reflections including 382 symmetry equivalent reflections (R_F†=0.033) and 1,443 Friedel reflections (R_F†=0.041) were collected. Atomic parameters for 26 C, N, O and Br atoms and for 24 H atoms were refined to the R value of 0.048. Comparison of $|F_O(hkl)|/|F_O(\bar{h}\bar{k}\bar{l})|$ and $F_C(hkl)/F_C(\bar{h}\bar{k}\bar{l})$ for 349 Friedel pairs for which both values differs more than 3% from unity, 343 pairs showed consistently the absolute configuration in Fig. 4. The final atomic parameters and the bond length of the free base and HBr salt of AB3217-A have been deposited at Cambridge Crystallographic Data Centre.

Thus, from the results of X-ray analysis and spectroscopic analysis, the absolute structure of AB3217-A was determined as (1*R*,3*S*,4*S*,7*R*,8*R*,11*R*,12*S*,13*R*)-4,12,13-trihydroxy-8-(4-methoxyphenyl)-6-aza-2,9,14-

† $R_F=2\sum||F_{O1}|-|F_{O2}||/\sum(|F_{O1}|+|F_{O2}|)$, where F_{O1} and F_{O2} are the crystal structure factors of symmetry equivalent reflections or Friedel reflections.

Fig. 4. A stereoscopic drawing of AB3217-A HBr salt.

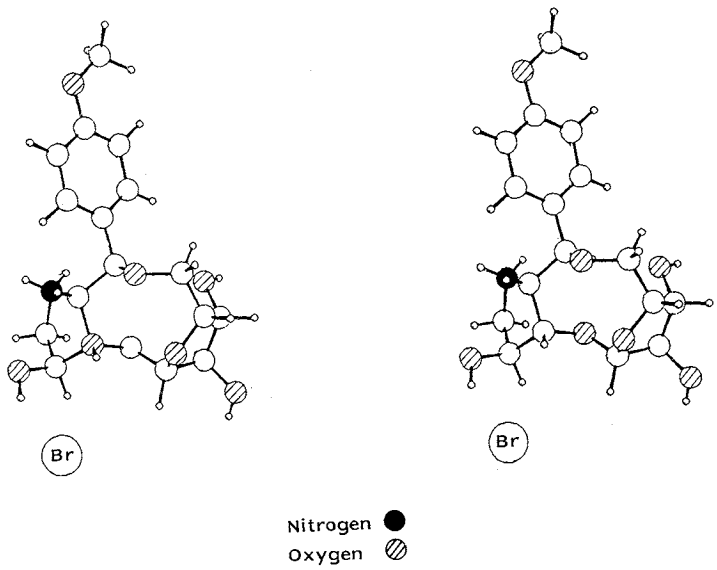


Table 4. Anti-mite activity of AB3217-A.

Concentration (μg/ml)	Protection coefficient (%)		
	AB3217-A	Anisomycin	Dicofol*
100	100	84	100
10	90	30	30
1	47	14	—
0.1	21	18	—

* Dicofol (an acaricide: 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol) 40% emulsified solution.

Table 5. Antimicrobial activity of AB3217-A.

Organism	Incubation type	MIC (μg/ml)
Bacillus subtilis PCI 219	a	>400
Staphylococcus aureus FDA 209P	a	>400
Klebsiella pneumoniae PCI 602	a	>400
Escherichia coli NIHJ	b	>400
Pseudomonas aeruginosa GN 315	c	>400
P. fluorescens IFO 12180	c	>400
Pyricularia oryzae P-2	d	50
Trichophyton asteroides 429	d	50
Saccharomyces cerevisiae ATCC 2366	e	25
Candida albicans 3147	e	>400

a: Nutrient agar, 37°C, 2 days.
b: Noken agar, 37°C, 2 days.
c: Noken agar, 24°C, 5 days.
d: Potato-sucrose agar, 24°C, 5 days.
e: Sabouraud agar, 27°C, 2 days.

trioxatricyclo[9.2.1.$0^{3,7}$]tetradecane.

Biological Properties

The activity of AB3217-A against the two spotted spider mite is shown in Table 4. The protection coefficient of AB3217-A in 10 ppm was more than that of dicofol. We also tested anisomycin and found that the compound showed anti-mite activity but less than that of AB3217-A.

AB3217-A showed no inhibitory activity at 400 μg/ml against Gram-positive and Gram-negative bacteria, but it showed weak activity against some fungi (Table 5).

Discussion

Several compounds belonging anisomycin group antibiotics were reported, such as anisomycin[4], deacetylanisomycin[5], isoanisomycin[5], anisomycin C[6], anisomycin D[6] and L-657,398[7]. While AB3217-A is structurally related to anisomycin, the compound has unique structure that deacetylanisomycin and β-D-xylofuranose linked through glycosidic bond and ether bond resulting in the formation of nine-

membered ring.

It is known that anisomycin shows anti-molds, anti-protozoa and herbicidal activities, but it has been not reported on anti-mite activity of anisomycin. We found that anisomycin showed anti-mite activity against two spotted spider mite although less than that of AB3217-A. AB3217-A has weak herbicidal activity against some weeds (data not shown). Therefore these similarity of biological activities between the two compounds were probably due to common partial structure (anisole ring and pyrrolidine ring).

It was found that the strain AB3217 produced not only AB3217-A but also two other anti-mite substances, AB3217-B and C, structurely closest to AB3217-A during improvement on purification method of AB3217-A. The isolation and structure determination of AB3217-B and C will be reported, separately.

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