ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1974, p. 578-581

Vol. 5, No. 6
Printed in U.S.A.

New Antibiotic Produced by Bacteria, 5-β-D-Xylofuranosylneamine

SATOSHI HORII, IKUO NOGAMI, NARIAKIRA MIZOKAMI, YOSHIAKI ARAI, AND MASAHIKO YONEDA

Microbiological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan

Received for publication 18 March 1974

A new aminoglycoside antibiotic was isolated from the fermentation broths of two strains of *Bacillus* species. The antibiotic is active against gram-positive and some gram-negative bacteria, and its antimicrobial spectrum is similar to that of ribostamycin. The chemical structure was determined to be 5-β-D-xylofuranosylneamine, which is identical to the deacylated product obtained from butirosin A.

In the course of a search for new antibiotics produced by bacteria, a water-soluble, basic antibiotic, which was differentiated from the related known antibiotics by means of chromatographic, electrophoretic, and bioautographic comparison, was isolated. The structure of this antibiotic was determined to be 5-β-D-xylofuranosylneamine, and the antibiotic was tentatively named xylostasin.

Shortly afterwards, this same compound was described as a chemical degradation product of butirosin A by Tsukiura et al. (9) and Haskell et al. (4). This paper deals with the fermentation process, isolation, characterization, and structural elucidation of xylostasin.

MATERIALS AND METHODS

Organism. *Bacillus* sp. V-7 (ATCC 21933; American Type Culture Collection, Rockville, Md.) and *Bacillus* sp. Y-399 (ATCC 21932) were isolated from soil samples collected at Tokyo and Kyoto in Japan, respectively, and used for the production of xylostasin. *Bacillus* sp. V-7 belongs to *Bacillus circulans*, whereas *Bacillus* sp. Y-399 differs from *B. circulans* in at least pigmentation and utilization of carbohydrates and shows a marked resemblance to *B. vitellinus* which was described as a butirosin producer by Hasegawa et al. (3).

Fermentation. A loopful of cells grown on a glycerin-bouillon agar was used to inoculate a 2-liter shaking flask containing 500 ml of a sterile medium (pH 7.2) composed of 3% peptone, 1% meat extract, and 0.5% sodium chloride. The flask was incubated on a reciprocal shaker at 28 C for 24 h.

One liter of inoculum from these cultures was transferred to a 30-liter seed fermentor containing 20 liters of the same medium. The cultivation was carried out at 28 C for 24 h under agitation at 200 rpm and aeration of 20 liters per min. Ten liters of the seed culture broth thus obtained was transferred to a 200-liter fermentor containing 100 liters of the sterile medium (pH 7.5) composed of 2% dextrin, 1% peptone, 0.7% meat extract, 0.5% $MgSO_4 \cdot 7H_2O$, and 0.05% antifoam agent (Actcol, Takeda Chemical Industries, Ltd.). The fermentor was aerated at 100 liters per min and stirred at 200 rpm at 28 C for 66 h.

Assay. Samples obtained during the fermentation and purification procedures were assayed by a paper-disk agar-diffusion method using *B. subtilis* PCI 219 as a test organism and crystalline xylostasin base as a standard.

Dextrin was determined by the method of Dubois et al. (2).

Bacterial growth was measured on absorbancy of a 10-fold diluted broth at 650 nm.

Isolation. Prior to filtering, 850 g of Primafloc C-7 (Rohm & Hass Co.) and 850 g of Hyflo-Super-Cel (Johns-Manville Products Co.) were added to the whole broth. The filtrate was passed through a column containing 5 liters of Amberlite IRC-50 (NH_4^+ form). The resin was eluted with 5% aqueous ammonia. The active effluent fractions were pooled and run onto a column of 2 liters of activated carbon. The carbon column was eluted with 0.3 N hydrochloric acid. The active fractions were neutralized with Amberlite IR-45 (OH^- form) and the antibiotics were adsorbed on a column of 500 ml of Amberlite CG-50 (NH_4^+ form). After washing with water, the column was eluted with 0.2 N ammonia water. The active fractions were collected and concentrated under reduced pressure. The concentrate was allowed to stand overnight in a refrigerator to give crystalline xylostasin as free base. Thin-layer chromatographic and high-voltage paper electrophoretic analyses indicated it to be homogeneous.

RESULTS AND DISCUSSION

The time course of a typical fermentation by *Bacillus* sp. Y-399 is given in Fig. 1. The cells grew logarithmically for the first 24 h of fermentation with increasing consumption of dextrin,

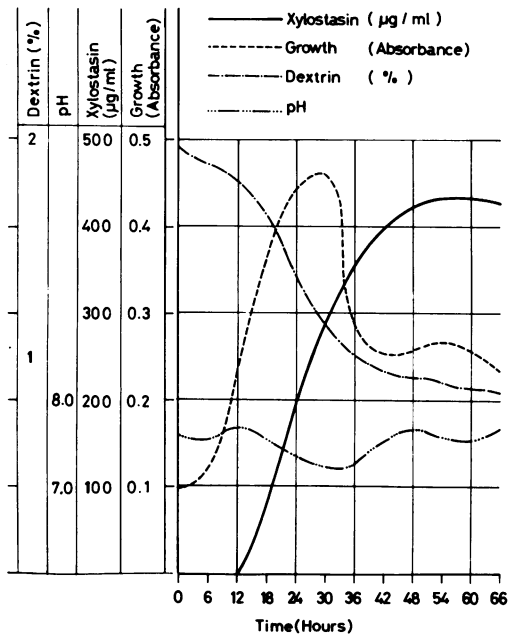


FIG. 1. *Time course of xylostasin production by Bacillus sp. Y-399.*

and the absorbancy at 650 nm rapidly decreased during the time period 30 to 36 h probably because of cell lysis. The antibiotic titer began to increase after 18 h of incubation and showed a maximal value (430 μg/ml) at 48 h, remaining unchanged up to 66 h. The pH was approximately constant near 7.5 during the course of the fermentation except for a slight decrease in the biosynthetic phase of the fermentation.

Xylostasin was also produced by *Bacillus* sp. V-7 under these same fermentation conditions but less efficiently than by the strain Y-399.

Xylostasin is a white crystalline compound which melts with decomposition at about 145 C. Analysis calculated for $C_{17}H_{34}N_4O_{10}$: C, 44.93; H, 7.54; N, 12.33; molecular weight (MW), 454.5. Found: C, 44.18; H, 7.58; N, 11.98; MW, 451 (neutralization equivalent); $[\alpha]_D^{21}$ +34° (c 1, H_2O); nuclear magnetic resonance (NMR), (D_2O); δ 1.33 (q, 1 H, axial methylene), δ 2.1 (m, 1 H, equatorial methylene) δ 5.43 (1 H, J = <1, anomeric proton), and δ 5.61 (d, 1 H, J = 3.5, anomeric proton); infrared spectrum (KBr), 3,320, 2,920, 1,590, 1,465, 1,340, 1,100 and 1,020 cm^{-1}, but no absorption in the 1,640 to 1,650 cm^{-1} region (no -NH-CO-group); ultraviolet spectrum (H_2O), no maximum except end-absorption. The antimicrobial spectrum of xylostasin by the agar dilution method is shown in Table 1. Xylostasin was cross-resistant with neomycin and kanamycin, and its spectrum was similar to that of ribostamycin (1, 6, 7). Xylostasin (free base) has mean lethal dose in mice of 1,000 mg/kg intravenously, and mice infected with *Escherichia coli* 0-111 were protected by 1 to 10 mg/kg by the subcutaneous route.

Mild acid hydrolysis of xylostasin (0.5 N H_2SO_4 at 90 C for 6 h) gave D-xylose, $[\alpha]_D^{22}$ +19.4° (c 1, H_2O), which was identified by automatic liquid chromatography, gas liquid chromatography (as a per-trimethylsilylated sugar), paper electrophoresis, and paper chromatography. Methanolysis of xylostasin (1 N HCl in MeOH at 22 C for 6 days) gave neamine and methyl D-xyloside.

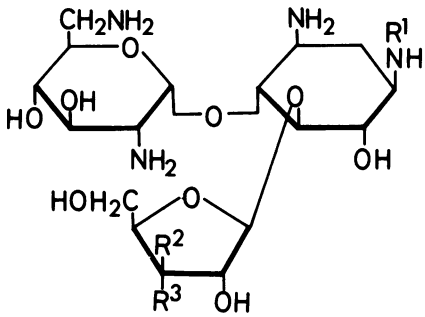
Xylostasin gave neamine, but no 4-amino-2-hydroxybutyric acid by strong acid hydrolysis (6 N HCl at 100 C for 16 h), whereas butirosin A (10-12) gave both neamine and 4-amino-2-hydroxybutyric acid, which was analyzed by using an amino acid autoanalyzer and by gas liquid chromatography (as a per-trimethylsilylated amino acid).

In thin-layer chromatography (Silica Gel G) using the upper phase of chloroform-methanol-17% ammonia water (2:1:1, vol/vol/vol), xylostasin, ribostamycin, and butirosins show retardation factors (R_f) of 0.61, 0.69, and 0.31, respectively, and using the upper phase of chloroform-methanol-28% ammonia water-0.1% boric acid (5:3:2:1, vol/vol/vol/vol), xylostasin, ribostamycin, and butirosin A and B (10-12) show R_f's of 0.69, 0.80, 0.35, and 0.56,

TABLE 1. *Antibacterial spectrum of xylostasin*

Test organisms	MIC[a] (μg/ml)
Escherichia coli IFO 3044	3.13
E. coli NIHJ	3.13
E. coli JR66/W677	>100.0
Shigella flexneri EW-10	12.5
Proteus vulgaris IFO 3045	50.0
Pseudomonas aeruginosa IFO 3080	>100.0
Klebsiella pneumoniae	0.78
Staphylococcus aureus FDA 209P	6.25
Bacillus subtilis PCI 219	3.13
B. cereus IFO 3466	50.0
B. brevis IFO 3331	25.0
Sarcina lutea IFO 3232	25.0
Mycobacterium avium IFO 3153	1.6
M. avium (streptomycin-resistant)	3.13
M. avium (neomycin-resistant)	50.0
M. phlei IFO 3158	1.6
M. smegmatis IFO 3083	1.6

[a] MIC, minimal inhibitory concentration.



	R^1	R^2	R^3
Xylostasin	H	OH	H
Butirosin A	4-amino-2-hydroxybutyryl	OH	H
Ribostamycin	H	H	OH
Butirosin B	4-amino-2-hydroxybutyryl	H	OH

FIG. 2. *Structure of xylostasin and its related antibiotics.*

respectively. In paper electrophoresis using borate buffer (pH 9.5, 0.025 M, 2 kV/36 cm, 90 min), xylostasin moves to the cathode by 5.5 cm, whereas butirosin A and ribostamycin move likewise by 10 cm and 2 cm, respectively.

Acetylation (acetic anhydride in methanol) of xylostasin gave a tetra-*N*-acetyl derivative. Analysis calculated for $C_{25}H_{42}N_4O_{14}\cdot H_2O$: C, 46.87; H, 6.92; N, 8.75. Found: C, 46.86; H, 6.83; N, 8.34; melting point, 203 to 207 C (decomposition); $[\alpha]_D^{27}$ +33.4° (c 1, H_2O); NMR (D_2O); δ 5.65 (d, J = 3.5, anomeric proton of *N*-acetyl neosamine C moiety) and δ 5.33 (J = <1, anomeric proton of D-xylose moiety). Per-trimethylsilylation (trimethylchlorosilane and hexamethyldisilazane in pyridine) of tetra-*N*-acetyl xylostasin gave a tetra-*N*-acetyl-hexa-*O*-trimethylsilyl derivative whose mass spectrum gave the characteristic peaks (10) at *m/e* 1054 (M^+, $C_{43}H_{90}N_4O_{14}Si_6$, 1.3%, all peak intensities are relative to *m/e* 73, $(CH_3)_3Si^+$), 1039 ($(M - CH_3)^+$, 13%), 807 (78%), 767 (36%), 649 (16%), 389 (59%), 259 (31%), 217 (59%), 173 (26%), and 103 (33%).

The point of attachment of D-xylose to neamine was elucidated as follows: periodate oxidation of tetra-*N*-acetyl xylostasin consumed 2 mol of the reagent, and 2-deoxystreptamine was obtained in good yield after vigorous acid hydrolysis of the periodate oxidation products, but no neosamine C was obtained. Furthermore, tetra-*N*-acetyl-hexa-*O*-methyl xylostasin, prepared by the method of Kuhn and others (CH_3I and BaO in *N*,*N*-dimethylformamide), was hydrolyzed with 6 N HCl to give 6-*O*-methyl-2-deoxystreptamine (5, 8) and with 0.5 N H_2SO_4 to give 2,3,5-tri-*O*-methyl-D-xylofuranose.

Alkaline hydrolysis (0.5 N $Ba(OH)_2$ at 100 C for 3 h, or 80% hydrazine hydrate in a sealed tube at 100 C for 12 h) of butirosin A gave de-(4-amino-2-hydroxybutyryl) compound which is identical with xylostasin on the basis of its antibacterial spectrum and its physical-chemical characteristics. Thus, we assigned the structure of xylostasin as shown in Fig. 2.

Xylostasin was also synthesized by the Koenigs-Knorr reaction of 4-*O*-(3,4-di-*O*-benzyl-2 , 6-dicarbobenzoxyamino-2 , 6-dideoxy-α-D-glucopyranosyl)-*N* , *N'*-dicarbobenzoxy-2-deoxystreptamine and 2,3,5-tri-*O*-benzoyl-D-xylofuranosyl bromide by using the procedure of Ito and others for ribostamycin (6).

Although some minor components, including 5-β-D-xylofuranosylparomamine, neamine, and paromamine, were also detected in the same culture filtrates, xylostasin was always the major component.

ACKNOWLEDGMENTS

The authors are grateful to R. Takeda, A. Miyake, M. Isono, S. Yamatodani, T. Yamano, and J. Ueyanagi. We are also indebted to I. Banno, T. Hasegawa, and S. Tanida for taxonomic studies of the organisms and to Y. Yasumura for his skillful technical assistance.

LITERATURE CITED

1. Akita, E., T. Tsuruoka, N. Ezaki, and T. Niida. 1970. Studies on antibiotic. II. Chemical structure of antibiotic SF-733. J. Antibiot. **23**:173-183.
2. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350-356.
3. Hasegawa, T., S. Tanida, H. Fukase, and H. Iwasaki. 1973. Japanese patent 73-62993.
4. Haskell, T. H., R. Rodebaugh, N. Plessas, D. Watson, and R. D. Westland. 1973. The preparation and biological activity of novel amino acid analogs of butirosin. Carbohyd. Res. **28**:263-280.
5. Horii, S., H. Hitomi, and A. Miyake. 1964. Structure of zygomycin A_1, zygomycin A_2 and dextromycin. Annu. Rep. Takeda Res. Lab. **23**:18-29.
6. Ito, T. E. Akita, T. Tsuruoka, and T. Niida. 1971. Synthesis of an aminocyclitol antibiotic, SF-733 (ribostamycin), p. 33-37. Antimicrob. Ag. Chemother. 1970.
7. Shomura, T., N. Ezaki, T. Tsuruoka, T. Niwa, E. Akita, and T. Niida. 1970. Studies on antibiotic SF-733, a new antibiotic. I. Taxonomy, isolation and characterization. J. Antibiot. **23**:155-161.
8. Tatsuoka, S., and S. Horii. 1963. The absolute configuration of zygomycin A, neomycin, and dihydrostreptomycin. Proc. Jap. Acad. **39**:314-318.
9. Tsukiura, H., K. Fujisawa, M. Konishi, K. Saito, K. Numata, H. Ishikawa, T. Miyaki, K. Tomita, and H.

Kawaguchi. 1973. Aminoglycoside antibiotics. III. Bioactive degradation products from butirosins and semisynthesis of butirosin analogs. J. Antibiot. **26**:351-357.
10. Woo, P. W. K. 1971. Butirosins A and B, aminoglycoside antibiotics. II. Mass spectrometric study. Tetrahedron Lett. 2621-2624.
11. Woo, P. W. K., H. W. Dion, and Q. R. Bartz. 1971. Butirosins A and B, aminoglycoside antibiotics. I. Structural units. Tetrahedron Lett. 2617-2620.
12. Woo, P. W. K., H. W. Dion, and Q. R. Bartz. 1971. Butirosins A and B, aminoglycoside antibiotics. III. Structures. Tetrahedron Lett. 2625-2628.