ISOLATION AND CHARACTERIZATION OF A NEW NUCLEOSIDE ANTIBIOTIC, AMIPURIMYCIN

SETSUO HARADA and TOYOKAZU KISHI

Medicinal Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan

(Received for publication October 25, 1976)

A new antibiotic amipurimycin, active against *Pyricularia oryzae in vitro* and *in vivo,* was isolated from the culture filtrate of *Streptomyces novoguineensis* nov. sp. The antibiotic was purified by a combination of ion-exchange and adsorption chromatography based on its amphoteric water-soluble characteristics. Its molecular formula was estimated to be $C_{20}H_{27\sim31}N_7O_8 \cdot H_2O$. Characteristic maxima in the UV spectrum and signals in the PMR and CMR spectra were similar to those of 2-aminopurine 9-(β-D)-riboside. These findings indicated that amipurimycin is a new nucleoside antibiotic and the first example of a natural product containing 2-aminopurine.

In our screening program for useful agricultural antibiotics, *Streptomyces novoguineensis* (No. T-36496) was isolated from a soil sample collected in Rae, Papua, New Guinea. The organism produced an antibiotic which displayed remarkable activity against *Pyricularia oryzae in vitro* and *in vivo.*[1] Isolation and purification studies were performed based on the results of *in vitro* and *in vivo* tests. The crystalline compound isolated from the culture filtrate of *S. novoguineensis* was deduced to be a new nucleoside antibiotic from their physico-chemical data. Since its ultraviolet (UV), proton magnetic resonance (PMR) and ^{13}C magnetic resonance (CMR) spectra showed that the base present was 2-aminopurine, the antibiotic was named amipurimycin. This paper deals with the isolation procedure and chemical characterization of amipurimycin.

Isolation Procedure

In preliminary experiments, amipurimycin (APM) was found to be retained on ion-exchange resins, activated charcoal and silica gel, and to be eluted with proper solvents. APM was purified by the procedure as shown in Chart 1.

APM in the culture filtrate was exchanged on to Amberlite IRA-410 and eluted with 0.5 N hydrochloric acid. The eluate was neutralized, adsorbed on a column of activated charcoal to remove inorganic salts and eluted with acetone-water (2:8). The concentrated eluate was adsorbed on Amberlite IRC-50 and eluted with 1% aqueous ammonia. The crude powder obtained from the eluate was again fractionated on a column of activated charcoal. Active fractions eluated with acetone - water (1:9 and 2:8) were concentrated to dryness. The product was chromatographed on silica gel and eluted with methanol containing methylamine. When the active fractions were concentrated a pale yellow substance precipitated. The dried powder was dissolved in water, adsorbed on a column of Amberlite CG-50 and fractionated with 0.5 and 1.0% aqueous ammonia, successively. The concentrated residue from active fractions was

crystallized from ethanol - water (1:1) to afford colorless prisms of amipurimycin.

Active fractions were detected by *in vitro* and *in vivo* tests using *P. oryzae* as test organism, by thin-layer chromatography (TLC) using UV light and by paper partition chromatography (PPC) using GREIG-LEABACK reagent[2] to visualize the compound.

Chemical Characterization

APM was recrystallized from ethanol - water (1:1) as colorless prisms. The melting point was 217°C (decomp.). The specific rotation was −3.2° (*c* 0.62) in water, −18.1° (*c* 0.61) in 0.1 N hydrochloric acid and +8.2° (*c* 0.63) in dimethyl sulfoxide - water (1:1). The mobilities of APM by TLC on silica gel, PPC and paper electrophoresis (PE) are shown in Table 1. APM gave a single spot in all solvent systems used. Its measurable pKa′ values were estimated as 3.7 and 9.1 by titration. APM was assumed to be amphoteric, but the basic character predominated, judging from the results of PE and its behavior towards cation- and anion-exchange resins. It was very easily soluble in water, but sparingly soluble in dimethyl sulfoxide, alcohols, pyridine or acetic acid, and insoluble in less polar organic solvents. APM was detectable by its UV absorbance at 254 or 365 nm on silica gel TLC plates. In color reactions, it was positive to GREIG-LEABACK (violet), EHRLICH (yellow) and ninhydrin (brown) reagents and negative to SAKAGUCHI, BARTON, MOLISCH, DRAGENDORFF, PAULY and basic potassium permanganate reagents.

The molecular weight of APM was 472 or 539 by titration or vapor pressure osmometry,

Chart 1. Isolation procedure for amipurimycin

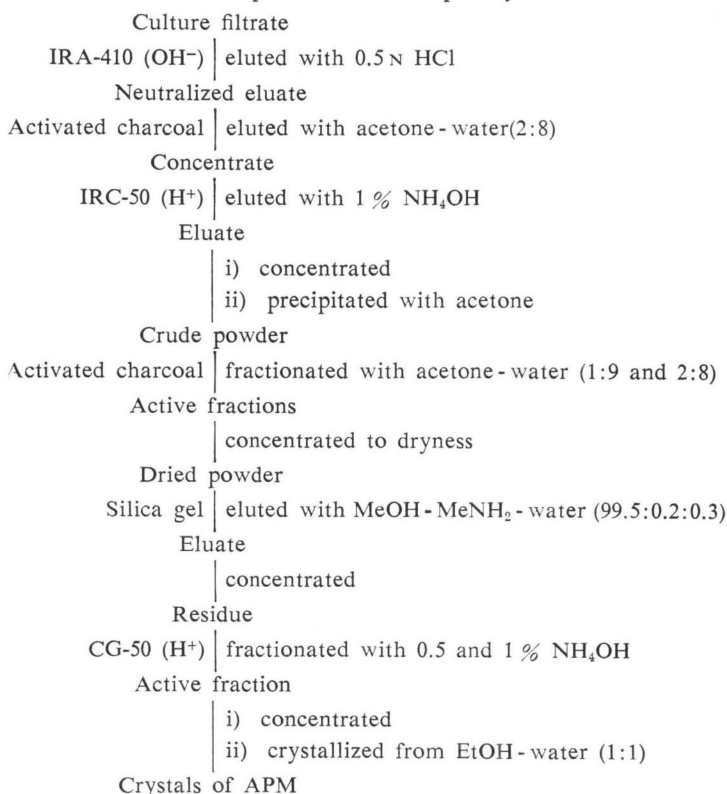
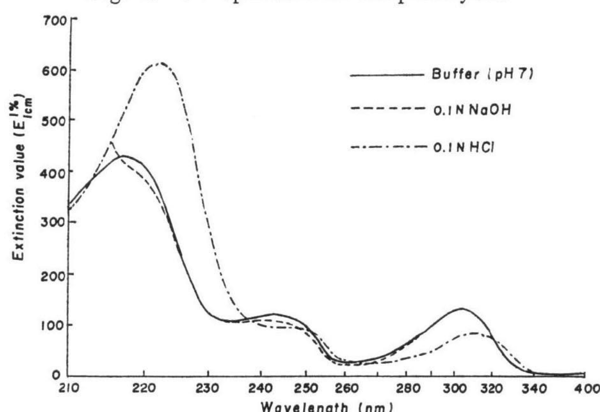


Table 1. Mobility of amipurimycin on TLC, PPC and paper electrophoresis (PE)

	Solvent system	Rf
TLC*[1]	MeOH - $MeNH_2$ - H_2O (99.5:0.2:0.3)	0.20
	PrOH - AcOH - Pyr - H_2O (15:3:10:10)	0.39
	$CHCl_3$ - MeOH - 17 % NH_4OH (2:1:1, upper layer)	0.84
PPC*[2]	iso-PrOH - 2 % HCOOH (6:4)	0.45
	BuOH - AcOH - H_2O (2:2:1)	0.41
	PrOH - H_2O (7:3)	0.15
	iso-PrOH - 5 % NH_4OH (6:4)	0.60
	BuOH - Pyr - H_2O (1:1:1)	0.21
		(cm)
PE*[3]	M/10 Citrate buffer (pH 3.6)	−2. 1
	M/15 Phosphate buffer (pH 7.0)	−1. 5
	M/10 Glycine/NaCl - NaOH (pH 9.15)	−0. 5

*[1] Kieselgel F_{254} (Merck AG).
*[2] Whatmann No. 1 (W. & R. Balston Ltd.).
*[3] Whatmann No. 1, 500 v, 2 hr.

Fig. 1. UV spectrum of amipurimycin.



respectively. The water content of crystals was 3.64 % (1.04 mole) by thermo-gravitational analysis, and elemental analysis of the same material showed C, 46.68; H, 6.08; N, 18.96; O, 30.09 (%). The number of carbon atoms in APM was determined to be 20 from its CMR spectrum in deuterium oxide. From these data the molecular formula was assumed to be $C_{20}H_{27\sim31}N_7O_8 \cdot H_2O$.

The UV spectrum of APM showed the maxima at 218 nm ($E^{1\%}_{1cm}$ 429), 243 (116), 305 (130) in phosphate buffer (pH 7), 243 nm ($E^{1\%}_{1cm}$ 108), 305 (136) in 0.1 N sodium hydroxide and 222 nm ($E^{1\%}_{1cm}$ 634), 244 (shoulder), 313 (78) in 0.1 N hydrochloric acid as shown in Fig. 1. The infrared (IR) spectrum of APM is shown in Fig. 2.

Discussion

APM was active against some phytopathogenic fungi including *P. oryzae* and *Trichophyton mentagrophytes*, but inactive against bacteria *in vitro*. It showed strong activity at a concentra-

Fig. 2. IR spectrum of amipurimycin.

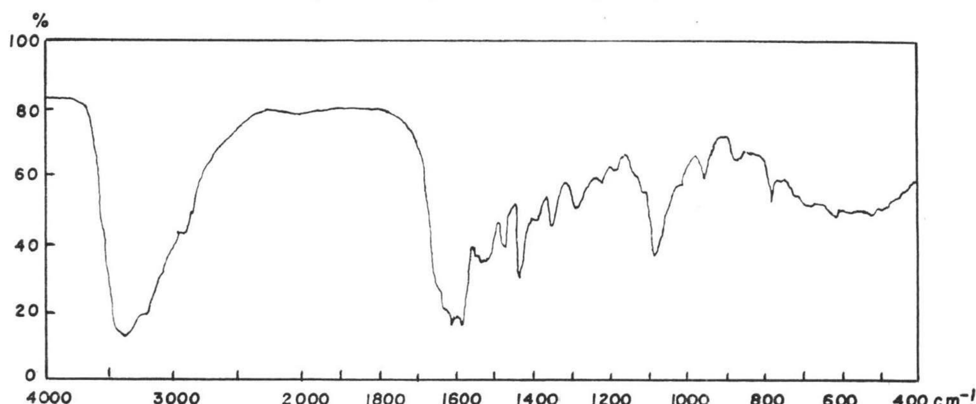


Table 2. Comparison of amipurimycin with miharamycins and 2-aminopurine 9-(β-D)-riboside (APR)

	Amipurimycin	Miharamycin[6] A·2HCl	Miharamycin[6] B·HCl	APR
$[\alpha]_D$	−3.2°(H_2O)	−59° (H_2O)	−63° (H_2O)	−42° (H_2O)
Anal. (Found)	C, 46.68	C, 40.07	C, 39.89	C, 44.68
	H, 6.08	H, 6.02	H, 5.97	H, 4.94
	N, 18.96	H, 21.59	N, 22.08	N, 25.91
		Cl, 9.12	Cl, 4.98	
M. W.	472 (Tit.)	314 (Tit.)	619 (Tit.)	267 (Mass)
M. F.	$C_{20}H_{27\sim31}N_7O_8\cdot$ H_2O	$C_{22}H_{35}N_{10}O_{10}\cdot$ 2HCl (674)	$C_{21}H_{36}N_{10}O_{11}\cdot$ HCl (640)	$C_{10}H_{13}N_5O_4\cdot$ (267)
$UV\lambda_{max}$	*1 218 (429)	217 (365)	218 (368)	219 (742)
	243 (116)	244 (105)	244 (106)	244 (208)
	305 (130)	307 (108)	307 (110)	303 (237)
$nm(E^{1\%}_{1cm})$	*2 222 (634)	223 (543)	223 (568)	225 (1159)
	244 (sh.)	244 (sh.)	242 (sh.)	244 (sh.)
	313 (78)	314 (66)	314 (66)	315 (144)
	*3 243 (108)	244 (105)	244 (105)	242 (sh.)
	305 (136)	307 (110)	307 (110)	303 (241)

*1 Neutral solution of pH 7 or H_2O.
*2 Acidic solution of N/10 HCl.
*3 Basic solution of N/10 NaOH

tion of 10 ppm in green house tests against *P. oryzae*, but phytotoxicity was observed at higher concentrations.[1] Preliminary LD_{50} values of APM in mice and rats were 1∼5, 1∼5 mg/kg intravenously and 10∼20, 20∼30 mg/kg orally, respectively.[1]

Many antibiotics, including blasticidin S,[3] kasugamycin,[4] bramycin,[5] miharamycins,[6,7] and aabomycin A[8] inhibit the growth of *P. oryzae in vitro* and *in vivo*. Of these, miharamycins A and B are similar to APM but differ in not giving SAKAGUCHI reaction and in specific rotation, elemental analysis (especially in nitrogen) and molecular formula[6] as shown in Table 2. Miharamycins showed antimicrobial activity against *Pseudomonas*,[7] whereas APM did not.[1] All these findings suggest that APM is a new antibiotic.

The UV spectrum of APM (Fig. 1) contains features characteristic of nucleosides. Examination of the UV spectra, pKa values[9] and fluorescence*[10] of known nucleoside analogs showed

* Weak fluorescence was observed in APM.

that the UV spectrum of APM was in good accord with that of 2-aminopurine 9-(β-D)-riboside[11] (APR) which has been enzymatically synthesized from 2-aminopurine and ribose-1-phosphate with a cell-free extract of *Escherichia coli*.[12] Table 2 shows physico-chemical data for this compound synthesized. In the PMR spectrum of APR, signals were observed at 8.38 ppm, 8.18 (H_6, H_8) and 5.93 ($H_{1'}$) in deuterium oxide; almost identical signals were observed in the PMR spectrum of APM at 8.38 ppm, 8.36 (H_6, H_8) and 5.98 (H_1) in deuterium oxide. Furthermore, the CMR spectrum of APM in deuterium oxide contained signals associated with the purine base at 159.9 ppm (s, C_2), 153.2 (s, C_4), 126.3 (s, C_5), 149.7 (d, C_6) and 142.8 (d, C_8). From all these data, the chromophore of APM is considered to be a 2-aminopurine.

Experimental

Isolation of amipurimycin (APM):

The culture broth of *S. novoguineensis* (850 liters)[1] was filtered with Hyflo Super-Cel (Johns-Manville Co.). The filtrate (600 liters, pH 8, 40 μg/ml) was adsorbed on a column of Amberlite IRA-410 (OH^- form, 100 liters, Rohm & Haas Co.) and eluted with 0.5 N HCl (250 liters) after washing with water (250 liters). The neutralized eluate was adsorbed on a column of activated charcoal (50 liters, Takeda Chem. Ind. Ltd.) and eluted with acetone-water (2:8, 250 liters). The concentrate of the eluate was adsorbed on a column of Amberlite IRC-50 (H^+ form, 20 liters, Rohm & Haas Co.) and eluted with 1 % NH_4OH (150 liters) after washing with water (100 liters). The eluate was concentrated *in vacuo* and the residue was precipitated with acetone to obtain a crude powder (60 g). The crude power (194 μg/mg) in water was chromatographed on activated charcoal (2 liters) and fractionated with acetone-water (1:9 and 2:8, 18 liters) after washing with water (10 liters) and acetone-water (5:95, 6 liters). The active fractions were collected and concentrated under reduced pressure to dryness. The dried powder (17 g, 480 μg/mg) in water was chromatographed on silica gel (0.05~0.20 mm, 1.7 liters, Merck AG) and eluted with MeOH-$MeNH_2$-water (99.5:0.2:0.3, 6 liters) after washing with MeOH (6 liters). The eluate was concentrated *in vacuo* and the residue was precipitated with MeOH to give a pale yellow powder (15.1 g). The powder in water was adsorbed on a column of Amberlite CG-50 (H^+ form, 0.75 liter, Rohm & Haas Co.) and fractionated with 0.5 and 1.0 % NH_4OH (4 liters) after washing with water (4 liters). The active fractions were collected and concentrated under reduced pressure. The residue was crystallized from EtOH-water (1:1) to yield colorless crystals of APM (8.5 g, 1,000 μg/mg); mp 217°C (decomp.).

Anal. Calcd. for $C_{20}H_{29}N_7O_8 \cdot H_2O$ (495.52): C, 46.78; H, 6.09; N, 19.09; O, 28.04.
Found: C, 46.68; H, 6.08; N, 18.96; O, 30.09.

Acknowledgement

The authors are indebted to Drs. R. TAKEDA and M. YONEDA for their encouragement throughout this work. Thanks are also due to the members of Microbiological and Chemical Research Laboratories for their tank fermentation and physico-chemical analysis.

References

1) IWASA, T.; T. KISHI, K. MATSUURA & O. WAKAE: *Streptomyces novoguineensis* sp. nov., an amipurimycin producer, and antimicrobial activity of amipurimycin. J. Antibiotics 30: 1~10, 1977
2) GREIG, C. G. & D. H. LEABACK: Use of chlorine in the detection of compounds on paper chromatograms. Nature 188: 310~311, 1960
3) TAKEUCHI, S.; K. HIRAYAMA, K. UEDA, H. SAKAI & H. YONEHARA; Blasticidin S, a new antibiotic. J. Antibiotics, Ser. A 11: 1~5, 1958
4) UMEZAWA, H.; Y. OKAMI, T. HASHIMOTO, Y. SUHARA, M. HAMADA & T. TAKEUCHI: A new antibiotic, kasugamycin. J. Antibiotics, Ser. A 18: 101~103, 1965
5) SAKAGAMI, Y.; H. SEKINE, S. YAMABAYASHI, Y. KITAURA, A. UEDA & Y. KOSAKA: The studies

on bramycin, a new antifungal antibiotic. I. Isolation, purification and properties of bramycin. J. Antibiotics, Ser. A 19: 99~103, 1966
6) TSURUOKA, T.; H. YUMOTO, N. EZAKI & T. NIIDA: New antibiotics, miharamycins A and B. I. Isolation and characterization of miharamycins A and B. Sci. Rep. Meiji Seika Kaisha 9: 1~4, 1967
7) SHOMURA, T.; K. HAMAMOTO, T. OHASHI, S. AMANO, J. YOSHIDA, C. MORIYAMA & T. NIIDA: New antibiotics, miharamycins A and B. II. Some biological characteristics of miharamycin. Sci. Rep. Meiji Seika Kaisha 9: 5~10, 1967
8) AIZAWA, S.; Y. NAKAMURA, S. SHIRATO, R. TAGUCHI, I. YAMAGUCHI & T. MISATO: Aabomycin, A, a new antibiotic. I. Production, isolation and properties of aabomycin A. J. Antibiotics 22: 457~462, 1969
9) MASON, S. F.: Purine studies. II. The ultra-violet absorption spectra of some mono- and poly-substituted purines. J. Chem. Soc. 1954: 2071~2081, 1954
10) WARD, D. C. & E. REICH: Fluorescence studies of nucleotides and poly nucleotides. J. Biol. Chem. 244: 1228~1237, 1969
11) FOX, J. J.; I. WEMPEN, A. HAMPTON & I. L. DOERR: Thiation of nucleosides. I. Synthesis of thioguanosine and related purine nucleosides. J. Amer. Chem. Soc. 80: 1669~1675, 1958
12) IMADA, A.: Metabolism of pyrimidine nucleosides and nucleotides by microorganisms. J. Takeda Res. Lab. 29: 722~775, 1970