Journal of Natural Products
Vol. 53, No. 4, pp. 825-829, Jul-Aug 1990

ISOLATION AND STRUCTURE DETERMINATION OF INOSTAMYCIN, A NOVEL INHIBITOR OF PHOSPHATIDYLINOSITOL TURNOVER

MASAYA IMOTO,[1] KAZUO UMEZAWA,[1,*] YOSHIKAZU TAKAHASHI, HIROSHI NAGANAWA, YOICHI IITAKA,[2] HIKARU NAKAMURA, YUMI KOIZUMI, YUMI SASAKI, MASA HAMADA, TSUTOMU SAWA, and TOMIO TAKEUCHI

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

ABSTRACT.—In the course of screening for phosphatidylinositol turnover inhibitors, we found that a culture broth of a *Streptomyces* strain strongly inhibited the incorporation of inositol into phosphatidylinositol and its phosphates. The active principle was isolated from the broth through EtOAc extraction, cc, centrifugation partition chromatography, and crystallization. Spectroscopic and crystallographic analysis revealed that it has a novel polyether structure, and we named it inostamycin. Inostamycin inhibited phosphatidylinositol turnover with an IC_{50} of 0.5 μg/ml in cultured A431 cells.

The binding of many hormones and growth factors to their specific cell surface receptors stimulates a rapid increase in phosphatidylinositol (PtdIns) turnover (1,2). Furthermore, the level of PtdIns turnover is elevated in a variety of oncogene-activated transformed cell lines (3–5). However it is unclear whether PtdIns turnover is actually important in growth factor- and oncogene-induced cell proliferation.

Therefore, we screened microorganisms to search for inhibitors of PtdIns turnover among their secondary metabolites to be used as a tool for the study of the biochemical significance of PtdIns turnover in the process of growth factor and oncogene product action. As a result we discovered psi-tectorigenin as an inhibitor of PtdIns turnover (6).

By continuation of our screening procedure we recently found another inhibitor of PtdIns turnover, one that we have named inostamycin. In this report we describe the isolation and structure detemination of this inhibitor, which was found to be a novel polyether compound (Figure 1).

RESULTS AND DISCUSSION

Inostamycin was purified from the culture broth of a microorganism whose taxonomic features indicated it to belong to the genus *Streptomyces* sp. MH816-AF15. This strain has been deposited with the Fermentation Research Institute of the Industrial Science and Technology, Tsukuba, Japan, under the collection number FERM P-10398.

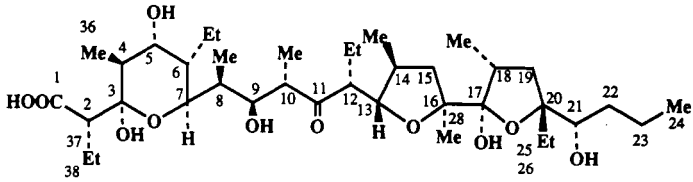

FIGURE 1. Structure of inostamycin. Note that the structure of inostamycin shown is the relative structure.

[1]Present address: Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 233, Japan.

[2]Laboratory of Biophysics, School of Pharmaceutical Sciences, Teikyo University, Sagamiko-machi, Tsukui-gun, Kanagawa 199-01, Japan.

For isolation of inostamycin, the producing strain was cultured in medium (pH 7.4) containing 2.0% galactose, 2.0% dextrin, 1.0% soy peptone, 0.5% corn steep liquor, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$ for 6 days at 27° on a rotary shaker (180 rpm/min). Then, 3 ml of the culture broth was inoculated into each of several 500-ml flasks containing 110 ml of 2.0% glycerol, 2.0% dextrin, 1.0% soy peptone, 0.3% yeast extract, 0.2% $(NH_4)_2SO_4$, and 0.02% $CaCO_3$ at pH 7.4, and the cultures were incubated for a further 5 days as described. The culture broth (5 liters) was centrifuged at 5000 rpm for 10 min, and the precipitate was extracted with 1.0 liter of Me_2CO. The Me_2CO extract was concentrated in vacuo and combined with the supernatant, then ex-

TABLE 1. ^{13}C- and ^{1}H-nmr Chemical Shifts[a] of Inostamycin Na salt in $CDCl_3$.

Position	δ C (ppm)	δ H (ppm)	Assignment
1	181.05	—	COONa
2	55.90	2.21	CH
3	100.69	—	C(O)(O)
4	38.22	2.13	CH
5	71.17	3.72	CH(O)
6	37.57	1.51	CH
7	74.75	4.16	CH(O)
8	32.30	1.80	CH
9	76.50	4.12	CH(O)
10	47.48	2.75	CH
11	214.96	—	CO
12	55.16	2.45	CH
13	83.62	3.99	CH(O)
14	34.73	2.23	CH
15	42.68	1.80	CH_2
16	86.29	—	C(O)
17	108.30	—	C(O)(O)
18	38.42	2.05	CH
19	37.57	1.51, 1.99	CH_2
20	87.26	—	C(O)
21	69.97	3.57	CH(O)
22	34.82	1.13, 1.27	CH_2
23	20.28	1.27, 1.67	CH_2
24	14.40	0.93	Me
25	31.11	1.56	CH_2
26	7.18	0.92	Me
27	14.74	0.98	Me
28	23.98	1.21	Me
29	15.56	1.03	Me
30	14.99	1.27, 1.99	CH_2
31	12.54	0.81	Me
32	12.84	0.87	Me
33	5.28	0.84	Me
34	18.41	1.21, 1.42	CH_2
35	10.85	0.92	Me
36	13.17	0.87	Me
37	20.08	1.42, 1.67	CH_2
38	12.37	0.93	Me
3-OH	—	8.26	—
5-OH	—	4.58	—
9-OH	—	4.04	—
17-OH	—	4.77	—
21-OH	—	7.75	—

[a]Chemical shifts downfield from TMS.

tracted with 3.5 liters of EtOAc. This extract was concentrated in vacuo, applied onto a Si gel column (50 g), and eluted with $CHCl_3$-MeOH (100:1). The active fraction was precipitated with MeCN, and the precipitate was further purified by centrifugation partition chromatography. The active fraction was crystallized from hexane/CH_2Cl_2 solution, and the crystals were washed with 1 N HCl, followed by 1 N NaOH and concentrated NaCl solution to obtain the Na salt. The active component was recrystallized from the same solution to give 112.4 mg of inostamycin Na salt: white powder, mp 181–182°, sims *m/z* 723 $[M+Na]^+$, $[\alpha]^{25}D$ +2.4° ($c=0.5$, $CHCl_3$), R_f in tlc 0.43 [Si gel, $CHCl_3$-MeOH (20:1)]. Inostamycin was soluble in MeOH, $CHCl_3$, and Me_2CO and insoluble in hexane and H_2O.

The molecular formula of inostamycin Na salt was deduced to be $C_{38}H_{67}O_{11}Na$ by mass spectrum and elemental analysis (found C 62.99, H 8.37, O 24.11, Na 3.54; calcd C 63.19, H 9.34, O 24.34, Na 3.18). The tentative structure of inostamycin was determined mainly by ^1H- and ^{13}C-nmr spectroscopy. The assignment of each proton and carbon in the ^1H- and ^{13}C-nmr spectra is shown in Table 1. Recently, the structure of portmicin (7) and of hidamicin (8), a member of the polyether antibiotic family, were determined by the use of advanced techniques of nmr spectroscopy such as ^1H-^1H shift correlation (COSY) spectrum and the heteronuclear multiple-bond correlation (HMBC) spectrum analysis. By these techniques, the tentative structure of inostamycin was proposed. To confirm the proposed structure and elucidate its stereochemistry, we also studied the inostamycin Na salt by X-ray crystallography.

A small colorless prismatic crystal of Na inostamycin grown in a hexane/CH_2Cl_2 solution was used for this study. The crystal dimensions were approximately 0.5 × 0.6 × 0.25 mm. Unit cell parameters and intensity data were collected on a Phillips PW1100 diffractometer using graphite-monochromated CuKα radiation. Reflections were measured within the 2θ range of 6° through 156°. The data were corrected for Lorentz and polarization factors, but no correction was made for absorption. Crystal data are summarized in Table 2. The crystal structure was determined by the direct method based on MULTAN (9) and refined by the method of least squares with block diagonal matrix approximations. The stereospecific structure of the molecule was drawn by the 5 PLUTO (10) program, from which the relative molecular structure was determined as shown in Figure 1. As shown in Figure 2, the molecule as a whole takes a folded conformation around the sodium ion and coordinates its carboxylate oxygens, two hydroxyl oxygens, and carbonyl and ether oxygen atoms with distances ranging from 2.225 to 2.676 Å. Thus, inostamycin was shown to be a novel polyether. It was shown to belong to the polyether antibiotic group which includes lysocellin (11), X-14873A, and related compounds from *Streptomyces* (12).

TABLE 2. Crystal Data of Inostamycin Na Salt.

Sodium inostamycin, $C_{38}H_{67}O_{11}Na$, MW = 722.9

Monoclinic, space group $C\,222_1$, Z = 8

Lattice constants:
$a = 26.404\,(14)$Å
$b = 10.458\,(6)$
$c = 33.661\,(18)$
$U = 9295$ Å^3

$Dx = 1.033$ g·cm^{-3}, μ for CuKα = 6.6 cm^{-1}

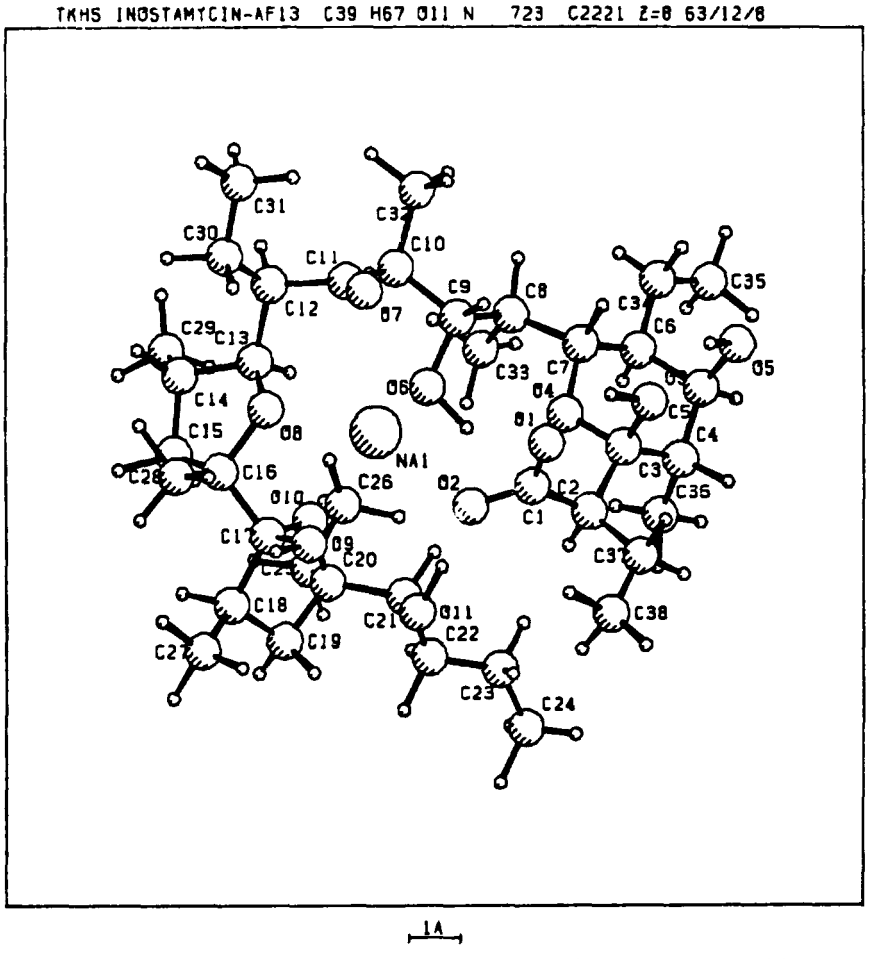

FIGURE 2. Relative molecular structure of inostamycin sodium salt determined by X-ray crystallography.

As shown in Figure 3, inostamycin inhibited EGF-induced inositol incorporation into inositol lipids with an IC_{50} of about 0.5 μg/ml in the A431 cell assay system. Lysocellin, which has a closely related structure, also inhibited PtdIns turnover; however, monensin, a polyether antibiotic having a considerably different structure, did not inhibit PtdIns turnover.

Inostamycin, having a totally different structure from psi-tectorigenin, may be an useful tool for study of the cellular role of PtdIns turnover. In situ and in vivo activities of inostamycin are currently being studied.

EXPERIMENTAL

MATERIALS.—EGF was purchased from Takara Shuzo, and [^{3}H]inositol (19.9 Ci/mmol) was obtained from Amersham. The A431 cell line was a gift from Prof. S. Kawai, Institute of Medical Science, University of Tokyo. Lysocellin was isolated in our institute, and monensin was purchased from Sigma.

PHOSPHATIDYLINOSITOL TURNOVER ASSAY (6).—A431 cells (3×10^5) grown for 16 h were preincubated for 30 min at 37° in 1 ml of Hepes-buffered saline (HBS: 20 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% glucose; pH 7.4) containing [^{3}H]inositol (1 μCi/ml). The test chemical and EGF (400 ng/ml) were added, and the incubation was continued at 37° for 60 min. Subsequently, 0.5 ml of 10% trichloroacetic acid containing 0.01 M sodium pyrophosphate was added, and the acid-insoluble fraction was scraped off from the dish in 1.0 ml of H_2O. The lipid was extracted from it

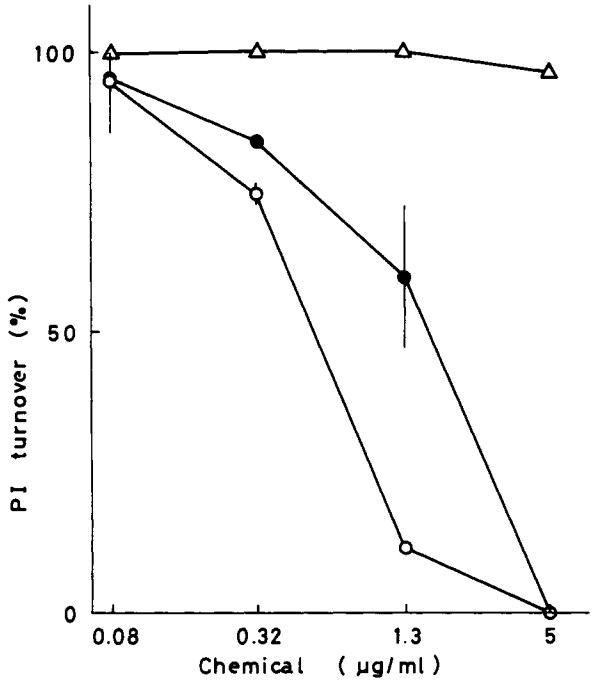

FIGURE 3. Inhibition of phosphatidylinositol turnover by inostamycin in cultured A431 cells. The cells were incubated with inostamycin (○), lysocellin (●), or monensin (Δ) for 1 h after preincubation with labeled inositol. The method is described in Experimental. Values are means of duplicate samples.

by the addition of $CHCl_3$-MeOH (1:1), and [^{3}H]inositol-labeled lipids were counted by liquid scintillation spectrophotometry.

LITERATURE CITED

1. R.H. Michell, *Biochim. Biophys. Acta*, **415**, 81 (1975).
2. R.V. Farese, *Mol. Cell. Endocrinol.*, **35**, 1 (1984).
3. L.T. Fleischman, S.B. Chahwala, and L. Cantley, *Science*, **231**, 407 (1986).
4. D.R. Kaplan, M. Whiteman, B. Schaffhausen, L. Raptis, R.L. Garcea, D. Pallas, T.M. Roberts, and L. Cantley, *Proc. Natl. Acad. Sci. USA*, **83**, 3624 (1986).
5. S. Jackowski, C.W. Rettenmier, C.J. Sherr, and C.O. Rock, *J. Biol. Chem.*, **261**, 4978 (1986).
6. M. Imoto, T. Yamashita, T. Sawa, S. Kurasawa, H. Naganawa, T. Takeuchi, Z. Bao-quan, and K. Umezawa, *FEBS Lett.*, **230**, 43 (1988).
7. H. Seto, K. Furihata, K. Saeki, N. Otake, Y. Kusakabe, C. Xu, and J. Clardy, *Tetrahedron Lett.*, **28**, 3357 (1987).
8. K. Dobashi, J. Yang, R. Ogata, Y. Takahashi, N. Matsuda, M. Hamada, H. Naganawa, T. Takita, and T. Takeuchi, *J. Antibiot.*, **42**, 629 (1989).
9. P. Main, S.E. Hull, L. Lessinger, G. Germain, J.-P. Declercq, and M.M. Woolfson, "Multan '78," University of York, England, and Leuven, Belgium, 1971.
10. "PLUTO," University Chemical Laboratory, Lensfield Road, Cambridge CB2 1 EW, England, 1983.
11. E. Ebata, H. Kasahara, K. Sekine, and Y. Inoue, *J. Antibiot.*, **28**, 118 (1975).
12. J.W. Westley, C.-M. Liu, J.F. Blount, L. Todaro, L.H. Sello, and N. Troupe, *J. Antibiot.*, **39**, 1704 (1986).

Received 27 November 1989