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ORIGINAL ARTICLE

Evaluation method for polyamine uptake by N^1-dansylspermine

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Abstract Polyamine uptake by the polyamine transport system (PTS) in HTC cells was studied without the use of radioisotope-labeled polyamines. N^1-Dansylspermine (DNS343) was selected as a candidate probe to examine the PTS. DNS343 was incorporated into HTC cells, and its distribution in the cells was confirmed by fluorescence microscopy. The incorporation of DNS343 via PTS was confirmed by a competition study with bis(3-aminopropyl)amine, which is incorporated into cells via the PTS. In addition, the temperature dependency of DNS343 uptake and studies with inhibitors of ornithine decarboxylase and proteoglycan synthesis supported the use of DNS343 as a fluorescent probe for the PTS. The kinetics studies for HTC cells treated with or without an ornithine decarboxylase inhibitor indicated that DNS343 uptake was saturable and that the apparent Km values for the PTS were approximately 1.5 μM in both types of cells at 37°C. Thus, we developed an assay method for the PTS by high-performance liquid-chromatography with DNS343. The inhibitory effect of polyamine analogs and related compounds on DNS343 uptake was then examined and discussed.

Keywords Polyamine · Polyamine transport system · HTC cells · N^1-dansylspermine · HPLC assay · Polyamine analog

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Abbreviations

17-DAH	1,7-Diaminoheptane
33	Bis(3-aminopropyl)amine
333	*N*,*N*′-Bis(3-aminopropyl)-1,3-diaminopropane
33333	1,19-Diamino-4,8,12,16-tetraazanonadecane
3334	1,16-Diamino-4,8,12-triazahexadecane
33433	1,20-Diamino-4,8,13,17-tetraazaeicosane
3434	1,17-Diamino-4,9,13-triazaheptadecane
34343	1,21-Diamino-4,9,13,18-tetraazahenicosane
3443	1,17-Diamino-4,9,14-triazaheptadecane
353	1,13-Diamino-4,10-diazatridecane
383	1,16-Diamino-4,13-diazahexadecane
4334	1,17-Diamino-5,9,13-triazaheptadecane
434	1,13-Diamino-5,9-diazatridecane
44	Homospermidine
444	1,14-Diamino-5,10-diazatetradecane
83	1,12-Diamino-4-azadidecane
AOAP	1-Aminooxy-3-aminopropane
Ac343	Acetylspermine
Boc_2O	Di-*tert*-butyl dicarbonate
DA343	Diacetylspermine
DA3443	N^1, N^{17}-Diacetyl-1,17-diamino-4,9,14-triazaheptadecane
DE33	Diethylnorspermidine
DE333	Diethylnorspermine
DFMO	α-Difluoromethylornithine
DNS333	N^1-Dansylnorspermine
DNS343	N^1-Dansylspermine
DNS-Cl	Dansyl chloride
FBS	Fetal bovine serum
GC7	N^1-Guanidino-1, 7-diaminoheptane
HTC cell	Morris hepatoma 7288c cell

MDL72527	N^1, N^4-Bis(2,3-butadienyl)-1, 4-butanediamine
MGBG	Methylglyoxalbisguanylhydrazone
NBD	N^1-(7-Nitro-2,1,3-benzoxadiazolyl)
NBD333	N^1-(7-Nitro-2,1, 3-benzoxadiazolyl)norspermine
ODC	Ornithine decarboxylase
Paraquat	1,1′-Dimethyl-4,4′-bipyridinium dichloride
PTS	Polyamine transport system
Put	1,4-Diaminobutane
Spd	Spermidine
Spm	Spermine
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
Xyl-PheNO$_2$	*p*-Nitrophenyl-*o*-*β*-D-xylopyranoside

Introduction

The cellular content of polyamines, putrescine, spermidine and spermine, is regulated by biosynthesis, degradation, and transport. The molecular biology of the biosynthesis, degradation and interconversion of polyamines has been well documented, and some properties of the polyamine transport system (PTS) in mammalian cultured cells have been determined (Tabor and Tabor 1984; Cohen 1998; Igarashi and Kashiwagi 2000; Seiler et al. 1996; Belting et al. 2003; Soulet et al. 2004; Hoshino et al. 2005; Phanstiel et al. 2007). The PTS is inducible and saturable. Depletion of the intracellular polyamine content led to a marked increase in the rate of polyamine uptake that scarcely affected the affinities to polyamines, while an increase in the intracellular polyamine level downregulated the PTS activity. A mechanism-based inhibitor of ornithine decarboxylase (ODC), α-(difluoromethyl)ornithine (DFMO) (Mitchell et al. 1992), or a competitive inhibitor of ODC, 1-aminooxy-3-aminopropane (AOAP) (Hyvonen et al. 1988), caused a substantial depletion of intracellular putrescine and spermidine that inhibited cell growth and increased PTS activity. The cellular polyamine content and cell growth were restored by supplementing exogenous polyamines to the culture medium (Mamont et al. 1981; Mitchell et al. 1992). Evidence from several studies supports the conclusion that the effectiveness of ODC inhibition could be increased by interfering with the PTS (Ask et al. 1992; Weeks et al. 2000; Belting et al. 2002). Extracellular sources of polyamines are important for the growth of tumor cells; thus, the identification of pharmacological agents that block polyamine transport is desired for the development of antineoplastic drugs (Marton and Pegg 1995).

The PTS activity is measured to screen for agents that block polyamine transport, and radioisotope-labeled polyamines are generally used in this assay (Hoshino et al. 2005). Although this method is sensitive and convenient, its use is limited due to radioisotope contamination. Recently, several groups have shown that fluorescent-labeled polyamines could be used as probes for the PTS in flow cytometry analysis (Guminski et al. 2009; Cullis et al. 1999; Aziz et al. 1998). In the present study, we developed a sensitive and accurate method that uses N^1-dansylspermine (DNS343) with a simple HPLC apparatus to evaluate PTS activity.

Materials and methods

Triethylamine, di-*tert*-butyl dicarbonate (BOC_2O), trifluoroacetyl ethylester, dansyl chloride (DNS-Cl), 1,4-Diaminobutane (Put) dihydrochloride, bis(3-aminopropyl)amine (norspermidine, 33) and 1,7-diaminoheptane (1,7-DAH) that were re-crystalized as hydrochloride salts and spermine (Spm) tetrahydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). *o*-Phthaldialdehyde, *p*-nitrophenyl-*o*-*β*-D-xylopyranoside (Xyl-PheNO$_2$), aminoguanidine bicarbonate salt, methylglyoxalbisguanylhydrazone (MGBG) dihydrochloride, 1,1′-dimethyl-4,4′-bipyridinium (Paraquat) dichloride and agmatine sulfate were purchased from Sigma (Tokyo, Japan). Trifluoroacetic acid (TFA) and 1-octanesulfonic acid sodium salt were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Spermidine (Spd) trihydrochloride and *N*,*N*′-Bis(3-aminopropyl)-1,3-diaminopropane (333) that was re-crystalized as hydrochloride salt were purchased from Aldrich (Tokyo, Japan). Other polyamines, polyamine analogs, and fluorescent polyamines were prepared according to the methods described previously (Takao et al. 2007, 2008; Samejima et al. 1984; Niitsu and Samejima 1986). All other reagents and organic solvents were of commercial analytical grade.

Synthesis of DNS343 tetrahydrochloride

DNS343 was synthesized by a previously described method (Takao et al. 2008). Briefly, spermine was protected on one of the primary amines with ethyl trifluoroacetate to afford mono-trifluoroacetamide derivative contaminated with di-trifluoroacetamide derivative. The remaining free amines were immediately protected with di-*tert*-butyl dicarbonate, and then the TFA-protecting group was removed with ammonia to afford tri-Boc-spermine that was purified by silica gel chromatography. The tri-Boc-spermine was fluorescent-labeled with DNS-Cl. The dansyl

derivative was subjected to silica gel column chromatography with a solvent system of chloroform:methanol:ammonia to obtain a single band of tri-Boc-dansylspermine. Boc groups of the pure derivative were then de-protected by trifluoroacetic acid to yield DNS343 trifluoroacetate that was exchanged to a hydrochloride salt. The DNS343 hydrochloride was re-crystalized from ethanol–water. DNS343 tetrahydrochloride, FAB-MS: 436 $[C_{22}H_{37}N_5O_2S]^+$ Anal Calcd for $C_{22}H_{37}N_5O_2S$ 4HCl: C 45.19; H 7.11; N 12.04. Found: C 45.19; H 6.98; N 11.97.

Cell culture

Morris hepatoma 7288c (HTC) cells and MEM-Eagle's medium were purchased from Dainippon Pharmaceuticals (Tokyo, Japan), and fetal bovine serum (FBS) was purchased from Nippon Biotest Laboratories, Inc., (Tokyo, Japan). Cells were cultured according to previously described methods (Beppu et al. 1996). HTC cells were grown as a monolayer in culture dishes. The HTC cells were maintained in MEM-Eagle medium containing 10% FBS at 37°C in an atmosphere of 5% CO_2 and 95% air.

DNS343 uptake experiment

Cells were seeded at 5×10^5 cells per 10-cm diameter culture dish. Cells grown for 24 h after the seeding were used for the experiments. The medium containing 1 mM aminoguanidine as an inhibitor of diamine oxidase and 10 μM DNS343 (10 ml) was added to the monolayer. Various compounds to be tested were added as described in the text. After incubation for 2 h at 37°C, the attached cells were washed three times with PBS, scraped, harvested, counted, and centrifuged by the usual method. A total of 200 μl of 10% trichloroacetic acid (TCA) was added to the resulting cell pellets; then, the mixture was vortexed and centrifuged. The supernatants were collected and subjected to HPLC analysis.

HPLC analysis

HPLC conditions for DNS343 were as follows: column, Tosoh ODS-80T_M (4.6 mmϕ × 150 mm); isocratic elution solution, methanol:water (75:25) containing 8 mM sodium octanesulfonate and 0.1% trifluoroacetic acid; flow rate 1 ml/min; and fluorescence detection by a Shimadzu fluorescence detector RF-10A_{XL}, Ex: 333 nm and Em 544 nm. The detection limit of DNS343 was approximately 30 fmol, and a linearity of the peak area of DNS343 was demonstrated at amounts greater than 20 pmol. The determination of 33 in Fig. 3 was performed using an ion-exchange *o*-phthalaldehyde-post-column HPLC as described previously (Shirahata et al. 1993). Aliquots of TCA supernatants from HTC cells were injected, and 33 was determined using the peak area.

Treatment with AOAP and Xyl-PheNO$_2$

Inhibitor treatments were performed according to the previously described methods (Takao et al. 2007; Belting et al. 1999). Briefly, cells were seeded at 5×10^5 cells per 10-cm diameter culture dish in the absence or presence of 100 μM AOAP, and 1 mM of Xyl-PheNO$_2$ (final concentration) was added to the culture 1 day after the seeding. After 2 days of culture with AOAP, cells were used for the DNS343 uptake experiment described above.

Fluorescence microscopy

Cells were seeded on a Lab-Tek2 chamber slide system (Nalge Nunc International, Tokyo, Japan). The cells attached to the slide were treated with 10 μM DNS343 for 6 h and fixed by formaldehyde in the usual manner. The fixed cells were viewed with an Olympus BX51WI upright microscope. We used a specific filter for DNS343 (Omega Optical, Ex/Em = 330/535 nm) to observe the fluorescence of compounds containing the dansyl group.

Results and discussion

To identify a suitable fluorescent probe for the PTS, dansyl derivatives and N^1-(7-nitro-2,1,3-benzoxadiazolyl) (NBD) derivatives of polyamines were examined instead of a large polyaromatic fluorophore, which might non-specifically interact with the lipid bilayer. Three compounds, DNS343, N^1-dansylnorspermine (DNS333), and N^1-NBD norspermine (NBD333), were prepared, and their incorporation into HTC cells, which have well-established PTS properties, was compared (Mamont et al. 1981; Mitchell et al. 1992). The results showed that the DNS343 and DNS333 uptakes were similar and greater than the NBD333 uptake (data not shown). Thus, DNS343, which has the natural polyamine (spermidine) structure, was used for the following study.

HPLC chromatograms of authentic DNS343 and sample solutions from HTC cells incubated in the presence or absence of DNS343 are shown in Fig. 1. DNS343 incorporated into HTC cells was clearly detected as a single peak.

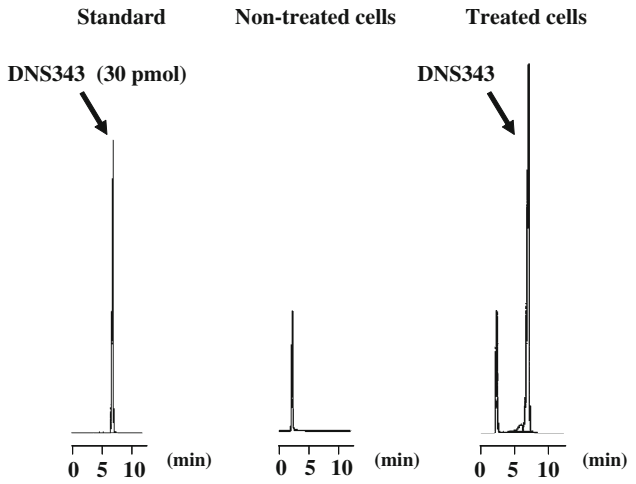

Fig. 1 HPLC chromatograms of authentic DNS343 and DNS343-treated or non-treated cells. HPLC conditions are described in the Materials and methods. Authentic DNS343 (30 pmol) was injected (standard). HTC cells were incubated in the absence (non-treated cells) or presence (treated cells) of 10 μM DNS343 for 2 h, and then samples for injection to HPLC were prepared

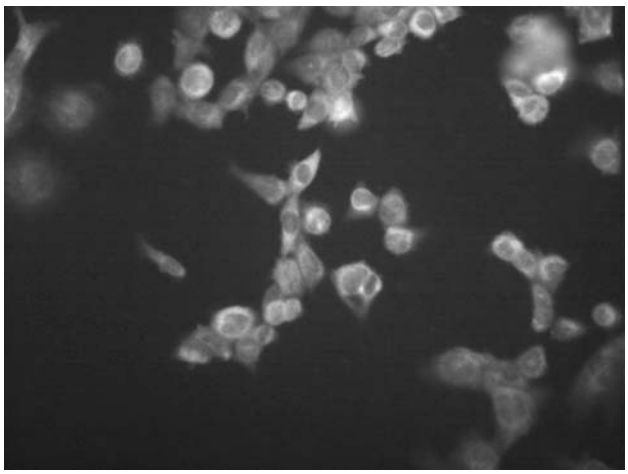

Fig. 2 Fluorescence photomicrograph of DNS343 accumulation in HTC cells seeded on a sterile glass chamber slide. Cells were grown on the Lab-Tek2 chamber slide system. After attachment, cells were incubated in the presence of 10 μM DNS343 and fixed with formaldehyde. The fluorescence of the dansyl group was detected by an Olympus BX51WI upright fluorescence microscope with filter for DNS343 (Omega Optical, Ex/Em = 330/535 nm)

As shown in the fluorescence photomicrograph of DNS343-treated cells (Fig. 2), the fluorescence was mainly located in the area of cytoplasm. This finding was similar to the results of previous studies that used *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-N′-{S-[spermidine-(N^4-ethyl)]thioacetyl}ethylenediamine (Kaneko et al. 2007; Soulet et al. 2002).

To assess the uptake of DNS343 via the PTS, a competition study was performed with bis(3-aminopropyl)amine

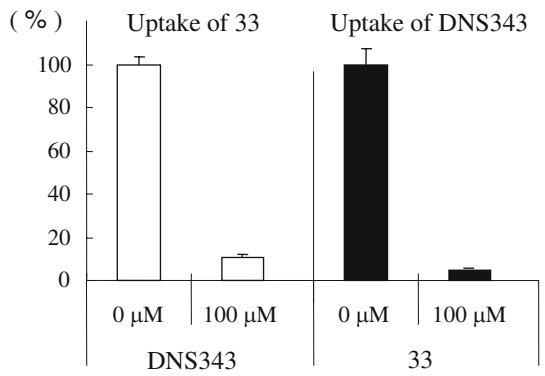

Fig. 3 Competition study of the uptake of 33 or DNS343 into HTC cells. Cells were incubated in the presence of 10 μM 33 alone or plus 100 μM DNS343 and in the presence of 10 μM DNS343 alone or plus 100 μM 33. Data are expressed as means ± SD ($n = 3$)

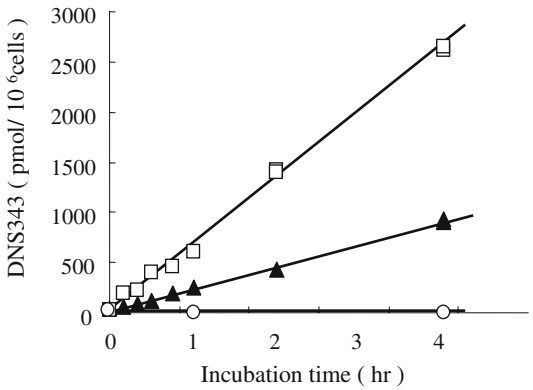

Fig. 4 Time-dependent uptake of DNS343 into HTC cells under different conditions. Cells were pre-cultured in the absence (control) or presence of 100 μM AOAP for 2 days and subjected to the method for the measurement of DNS343 uptake. Cell samples were collected at the indicated culture time. Control cells were cultured at 37°C (*filled triangle*) or 0°C (*open circle*), and AOAP-treated cells were cultured at 37°C (*open square*). Data are expressed as duplicate determinations

(33), which is incorporated into cells via the PTS as a spermidine analog (Porter 1983). The presence of higher concentrations of 33 in the media inhibited the cellular uptake of DNS343, and also the uptake of 33 was inhibited by DNS343 (Fig. 3). These results suggested that DNS343 was incorporated via the PTS.

DNS343 was incorporated into HTC cells in a linear manner for more than 4 h (Fig. 4). Although Seiler et al. reported that DNS343 was a potent calmodulin antagonist that interfered with the PTS (Seiler et al. 1998), the linear DNS343 uptake strongly suggested that DNS343 does not have any effect on the PTS as a calmodulin antagonist in our conditions. In addition, the uptake was temperature-dependent; only negligible uptake occurred at 0°C, as compared to the significant uptake at 37°C in control cultures. The incorporation of DNS343 into HTC cells treated

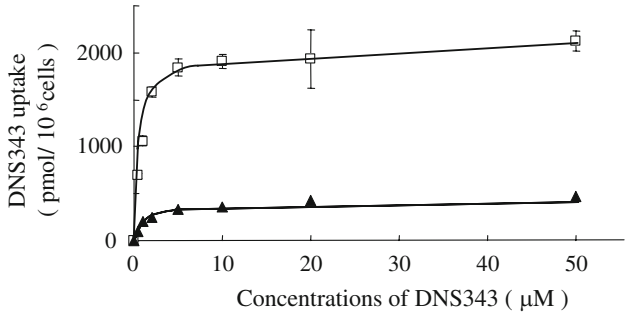

Fig. 5 Dose-dependent uptake of DNS343 into HTC cells. Cells were pre-cultured in the absence (control *filled triangle*) or presence of 100 μM AOAP (*open square*) for 2 days and subjected to the method for the measurement of DNS343 uptake. Cells were cultured with the indicated concentration (0.5–50 μM) of DNS343 for 2 h. Data are expressed as means ± SD ($n = 3$)

with AOAP, which prevents putrescine production at a concentration lower than DFMO (Hyvonen et al. 1988; Poulin et al. 1989; Takao et al. 2007) and increases the PTS activity (Hyvonen et al. 1988), also increased in a linear manner for more than 4 h.

A tentative kinetics study for HTC cells treated with or without AOAP indicated that DNS343 uptake was saturable and that the apparent K_m values for the PTS were approximately 1.5 μM in both types of cells at 37°C (Fig. 5). This K_m value is similar to a previously reported K_m value measured with ^{14}C-labeled spermidine in HTC cells (Mitchell et al. 1992).

Bolkenius and Seiler (1981) reported in vitro degradation of DNS343 by polyamine oxidase (PAO). Under the present incubation conditions for 2 h, there was no difference in the incorporation of DNS343 with or without 25 μM MDL72527, an irreversible inhibitor of PAO (data not shown). A PAO inhibitor, such as MDL72527, which does not have any effect on DNS343 uptake (Table 1), should be added to the incubation media in uptake experiments when DNS343 is applied to cells with high PAO activity, such as Chinese hamster ovary cells (Carper et al. 1991).

Belting et al. (1999) reported that proteoglycans were involved in polyamine uptake; this conclusion was based on their observation that spermine uptake into HEL cells was inhibited by Xyl-PheNO$_2$, an inhibitor of endogenous proteoglycan synthesis. The effect of Xyl-PheNO$_2$ on DNS343 uptake was then examined. The uptake decreased with Xyl-PheNO$_2$ and increased with AOAP (Fig. 6), assuring the use of DNS343 as a probe for the PTS.

Based on these observations, DNS343 was applied to examine the affinities of various polyamines and related compounds to the PTS. The inhibition of DNS343 uptake with these compounds is summarized in Table 1.

Compounds that showed no inhibition or weak inhibition of DNS343 uptake, such as MDL72527, AOAP and

Table 1 Inhibition of DNS343 uptake into HTC cells by polyamines or related compounds

Polyamines or related compounds	DNS343 uptake (percentage of control)		
	1 μM	10 μM	100 μM
Diamines			
MDL72527	>95	>95	>95
AOAP	>95	>95	>95
Put	>95	>95	82
1,7-DAH	70	42	10
Triamines			
DE33	>95	>95	74
83	90	60	11
33	81	32	9
Spd	71	16	<5
44	55	9	<5
Tetramines			
DA343	>95	>95	95
Ac343	>95	90	41
383	75	60	23
DE333	86	60	13
333	71	28	11
434	32	11	<5
444	30	9	<5
353	32	6	<5
Spm	29	6	<5
Pentamines			
DA3443	93	76	48
3334	56	12	6
3434	22	6	<5
4334	20	<5	<5
3443	14	<5	<5
Hexamines			
33333	35	8	<5
33433	31	8	<5
34343	20	<5	<5
Others			
MGBG	>95	95	81
Paraquat	>95	94	78
GC7	84	73	38
Agmatine	91	79	31

Cells were incubated for 2 h in the presence of 10 μM DNS343 and 1, 10, or 100 μM polyamines or related compounds. The number of control cells and treated cells remained equivalent during the experiments. Data are expressed as means ($n = 3$)

DA343 including Put, DE33, MGBG and Paraquat, might not necessarily enter cells via the PTS. Acetylation of the terminal amine group of polyamines significantly reduced their inhibitory effects on DNS343 uptake, as illustrated by the data from DA343, Ac343 and Spm and from DA3443 and 3443. Compounds with a terminal primary amine were

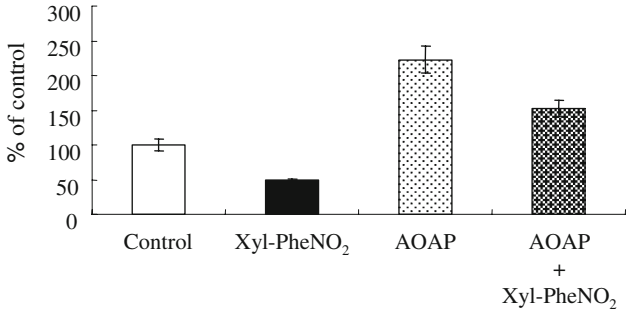

Fig. 6 Effect of Xyl-PheNO$_2$ on the uptake of DNS343 into HTC cells. Cells were seeded in the absence (*control*) or presence of 100 μM AOAP. The next day, 1 mM Xyl-PheNO$_2$ was added. After 1 day of incubation, the DNS343 uptake of cells was assayed. Data are expressed as means ± SD ($n = 3$)

highly potent inhibitors than compounds with a secondary amine, as illustrated by the data from DE33 and 33 and from DE333 and 333. Of the triamines, 44 inhibited more potently than Spd, as previously reported (Porter 1983). Potent inhibition was mediated by tetramines (434, 444, 353 and Spm), pentamines (3334, 3434, 4334 and 3443), and hexamines (33333, 33433 and 34343). Guanyl compounds, such as GC7 and agmatine, mediate only weak inhibition. Compounds of similar molecular size with a different charge provided similar levels of inhibition in the case of 1,7-DAH and 33, but such compounds provided different levels of inhibition in the case of 83 and Spm and that of 383 and 3334. The present non-radioactive evaluation method for the PTS with DNS343 will be helpful in identifying compounds that inhibit the PTS.

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