Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 6, pp 825–836, 1996

0968-0896/96 $15.00+0.00

S0968-0896(96)00072-7

Synthesis and Evaluation of a Polyamine Phosphinate and Phosphonamidate as Transition-State Analogue Inhibitors of Spermidine/Spermine-N^1-Acetyltransferase

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Abstract—Polyamine analogues such as bis(ethyl)norspermine and N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8-diazaundecane (CPENSpm) act as inhibitors of the enzyme spermidine/spermine-N^1-acetyltransferase (SSAT) in vitro and possess impressive antitumor activity against a number of cell lines. However, the propensity of these compounds to superinduce SSAT in intact cells limits their usefulness in studies aimed at elucidating the role of SSAT in cellular metabolism. The recently synthesized alkylpolyamine analogue N^1-ethyl-N^{11}-[(cycloheptyl)methyl]-4,8-diazaundecane (CHENSpm, **3**) is also an effective inhibitor of SSAT and has potent antitumor activity, but does not appear to superinduce SSAT. These findings suggest that it is possible to synthesize polyamine analogues that can be used for selective inhibition of the enzyme in cellular metabolic studies. Along these lines, the phosphate-based transition state analogues **4** and **5** were synthesized and evaluated as inhibitors of isolated SSAT. Phosphonamidate **4** was rapidly hydrolyzed under the assay conditions, and thus did not inhibit the enzyme. However, the phosphinate analogue **5** was an effective inhibitor of purified human SSAT, with a K_i value of 250 μM. The inhibitory activity of **5** was also compared with that of CHENSpm (IC_{50} = 13 μM), as well as a series of bis-substituted alkylpolyamine analogues. The unsymmetrically substituted polyamine analogue CHENSpm (**3**) and the phosphinate transition state analogue **5** represent the first functional, nonsuperinducing inhibitors of human SSAT.

Introduction

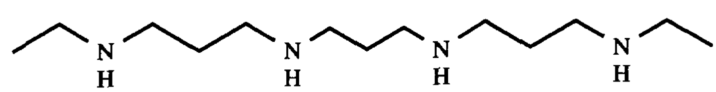
The enzymes involved in the polyamine biosynthetic pathway have been the subject of intensive study and a number of specific inhibitors for these enzymes have been designed as potential antitumor or antiparasitic agents.[1–3] Despite these efforts, only one of these inhibitors, α-difluoromethylornithine, has become a clinically useful agent. To date, most of the studies involving polyamine biosynthesis inhibitors have focused on the enzymes involved in the forward pathway, most notably the controlling enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMet-DC). Recently, however, there has been considerable interest generated in the enzyme spermidine/spermine-N^1-acetyltransferase (SSAT), the rate-limiting step in the back conversion of polyamines.[3] SSAT, in conjunction with polyamine oxidase (PAO), allows for the reversal of the biosynthetic pathway and thus aids in the finely controlled modulation of individual cellular polyamine levels.

We[4,5] and others[6–8] have recently described the synthesis and evaluation of a series of bis-alkyl-substituted polyamine analogues with impressive in vitro antitumor effects. These analogues were designed to enter the cell by the polyamine transport system,[9] and then to disrupt the biosynthesis and metabolic interconversion of cellular polyamines. One of the first of these agents to be described, bis(ethyl)norspermine (BENSpm, **1**, Fig. 1), exhibits phenotype-specific cytotoxicity in vitro against two human lung cancer cell lines,[10–13] the NCI H157 large cell lung carcinoma line (LCLC) and the NCI H82 small cell lung carcinoma (SCLC) line. The LCLC cell line is rapidly killed by treatment with the bis(ethyl)polyamines,[10] and this cytotoxic response is accompanied by a tremendous induction of SSAT activity, often to levels greater than 1000 times baseline.[11–13] By contrast, the SCLC cell line is not killed by bis(ethyl)polyamine analogues, is only moderately growth inhibited,[10,11] and minimal induction of SSAT activity is observed. It now appears that, as a rule, the nonSCLC lines are much more sensitive to the effects of the bis(alkyl)polyamine analogues.[13] There is now substantial data which suggests that superinduction of the polyamine catabolic enzyme SSAT is positively correlated with the observed cytotoxic response in the H157 and H82 cell lines.

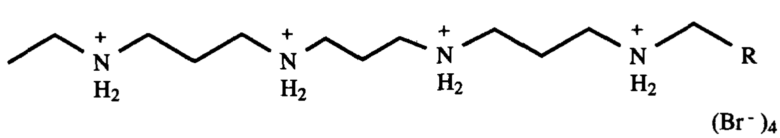
†Presented in part at the 209th National Meeting of the American Chemical Society, April 2–6, 1995 in Anaheim, California (Abstract MEDI 49).

Although SSAT induction has been observed in other cell systems, the correlation between SSAT induction and degree of cytotoxicity is less apparent.[14]

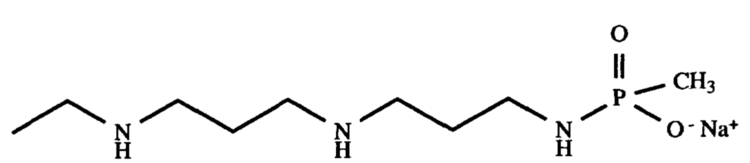
We have recently described the synthesis and evaluation of the unsymmetrically substituted alkylpolyamine analogue CPENSpm (**2**), shown in Figure 1, which is an effective inhibitor and superinducer of human SSAT and acts as a promising antitumor agent in vitro.[4,5] This analogue, like BENSpm, was found to be markedly cytotoxic at a concentration of 10 μM in the LCLC line, accompanied by nearly complete depletion of all intracellular polyamines and a decrease in ODC activity to undetectable levels. By contrast, rate of growth and cellular polyamine content in the SCLC cell line were minimally affected by CPENSpm. In the responsive LCLC line, CPENSpm was found to induce SSAT in a time- and dose-dependent manner to maximum levels 1200-fold greater than baseline. Conversely, in the unresponsive SCLC cell line, minimal (less than sevenfold) induction of SSAT was observed. These results support the contention that the differential induction of SSAT may play a role in determining cell-type specific sensitivity to the bis-alkylated polyamine analogues. However, we have recently synthesized the previously unreported unsymmetrically alkylated polyamine analogue N^1-ethyl-N^{11}-[(cycloheptyl)methyl]-4,8-diazaundecane (CHENSpm, **3**, Figure 1) and preliminary biological studies suggest that this analogue may act by a different mechanism (unpublished observations). When the LCLC and SCLC cell lines are treated with 1 μM CHENSpm, a cell-type specific response is observed, wherein the LCLC develop rapid cytotoxicity, while the SCLC are minimally effected. CHENSpm now appears to be the most potent alkylpolyamine antitumor analogue produced to date against the H157 cell line and exhibits the highest degree of cell-type specificity between H157 and H82 cells. It is also an effective inhibitor of isolated human SSAT (see below). However, growth inhibition following CHENSpm treatment does not appear to correlate with SSAT superinduction, since dramatically elevated levels of SSAT cannot be detected in either cell line. This data, which



BENSpm, 1



R = cyclopropyl CPENSpm, 2
R = cycloheptyl CHENSpm, 3



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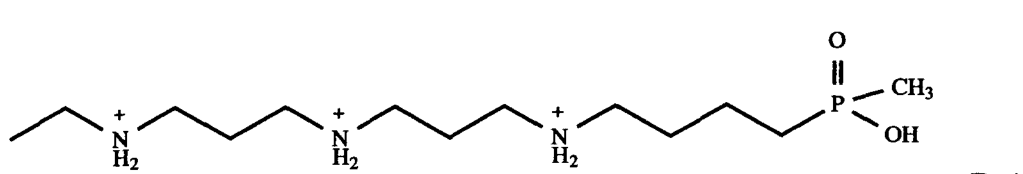


5 $(Br^-)_3$

Figure 1. Structures of the antineoplastic polyamine analogues BENSpm (**1**), CPENSpm (**2**), CHENSpm (**3**), phosphonamidate (**4**) and phosphinate (**5**).

is being published separately, suggests that it is possible to design polyamine analogues as antitumor agents which do not superinduce SSAT. Although one noninducing polyamine analogue with antitumor activity has been described,[14,15] its ability to inhibit or interact with SSAT has not been described. A potent SSAT inhibitor which is noninducing would be of great value in studying the cellular effects of selective inhibition of SSAT.[14]

The reaction catalyzed by SSAT has been shown to proceed via a sequential bi–bi mechanism in which the polyamine is the first substrate to add to the surface of the enzyme.[16] This data implies that a tetrahedral transition state is likely for SSAT mediated acetyl transfer, which does not involve an acetylated enzyme intermediate. In an effort to develop additional specific inhibitors for SSAT, the phosphonamidate and phosphinate transition state analogue inhibitors **4** and **5**, shown in Figure 1, were designed. Such compounds have been shown to act as potent and specific inhibitors of a wide variety of enzymatic reactions involving a tetrahedral transition state.[17,18] It is postulated that the proposed phosphate-based transition state mimics **4** and **5**, if active, could provide additional tools for study of the polyamine catabolic pathway and, more specifically, could be of great value in determining the cellular effects of selective inhibition of SSAT. We now report the synthesis of the title phosphate-based transition state mimics **4** and **5**, and describe the results of our preliminary biological studies.

Chemistry

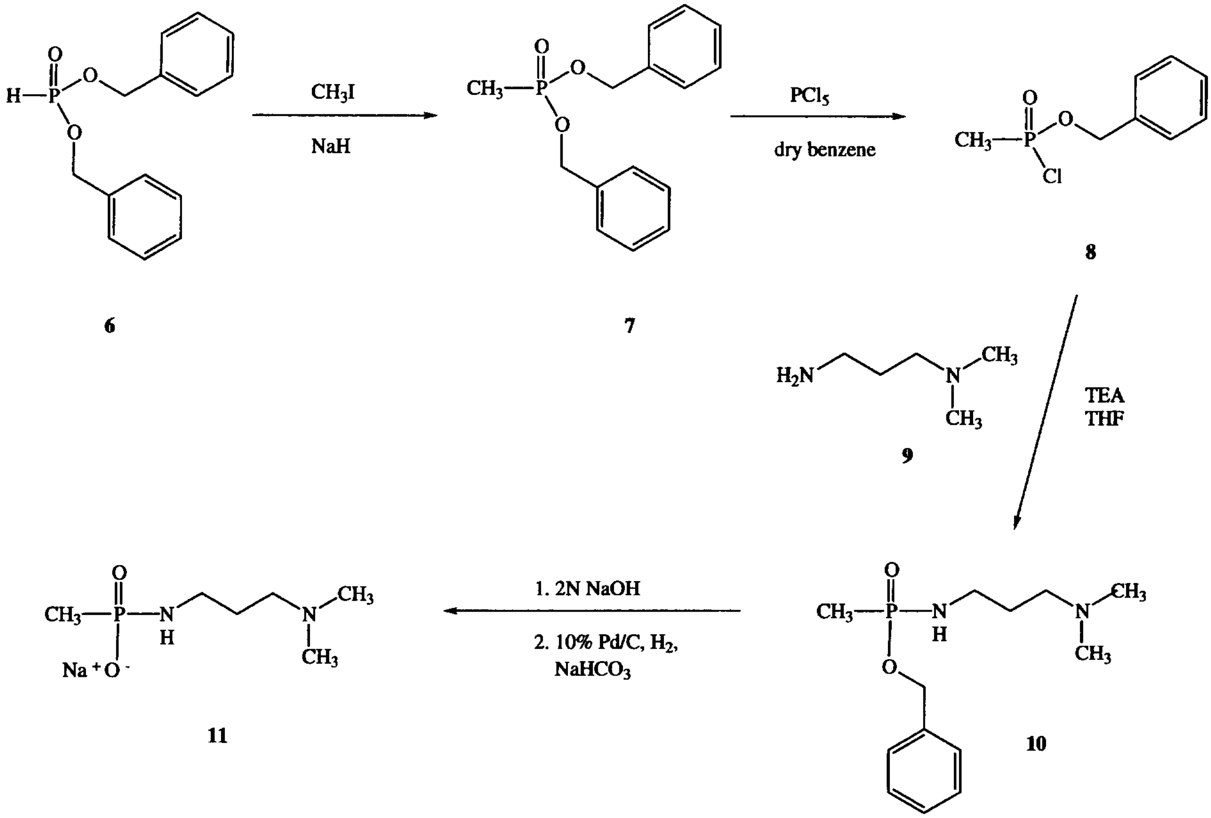
The synthesis of the proposed phosphonamidate transition state analogue **4** is outlined in Scheme 2. Initially, however, phosphonamidate **11** was synthesized in a series of model reactions that were used to determine the optimal conditions for the formation of the phosphonamidate bond and for the subsequent deprotection steps. The synthesis of this model phosphonamidate is outlined in Scheme 1. Thus, dibenzylphosphite **6** was treated with sodium hydride and iodomethane using the procedure of Portoghese[18] to afford dibenzyl(methyl)phosphonate (**7**) in 86% yield. Compound **7** was then treated with phosphorus pentachloride in dry benzene by a modification of the published procedure[18] to afford the requisite phosphonochloridate **8**. Compound **8** was found to be extremely unstable and was thus generated in situ immediately prior to reacting it with the appropriate amine, as outlined below. Prior to its use, the structure of **8** was assured by withdrawing a 0.1 mL aliquot of the reaction mixture, removing the solvent under nitrogen and immediately collecting an NMR spectrum of the residue. As a model reaction, phosphonochloridate **8** was regioselectively appended to the primary amino group of *N*,*N*-dimethyl-1,3-diaminopropane (**9**) to afford the protected phosphonamidate **10** in 72% yield. The *O*-benzyl protecting group was removed (2 N NaOH, then 10% Pd/C and H_2) to afford the target phosphonamidate **11**. Our attempts to deprotect **10** by hydrogenolysis without prior treatment with base resulted in a poor yield of **11**.[19]

Using conditions elucidated during the synthesis of the model phosphonamidate **11**, preparation of the target phosphonamidate analogue **4** was undertaken, as outlined in Scheme 2. The selectively protected intermediate **12**, previously reported by this laboratory,[4] was ethylated[4,7] (NaH, EtI) to afford compound **13**. The benzyl protecting group was then removed by hydrogenolysis[20] to yield **14**, followed by removal of the mesityl group (30% HBr)[21] to produce **15**. The secondary nitrogens were then simultaneously reprotected (Cbz-Cl)[22] to afford the bis-*N*-Cbz derivative **16**. Removal of the phthalimide (NH_2NH_2, MeOH)[23] then produced the primary amine analogue **17**. This intermediate was then coupled to phosphonochloridate **8**,[18] as described above, to afford the protected phosphonamidate **18**. The *O*-benzyl and *N*-Cbz protecting groups were sequentially removed (2 N NaOH, then 10% Pd/C and H_2) to afford the target phosphonamidate **4**. Our attempts to deprotect **18** by hydrogenolysis without prior treatment with base resulted in decomposition of the product. In addition, treatment of **18** with 2 N NaOH resulted in only partial removal of the *O*-benzyl protecting group, as determined by NMR spectroscopy, even following a two day reaction time.

The phosphinate transition state analogue **5** was synthesized by an analogous route, as shown in Scheme 3. Compound **14** (from Scheme 2) was mesitylated[24] at the free secondary nitrogen to yield **19**, and the phthalimide was removed from this intermediate (NH_2NH_2, MeOH)[23] to afford the primary amine **20**. Mesitylation[24] of the primary amine in **20** then produced the trimesitylated intermediate **21**, in which there remained a single acidic proton. Removal of this proton (NaH) followed by alkylation with 1,3-dibromobutane[4,7] then afforded the trimesitylated bromide **22**. Treatment of **22** with diethyl methylphosphonite under modified Arbuzov conditions[25] then yielded the fully protected phosphinate **23**. The mesityl and *O*-ethyl protecting groups were simultaneously removed (30% HBr)[21] to afford the target phosphinate transition state analogue **5**.

Biological Evaluation

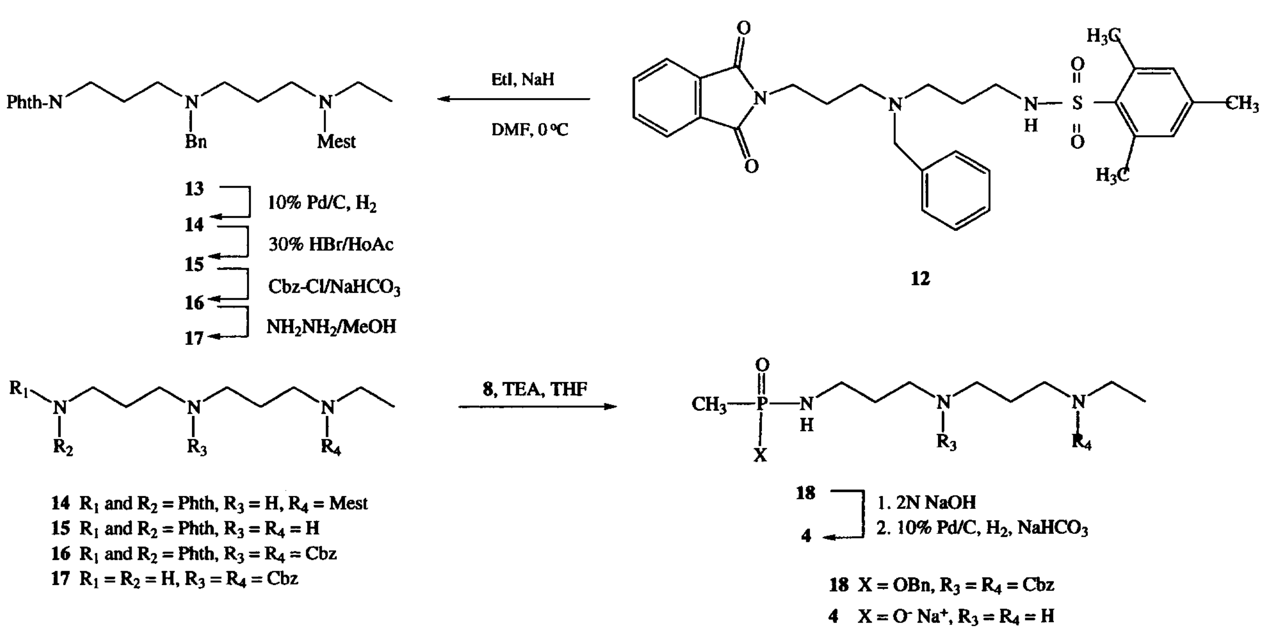
The target phosphonamidate **4** as well as its immediate precursor **18** and model phosphonamidate **11** were evaluated for their ability to inhibit isolated human SSAT in an in vitro assay system. None of these analogues appeared to act as inhibitors of the enzyme. Following the evaluation of **4**, HPLC analysis of the assay reaction mixture suggested that the phosphonamidate bond was hydrolyzed under the reaction conditions and that the hydrolysis product of **4**, 1-ethylnorspermidine, served as a substrate for the enzyme, producing 1-acetyl-7-ethyl-4-azaheptane (data not shown). These experiments are being repeated to determine whether conditions can be developed under



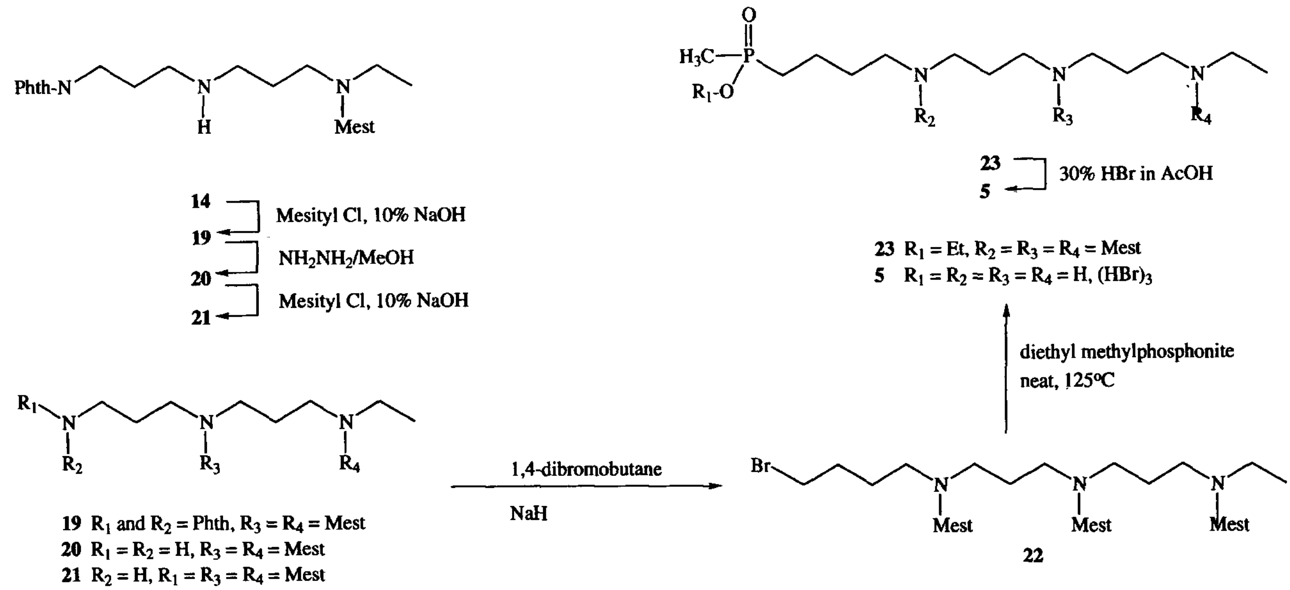
Scheme 1.

which the transition state analogue **4** will remain intact long enough to inhibit the enzyme.[19]

It is clear that the phosphonamidate linkages in **4**, **11** and **18** are extremely labile and thus structural modifications were required to produce a transition state analogue which could be used under physiological conditions. Thus, the phosphinate analogue **5**, in which the amino group present in the phosphonamidate bond had been replaced by a methylene moeity, was designed and synthesized as outlined above. In addition, **5** contains a norspermine backbone, reflecting



Scheme 2.



Scheme 3.

studies that have shown that SSAT has a greater affinity for analogues with a 3–3–3 (norspermine) or 3–4–3 (spermine) carbon–nitrogen skeleton than for 3–3 (norspermidine) or 3–4 (spermidine) analogues.[4,7] Transition-state analogue **5** proved to be an effective inhibitor of the enzyme, as shown in the Dixon plot in Figure 2, and reversibly inhibited purified human SSAT with a K_i value of 250 μM. A time-dependence experiment revealed that there was no increase in the inhibition of SSAT by phosphinate **5** with time. The ability of **5** to inhibit purified human SSAT was compared with that of the known inhibitors[5] CBENSpm (N^1-ethyl-N^{11}-[(cyclobutyl)methyl]-4,8-diazaundecane), BENSpm (**1**), CPENSpm (**2**) and CHENSpm (**3**), as shown in Figure 3. At a 50 μM concentration of the natural substrate spermidine, **5** was found to inhibit the enzyme with an IC_{50} value of 58 μM. At the same concentration of spermidine, CBENSpm, BENSpm, CPENSpm and CHENSpm inhibited SSAT with IC_{50} values of 5, 8, 7 and 13 μM, as shown in Figure 3. In this experiment **5** was evaluated following a 10 min preincubation, while the alkylpolyamine analogue data points were generated following direct addition of the inhibitor. However, related experiments have shown that nearly identical inhibition curves are produced by **5** following 0, 10 and 30 min preincubation periods. The IC_{50} for **5** was also determined with no preincubation (100 μM spermidine concentration) and was found to be 90 μM under these conditions (data not shown). As shown in Figure 3, SSAT is activated at low concentrations of the alkylpolyamine derivatives, followed by inhibition at higher concentrations. The phosphinate transition state analogue **5** did not appear to have the same stimulatory effect on the enzyme.

A series of experiments was next conducted to determine the inhibitory effects of **5** in intact NCI H157 cells. Treatment for 96 h with 10 μM **5** inhibited growth less than 20% and had no significant effect on intracellular polyamine pools in H157 cells, as shown in Table 1. It is important to note that compound **5** was not detectable by standard polyamine HPLC analysis[26] and, therefore, it was not possible to determine how much of the analogue was accumulated by H157 cells. To determine whether **5** was an effective inhibitor of the inducible enzyme SSAT in intact cells, they were treated with 10 μM **5** for 6 h, then exposed to increasing concentrations of bis(ethyl)spermine (BESpm) for an additional 24 h. Following this treatment, BESpm accumulation and SSAT activity were determined. The greatest inhibition of SSAT induction occurred at a 2 μM concentration of BESpm, as shown in Figure 4. Without compound **5**, SSAT activity was induced to 21036 pmol/mg protein/min, while in the

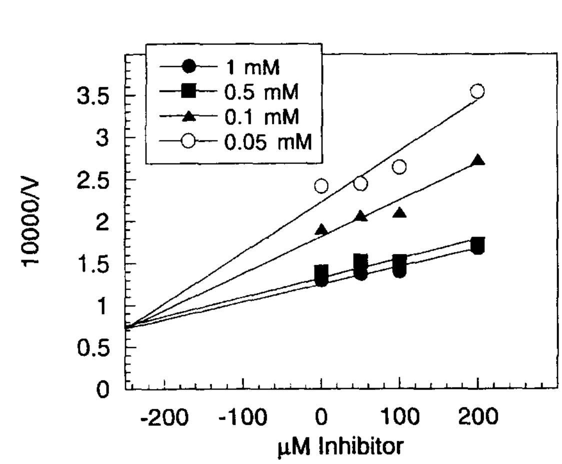


Figure 2. Dixon plot determination of K_i value for inhibition of human SSAT by the phosphinate transition-state analogue **5**. Inhibition curves were determined at 1.0, 0,1 and 0.05 mM spermidine. y-axis values are expressed as $10{,}000/V$, where V is the cpm of [^{14}C]acetylspermidine detected in the assay. Each data point is the result of two determinations that in each case differed by 5% or less.

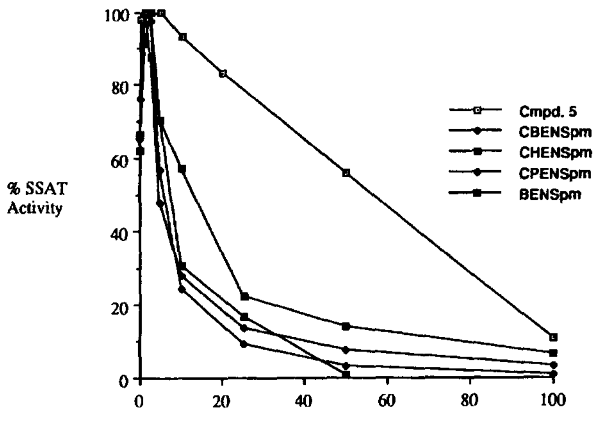


Figure 3. Inhibition of purified human SSAT by **5** and by alkylpolyamine analogues CBENSpm, CHENSpm, CPENSpm and BENSpm. All inhibition curves were generated at a 50 μM spermidine concentration. Each data point is the average of two determinations that in each case differed by 5% or less.

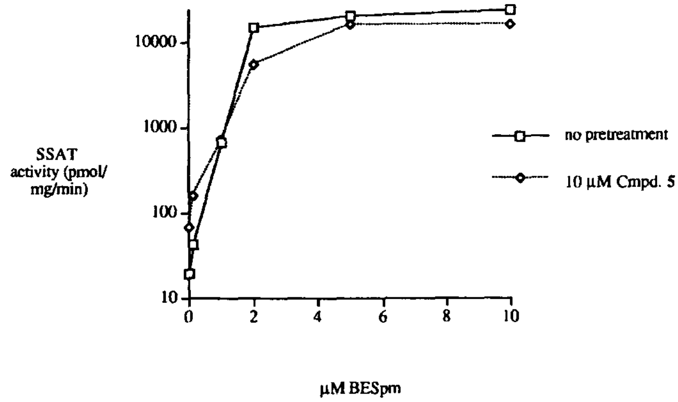


Figure 4. Induction of SSAT by BESpm in cultured NCI H157 cells in the presence and absence of 10 μM of **5**. Each data point represents the average of two determinations that in each case differed by 5% or less.

presence of **5** SSAT activity was induced to only 5496 pmol/mg protein/min. Since this effect could result from a lowered intracellular level of BESpm, compound **5** was also examined for the ability to inhibit the uptake of BESpm. BESpm import into cells is known to be mediated by the polyamine uptake system.[9] Cells which were pretreated with a 10 μM concentration of **5** took up significantly less BESpm than cells which had not been pretreated, as shown in Figure 5.

Discussion

As was stated above, polyamine analogues that inhibit but do not superinduce SSAT could be of great value in studying the metabolic effects of selective inhibition of SSAT. Although analogues such as BENSpm and CPENSpm act as reversible inhibitors of isolated SSAT, this inhibition becomes biologically insignificant in intact cells, since they induce SSAT to levels as much as 1700 times baseline. Following treatment of cultured cells with CPENSpm, the induction of SSAT is sufficient to overcome analogue-induced enzyme inhibition and polyamines are depleted by rapid acetylation and transport of the resulting excess acetylpolyamines from the cell.[3] In treated cells, the analogue becomes the major intracellular polyamine and acts to down regulate ODC and AdoMet-DC; however, the analogue cannot support the growth functions of the natural polyamines. For this reason, none of the previously described alkylpolyamines which interact with SSAT can be considered functional inhibitors for use in cellular metabolic studies. Thus, the alkylpolyamine analogue CHENSpm (**3**) and the phosphinate transition state analogue **5** represent the first examples of SSAT inhibitors which do not superinduce the enzyme in cultured H157 cells. Additional experiments are required to determine the utility of these analogues in cellular metabolic studies and these studies are now being conducted.

Table 1. Effects of **5** on NCI H157 cell growth and intracellular polyamine pools

96 h treatment	% of control growth	Polyamines (ng/mg of protein)		
		Putrescine	Spermidine	Spermine
No treatment	100	2.1	5.8	9.7
10 μM **5**	82	1.4	5.7	11.8

Cells were seeded at 2 × 106 cells per 75 cm^2 flask. Each data point represents the mean of 2 determinations which in each case differed by 5% or less.

Compound **5** did not cause any adverse growth or survival effects in cultured NCI H157 lung carcinoma cells. Thus, although it is an effective inhibitor of purified human SSAT in an in vitro assay, it appears to be an ineffective growth inhibitor in cultured tumor cells. This observation is in sharp contrast to the activity of the potent SSAT inhibitor CHENSpm (**3**), which is an effective growth inhibitor in the H157 cell line. The observed lack of growth inhibitory effects in intact cells following treatment with **5** may be the result of poor transport of the analogue. Since current methodologies do not allow the quantitation of **5** in cells, this hypothesis remains untested. Preliminary studies suggest that phosphinate **5** alone does not induce SSAT in cultured NCI H157 cells following treatment at concentrations up to 100 μM (data not shown). When cells were pre-treated with compound **5** for 6 h prior to exposure to increasing concentrations of BESpm, the greatest inhibition of SSAT (74%) was observed at a 2 μM concentration of BESpm (Fig. 4). When cells were treated with higher concentrations of BESpm, the effect was less pronounced. It is currently unclear whether the observed decrease in SSAT activity following pretreatment of H157 cells with **5** is produced by direct inhibition of the enzyme or is simply a result of diminished uptake of BESpm. However, as shown in Figure 5, the data suggests that **5** may interfere with the uptake of polyamine analogues such as BESpm. It is also possible that the reduced SSAT activity produced by **5** is a result of destabilization of SSAT, since it may compete at a putative stimulatory binding site. Alkylpolyamine analogues such as BESpm are thought to stabilize SSAT in a fashion similar to that of the natural polyamines and it is possible that **5** interferes with this effect. Again, additional experiments must be conducted to test this hypothesis.

As outlined above, although CHENSpm (**3**) and the phosphinate **5** are both effective inhibitors of isolated SSAT, they have significantly different potencies and also differ in their effects on intact cells. One fundamental difference between these two compounds is in their charges, since the phosphinate **5** would have three positive charges and one negative charge, while CHENSpm would possess four positive charges, at physiological pH. On this basis, it is plausible to assume that **5** would bind to SSAT and to the polyamine transport protein, less effectively than CHENSpm. In order to test this hypothesis, a series of analogues related to **5** is being synthesized in which the negative charge has been masked or eliminated. The synthesis and evaluation of these compounds, and of related analogues, are the subject of ongoing concern in our laboratories.

Experimental

The selectively triprotected intermediate **12** was prepared as previously described.[4] All other reagents were purchased from Aldrich (Milwaukee, Wisconsin) or Sigma and were used without further purification, except as noted below. Pyridine was dried by passing it through an aluminum oxide column and then stored over KOH. Triethylamine was distilled from potassium hydroxide and stored in a nitrogen atmosphere. Methanol was distilled from magnesium and iodine under a nitrogen atmosphere and stored over molecular sieves. Methylene chloride was distilled from phosphorus pentoxide and chloroform was distilled from calcium sulfate. Tetrahydrofuran was purified by distillation from sodium and benzophenone. Dimethyl formamide was dried by distillation from anhydrous calcium sulfate and was stored under nitrogen. Preparative scale chromatographic procedures were carried out using E. Merck silica gel 60, 230–440 mesh. Thin-layer chromatography was conducted on Merck precoated silica gel 60 F-254. Ion exchange chromatography was conducted on Dowex 1X8-200 anion exchange resin.

All ^{1}H and ^{13}C NMR spectra were recorded on a General Electric QE-300 spectrometer and all chemical shifts are reported as δ values referenced to TMS or DSS. ^{31}P NMR spectra were acquired at 121 mHz on a General Electric GN-300 spectrometer and were proton decoupled. IR spectra were recorded on a Nicolet 5DXB FT-IR spectrophotometer and are referenced to polystyrene. In all cases, ^{1}H NMR, ^{13}C NMR and IR spectra were consistent with assigned structures. Melting points were recorded on a Thomas Hoover capillary melting point apparatus and are uncorrected. Mass spectra were recorded on a Kratos MS 80 RFA (EI and CI) or Kratos MS 50 TC (FAB) mass spectrometers. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee, and were within 0.4% of calculated values.

Dibenzyl(methyl)phosphonate (7). Dibenzylphosphite **6** (20.62 mL, 0.0786 mol) in 100 mL of dry THF was added dropwise under a nitrogen atmosphere to a cooled solution (−10 to −15 °C) of sodium hydride (60% mineral oil dispersion, 4.2 g, 0.105 mol) in 60 mL dry THF with vigorous stirring over a 15 min period. The resulting solution containing the sodium salt of dibenzylphosphite was added dropwise (via a transfer needle) to a cooled solution (−10 to −20 °C) of iodomethane (8.275 g, 0.0583 mol) in dry THF (60 mL) over a 10 min period. The reaction was allowed to stir at room temperature for 16 h, the solvent was removed in vacuo and the residue was diluted with 350 mL of $CHCl_3$. The organic layer was washed with 100 mL portions each of 5% citric acid, 5% $NaHCO_3$ and H_2O

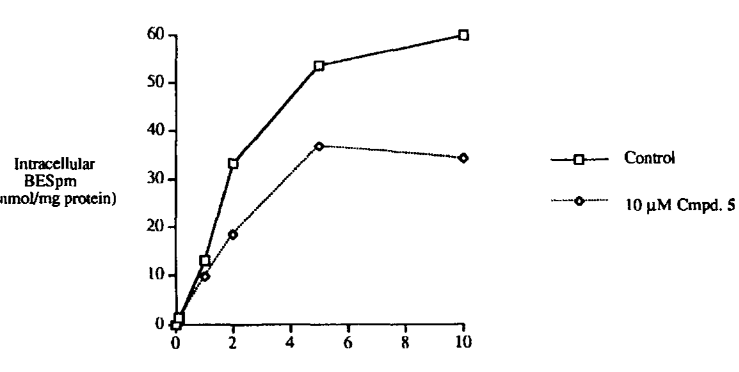


Figure 5. Intracellular levels of BESpm in cultured NCI H157 cells in the presence and absence of 10 μM of **5**. Each data point represents the average of two determinations that in each case differed by 5% or less.

(twice) and then dried over $MgSO_4$. Filtration and removal of the solvent in vacuo followed by chromatographic purification (silica gel, hexane:ethyl acetate 4:6) yielded 18.71 g of the pure product **7** as a yellow oil (86.1%). ^{1}H NMR ($CDCl_3$): δ 7.25 (s, 10H, benzyl aromatic), 4.95–5.09 (m, 4H, benzylic CH_2), 1.43–1.49 (d, $J = 17.7$ Hz, 3H, CH_3P); ^{13}C NMR ($CDCl_3$): δ 136.36, 136.28 (benzyl aromatic C-1), 128.39 (d, $J = 7.32$ Hz, benzyl aromatic C-2 and C-6), 127.87 (benzyl aromatic C-3 and C-5), 67.13, 67.03 (d, benzylic CH_2), 12.64, 10.73 (d, $J = 143.7$ Hz, PCH_3); IR cm^{-1} (neat) 1243 (P=O), 1046, 976, 920 (P-O-C).

1-{*N*-[Benzyloxy(methyl)phosphinyl]amino}-3-[(*N,N*-dimethyl)amino]propane (10). Dibenzyl(methyl)phosphonate, **7** (0.500 g, 0.0018 mol, previously dried over P_2O_5) and PCl_5 (0.410 g, 0.00197 mol) were dissolved in 9 mL of dry benzene and the reaction was allowed to reflux in an 85 °C oil bath under nitrogen for 2.5 h. While still hot, the benzene was removed under a fast stream of nitrogen to afford crude benzyl(methyl)phosphonyl chloride (**8**). The warm chloride was quickly characterized by ^{1}H NMR ($CDCl_3$): δ 7.40 (s, 5H, benzyl aromatic), 5.29–5.14 (m, 2H, benzylic CH_2), 2.03–1.98 (d, $J = 17.4$ Hz, 3H, PCH_3). Following characterization, the phosphonyl chloride **8** was redissolved in 3 mL of dry CH_2Cl_2 and the resulting solution was added dropwise via transfer needle to a round bottom flask containing **9** (0.369 g, 0.00362 mol) and dry triethylamine (0.237 g, 0.326 mL, 0.00233 mol) dissolved in 3 mL of dry CH_2Cl_2. The mixture was allowed to stir under nitrogen at room temperature for 20 min, after which the solvent was removed and the residue was dried on high vacuum to afford crude **10**. Chromatographic purification (silica gel, chloroform: methanol:NH_4OH 900:600:3) then yielded pure **10** (0.353 g, 72.5%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 7.30 (s, 5H, aromatic), 4.96 (m, 2H, benzylic CH_2), 3.79 (broad m, 1H, NH), 2.96 (m, 2H, H-1), 2.45 (t, 2H, H-3), 2.27 (s, 6H, N—CH_3), 1.66 (p, 2H, H-2), 1.49 (d, $J = 16.5$ Hz, 3H, PCH_3); ^{13}C NMR ($CDCl_3$): δ 136.9, 128.5, 128.1, 127.6 (aromatic carbons), 65.1 and 64.9 (d, $J = 21$ Hz, benzylic CH_2), 57.4 (C-1), 45.0 (N-$\underline{C}H_3$), 39.5 (C-3), 28.3 (C-2), 12.81 (d, $J = 521.7$ Hz, P-$\underline{C}H_3$); ^{31}P NMR ($CDCl_3$): δ 34.255, IR cm^{-1} (neat) 3416 (NH), 2945 (aliphatic), 1301, 1201 (P=O), 1018, 927 (P-O-C). Anal. ($C_{13}H_{23}N_2O_2P$) C, H, N.

1-{*N*-[Hydroxy(methyl)phosphinyl]amino}-3-[(*N,N*-dimethyl)amino]propane, sodium salt (11). A 0.1 g portion of **10** (0.0004 mol) in 1.5 mL of 2 N NaOH was stirred at room temperature for 24 h. The water layer was then lyophilized to give a white powder, which was redissolved in 15 mL of 50% aqueous EtOH containing 0.030 g (0.0004 mol) of $NaHCO_3$. The mixture was then added to 0.045 g of 10% Pd/C, which had been previously wetted with 1 mL of dry ethanol, and the resulting suspension was hydrogenated at room temperature and atmospheric pressure in a Parr apparatus for 6 h. The catalyst was filtered off (0.45 μ Zetapore filter), washed with methanol and the combined washings were concentrated in vacuo to give crude **11**. Chromatography on silica gel ($CHCl_3$:CH_3OH:NH_4OH, 900:600:3) then afforded pure **11** as the ammonium salt. The product was converted to the more stable sodium salt by applying it to an ion exchange column (Dowex 50X8-200, Na^+ form). Yield = 0.051 g (63.1%); ^{1}H NMR (D_2O): δ 2.78 (q, 2H, H-1), 2.35 (t, 2H, H-3), 2.17 (s, 6H, N-CH_3), 1.64 (p, 2H, H-2), 1.25 (d, $J = 15.3$ Hz, 3H, P-CH_3); ^{31}P NMR (D_2O): δ 28.37; IR cm^{-1} (KBr pellets): 3455 (NH), 2957 (aliphatic), 1456, 1303 (P=O), 1177, 1058 (P—O).

1-Phthalimido-4-(*N*-benzyl)-7-{*N*-ethyl-*N*-[(2-mesitylene)sulfonyl]}amino-4-azaheptane (13). Sodium hydride (0.0084 mol, 0.337 g of a 60% mineral oil dispersion) in 13 mL of dry DMF was added to solution of **12** (2.3 g, 0.0043 mol) in dry DMF (23 mL) at 0 °C under a nitrogen atmosphere and the solution was stirred at 0 °C for 30 min. After the evolution of hydrogen had subsided, iodoethane (1.9 g, 0.0125 mol) in 7 mL of dry DMF was added. The reaction was maintained at 0 °C for another 30 min, warmed to room temperature and stirred for an additional 45 min. The reaction was recooled to 0 °C and cold $CHCl_3$ (3 mL) and H_2O (3 mL) were added. The solvents were removed under reduced pressure and the resulting yellow powder was chromatographed on silica gel ($CHCl_3$:MeOH 95:5) to yield 1.93 g (80%) of pure **13** as white crystals. ^{1}H NMR ($CDCl_3$): δ 7.82 (m, 2H, phthalimide aromatic H-3 and H-6), 7.73 (m, 2H, phthalimide aromatic H-4 and H-5), 7.20 (m, 5H, benzyl aromatic), 6.90 (s, 2H, mesityl aromatic H-3 and H-5), 3.63 (t, 2H, H-1), 3.43 (s, 2H, benzyl), 3.22 (q, 2H, ethyl CH_2), 3.1 (t, 2H, H-7), 2.56 (s, 6H, mesityl CH_3-2 and CH_3-6), 2.38 (t, 2H, H-3), 2.29 (t, 2H, H-5), 2.25 (s, 3H, mesityl CH_3-4), 1.76 (q, 2H, H-2), 1.6 (q, 2H, H-6), 1.06 (t, 3H, ethyl CH_3); ^{13}C NMR ($CDCl_3$): δ 168.28 (phthalimide C=O), 141.76 (mesityl aromatic C-2 and C-6), 139.01 (mesityl aromatic C-3 and C-5), 139.27 (benzyl aromatic C-1), 133.9 (phthalimide aromatic C-1 and C-2), 132.05 (phthalimide aromatic C-3 and C-6), 131.83 (mesityl aromatic C-4), 128.51 (benzyl aromatic C-2 and C-6), 128.18 (benzyl aromatic C-3 and C-5), 127.21 (benzyl aromatic, C-4), 123.17 (phthalimide aromatic C-4 and C-5), 58.33 (benzylic C), 51.11 (C-3–5), 43.54 (C-7), 40.37 (C-ethyl CH_2), 36.22 (C-1), 25.9 (C-2), 25.2 (C-6), 22.75 (C-mesityl CH_3-2 and CH_3-6), 20.89 (C- mesityl CH_3-4), 13.0 (C-ethyl CH_3); IR cm^{-1} (KBr pellets): 1771, 1700 (phthalimide C=O), 1602 (aromatic), 1461, 1314, 1152 (SO_2). Anal. ($C_{32}H_{39}N_3O_4S$) C, H, N.

1-Phthalimido-7-[[*N*-ethyl-*N*-[(2-mesitylene)sulfonyl]-amino]-4-azaheptane (14). A 1.0 g portion of **13** (0.0017 mol) was dissolved in 23 mL of MeOH and added to a suspension of 10% Pd/C (0.416 g), which had been previously wetted with 2 mL of ethanol. The resulting suspension was hydrogenated at 50 psi in a Parr apparatus at room temperature for 2 days. The catalyst was filtered off (0.45 μ Zetapore filter), washed with methanol and concentrated in vacuo to afford the crude product. Chromatographic purification on silica gel (chloroform:methanol:ammonium hydroxide 900:50:3) yielded the pure product **14** (0.615 g, 60%) as a yellow oil. This product was used immediately in the subsequent reaction without further purification. ^{1}H NMR ($CDCl_3$): δ 7.82 (m, 2H, phthalimide H-3 and H-6), 7.73 (m, 2H, phthalimide H-4 and H-5), 6.93 (s, 2H, mesityl aromatic H-3 and H-5), 3.72 (t, 2H, H-1), 3.24 (m, 4H, H-7 and ethyl CH_2), 2.59 (s, 6H, mesityl 2-CH_3 and 6-CH_3), 2.54 (t, 2H, H-3), 2.48 (t, 2H, H-5), 2.28 (s, 3H, mesityl 4-CH_3), 1.79 (q, 2H, H-2), 1.65 (q, 2H, H-6), 1.07 (t, 3H, ethyl CH_3); ^{13}C NMR ($CDCl_3$): δ 168.3 (phthalimide C=O), 142.18 (mesityl aromatic C-2 and C-6), 140.08 (mesityl aromatic C-3 and C-5), 133.9 (phthalimide aromatic C-1 and C-2), 131.83 (phthalimide aromatic C-3 and C-6), 130.83 (mesityl aromatic C-4), 123.13 (phthalimide aromatic C-4 and C-5), 46.77 (C-3 and C-5), 42.92 (C-7), 40.1 (ethyl $\underline{C}H_2$), 35.81 (C-1), 28.72 (C-2), 27.75 (C-6), 22.73 (mesityl 2-$\underline{C}H_3$ and 6-$\underline{C}H_3$), 20.86 (mesityl 4-$\underline{C}H_3$), 12.81 (ethyl $\underline{C}H_3$); IR cm^{-1} (neat) 2939 (NH), 1771, 1714 (phthalimide C=O), 1397, 1314, 1146 (SO_2).

1-Phthalimido-7-[*N*-(ethyl)amino]-4-azaheptane dihydrobromide (15). A 4.5 g portion of phenol (0.0479 mol) was dissolved in 45 mL of 30% HBr/HOAc in a stoppered flask and to this mixture a solution of **14** (0.7 g, 0.001578 mol) in 15 mL of ethyl acetate was added in three portions over a period of 3 h. After addition was complete, the reaction mixture was stirred for an additional 18 h at room temperature, then cooled to 0 °C and diluted with 45 mL of water. The aqueous phase was washed with three 60 mL portions of ethyl acetate before being lyophilized to give crude **15** as a dark yellow solid (0.659 g, 92% yield). This product was used immediately in the subsequent reaction without further purification. ^{1}H NMR (D_2O): δ 7.82 (m, 2H, phthalimide H-3 and H-6), 7.8 (m, 2H, phthalimide H-4 and H-2), 3.72 (t, 2H, H-1), 3.06 (m, 8H, H-3, H-5, H-7, and ethyl CH_2), 2.03 (q, 4H, H-2, and H-6), 1.21 (t, 3H, ethyl CH_3).

1-Phthalimido-4-*N*-(carbobenzyloxy)-7-[*N*-ethyl-*N*-(carbobenzyloxy)amino]-4-azaheptane (16). A 0.659 g portion of **15** (0.0015 mol) was added to a solution of 0.723 g (0.0086 mol) of $NaHCO_3$ in 40 mL of H_2O and to this solution was added 0.615 g (0.01 mol) of NaCl with stirring. The mixture was cooled to 0 °C and 0.483 g (0.0028 mol) of benzylchloroformate in 66 mL of $CHCl_3$ was added. The reaction was heated to reflux for 3 h, then cooled to room temperature. The $CHCl_3$ layer was then separated, dried over $MgSO_4$, filtered and the solvent was removed in vacuo. The resulting crude product was chromatographed on silica gel (chloroform:methanol 95:5) to yield the pure product as yellow oil (0.489 g, 60%). ^{1}H NMR ($CDCl_3$): δ 7.82 (m, 2H, phthalimide H-3 and H-6), 7.73 (m, 2H, phthalimide H-4 and H-5), 7.32 (s, 10H, carbobenzyloxy aromatic), 5.08 (s, 4H, benzylic CH_2), 3.66 (broad m, 2H, H-1), 3.24 (broad m, 8H, H-3, H-5, H-7 and ethyl CH_2), 1.9 (broad m, 2H, H-2), 1.77 (broad m, 2H, H-6), 1.65 (q, 2H, H-6), 1.07 (broad t, 3H, ethyl CH_3); ^{13}C NMR ($CDCl_3$): δ 168.3 (phthalimide C=O), 155.93 (carbobenzyloxy C=O), 136.65 (d, carbobenzyloxy aromatic C-1), 133.91 (phthalimide aromatic C-1 and C-2), 132.05 (phthalimide aromatic C-3 and C-6), 128.43 (carbobenzyloxy aromatic C-2, C-3, C-5 and C-6), 127.86 (carbobenzyloxy aromatic C-4), 123.23 (phthalimide aromatic C-4 and C-5), 67.07 and 66.85 (benzylic CH_2), 44.44 (C-3, C-5 and C-7), 41.57 (ethyl $\underline{C}H_2$), 35.68 (C-1), 27.67 (C-2 and C-6), 13.78 (ethyl $\underline{C}H_3$); IR cm^{-1} (neat): 1771, 1714 (phthalimide C=O). Anal. ($C_{32}H_{35}N_3O_6$) C, H, N.

1-Amino-4-*N*-(carbobenzyloxy)-7-[*N*-ethyl-*N*-(carbobenzyloxy)amino]-4-azaheptane (17). A 0.033 mL portion of dry hydrazine (0.034 g, 0.0011 mol) was added via syringe to a dry methanol solution (7 mL) containing 0.489 g (0.00088 mol) of **16** under a nitrogen atmosphere. The solution was heated to 50 °C for 18 h, then concentrated, redissolved in CH_2Cl_2 (12 mL), filtered and the solvent removed in vacuo to give crude **17** (0.374 g, 100%) as a yellow oil. This product was used immediately without further purification in the subsequent step. ^{1}H NMR ($CDCl_3$): δ 7.32 (s, 10H, carbobenzyloxy aromatic), 5.11 (s, 4H, carbobenzylic CH_2), 3.24 (broad m, 8H, H-3, H-5, H-7 and ethyl CH_2), 2.64 (broad m, 2H, H-1), 1.77 (broad m, 2H, H-6), 1.65 (q, 2H, H-2), 1.07 (t, 3H, ethyl CH_3); ^{13}C NMR ($CDCl_3$): δ 155.93 (carbobenzyloxy C=O), 136.65 (carbobenzyloxy aromatic C-1), 128.43 (carbobenzyloxy aromatic C-2, C-3, C-5 and C-6), 127.86 (carbobenzyloxy aromatic C-4), 67.07–66.85 (benzylic CH_2), 44.44 (C-3, C-5 and C-7), 41.57 (ethyl $\underline{C}H_2$), 39.7 (C-1), 30.7 (C-2), 27.67 (C-6), 13.78 (ethyl $\underline{C}H_3$); IR cm^{-1} (neat): 3374 (NH_2), 1693 (NCOO).

1-{*N*-[Benzyloxy(methyl)phosphinyl]amino}-4-*N*-(carbobenzyloxy)-7-[*N*-ethyl-*N*-(carbobenzyloxy)amino]-4-azaheptane (18). A 0.250 g (0.00058 mol) portion of **17** was converted to the protected phosphonamidate **18** exactly as described for the synthesis of compound **10** above. Chromatographic purification (silica gel, chloroform:methanol 95:5) yielded pure **18** (0.200 g, 57%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 7.32 (s, 10H, carbobenzyloxy aromatic), 7.25 (s, 5H, benzyl aromatic), 5.11 (s, 4H, benzylic CH_2), 4.96 (broad m, 2H, benzylic CH_2), 3.21 (broad m, 8H, H-3, H-5, H-7 and ethyl CH_2), 2.84 (broad m, 2H, H-1), 1.74 (broad m, 2H, H-6), 1.61 (q, 2H, H-2), 1.49–1.43 (d, $J = 17.7$ Hz, 3H, PCH_3), 1.05 (broad, 3H, ethyl CH_3); ^{13}C NMR ($CDCl_3$): δ 155.93 (carbobenzyloxy C=O), 136.79 (carbobenzyloxy aromatic C-1), 136.48 (benzyl aromatic C-1), 128.37 (carbobenzyloxy aromatic C-2, C-3, C-5 and C-6), 128.01 (benzyl aromatic C-2 and C-6), 127.87 (benzyl aromatic C-3 and C-5), 127.86 (carbobenzyloxy aromatic C-4), 67.07 and 66.85 (carbobenzylic CH_2), 65.03, 64.97 (d, $J = 4.6$ Hz, benzylic CH_2), 44.54–43.97 (C-3, C-5 and C-7), 41.77 (ethyl $\underline{C}H_2$), 37.3 (C-1), 30.26 (C-2), 27.13 (C-6), 13.22 (ethyl $\underline{C}H_3$), 13.96, 12.22 (d, $J = 131.7$ Hz, PCH_3); ^{31}P NMR ($CDCl_3$): δ 33.997; IR cm^{-1} (neat): 1696 (NCOO), 1273, 1203 (P=O), 1118, 1006, 914 (P—O—C). Anal. ($C_{32}H_{42}N_3O_6P$) C, H, N.

1-{*N*-[Hydroxy(methyl)phosphinyl]amino}-7-(*N*-ethyl)-amino-4-azaheptane, sodium salt (4). A 0.060 g portion of **18** (0.0001 mol) in 0.6 mL of 2 N NaOH was stirred at room temperature for 1 day. The water layer was then lyophilized to give a white powder, which was redissolved in 15 mL of 50% aqueous EtOH containing 0.018 g of $NaHCO_3$. The mixture was added to 10% Pd/C (0.045 g), which had been previously wetted with 1 mL of dry ethanol and the resulting suspension was hydrogenated at atmospheric pressure in a Parr apparatus at room temperature for 6 h. The catalyst was filtered off (0.45 μ Zetapore filter), washed with methanol and the combined organic layers were concentrated in vacuo to give reasonably pure **4** (0.100 g). Attempts to purify **4** by column chromatography on silica gel resulted in decomposition of the product. ^{1}H NMR (D_2O): δ 3.21 (q, 2H, H-1), 2.95 (m, 6H, H-3, H-5, H-7), 2.84 (q, 2H, ethyl CH_2), 1.69 (m, 4H, H-2, and H-6), 1.32–1.27 (d, J = 15.3 Hz, 3H, P—CH_3), 1.08 (t, 3H, ethyl CH_3); ^{31}P NMR (D_2O): δ 28.42.

1-Phthalimido-4-[*N*-(2-mesitylene)sulfonyl]-7-{*N*-ethyl-*N*-[(2-mesitylene)sulfonyl]}amino-4-azaheptane (19). A 1.00 g portion of **14** (0.0021 mol) was added to a stirred mixture of 5 mL of dry dichloromethane and 5 mL of 10% NaOH and the mixture was cooled to 0 °C in an ice bath. A 0.605 g portion of 2-mesitylenesulfonyl chloride (0.0028 mol) in 10 mL of dry dichloromethane was then introduced by dropwise addition and the reaction was allowed to stir for 1 h at 0 °C. After 1 h, the mixture was warmed to room temperature stirred for an additional 2 h. The reaction was diluted with 20 mL of H_2O and 50 mL of $CHCl_3$ and the layers were separated. The aqueous phase was further extracted with 25 mL $CHCl_3$ and the combined organic layers were dried ($MgSO_4$) and evaporated to give 1.985 g of crude product. Purification on silica gel (hexane:ethyl acetate 3:2) then afforded pure **19** (1.091 g, 79% yield) as white foam. ^{1}H NMR ($CDCl_3$): δ 7.85 (m, 2H, phthalimido H-3 and H-6), 7.75 (m, 2H, phthalimido H-4 and H-5), 6.92 (s, 2H, mesityl aromatic H-3 and H-5), 6.79 (s, 2H, mesityl aromatic H-3 and H-5), 3.47 (t, 2H, H-1), 3.08–3.19 (m, 8H, H-3, H-5, H-7, ethyl CH_2), 2.56 (s, 6H, mesityl 2-CH_3 and 6-CH_3), 2.49 (s, 6H, mesityl 2-CH_3 and 6-CH_3), 2.29 (s, 3H, mesityl 4-CH_3), 2.23 (s, 3H, mesityl 4-CH_3), 1.71 (m, 4H, H-2 and H-6), 1.00 (t, 3H, ethyl CH_3); ^{13}C NMR ($CDCl_3$): δ 168.0 (phthalimido C=O), 142.3 (mesityl aromatic C-1), 140.0 (phthalimido aromatic C-1 and C-2), 135.1 (mesityl aromatic C-4), 133.0 (mesityl aromatic C-2 and C-6), 130.9 (phthalimido aromatic C-3 and C-6), 124.3 (mesityl aromatic C-3 and C-5), 122.1 (phthalimido aromatic C-4 and C-5), 43.4, 42.6 (C-1, C-3, C-5, C-7), 40.2 (ethyl CH_2), 25.6 (C-2), 23.7 (C-6), 21.9 (mesityl 2-CH_3 and 6-CH_3), 11.9 (ethyl CH_3). Anal. ($C_{34}H_{43}N_3O_6S_2$) C, H, N.

1-Amino-4-[*N*-(2-mesitylene)sulfonyl]-7-{*N*-ethyl-*N*-[(2-mesitylene)sulfonyl]}amino-4-azaheptane (20). A solution of **19** (1.00 g, 0.0015 mol) and 0.130 g (0.0041 mol) of hydrazine in 15 mL of dry methanol was stirred at 50 °C for 17 h under a nitrogen atmosphere. The solvent was removed and the residue was taken up in 100 mL of $CHCl_3$. The solution was filtered to remove the phthalhydrazide by-product, and the solvent was removed in vacuo to afford 0.838 g (100%) of the crude amine **20**. This product was used without further purification in the subsequent step. ^{1}H NMR ($CDCl_3$): δ 6.93 (s, 4H, mesityl aromatic H-3 and H-5), 3.01–3.25 (m, 10H, H-1, H-3, H-5, H-7, ethyl CH_2), 2.56 (s, 12H, mesityl 2-CH_3 and 6-CH_3), 2.30 (s, 6H, mesityl 4-CH_3), 1.71 (m, 2H, H-6), 1.56 (m, 2H, H-2), 0.98 (t, 3H, ethyl CH_3).

1-[*N*-(2-mesitylene)sulfonyl]amino-4-[*N*-(2-mesitylene)-sulfonyl]-7-{*N*-ethyl-*N*-[(2-mesitylene)sulfonyl]}amino-4-azaheptane (21). A 0.771 g portion of **20** (0.0015 mol) was mesitylated exactly as described for the synthesis of compound **19** above. The crude product was chromatographed on silica gel (hexane:ethyl acetate 3:2) to yield 0.797 g of **21** (77% yield) as a white foam. ^{1}H NMR ($CDCl_3$): δ 6.93 (s, 6H, mesityl aromatic H-3 and H-5), 3.23 (t, 2H, H-3), 3.00–3.12 (m, 8H, H-1, H-5, H-7, ethyl CH_2), 2.60 (s, 6H, mesityl 2-CH_3 and 6-CH_3), 2.53 (s, 12H, mesityl 2-CH_3 and 6-CH_3), 2.30 (s, 9H, mesityl 4-CH_3), 1.65 (m, 4H, H-2 and H-6), 0.91 (t, 3H, ethyl CH_3). Anal. ($C_{35}H_{51}N_3O_6S_3$) C, H, N.

1-Bromo-5,9-bis[*N*-(2-mesitylene)sulfonyl]-12-{*N*-ethyl-*N*-[(2-mesitylene)sulfonyl]}amino-5,9-diazadodecane (22). To a cooled (0 °C), stirring solution of **21** (0.310 g, 0.00044 mol) in 1 mL of dry DMF was added a 0.167 g portion of sodium hydride (60% mineral oil dispersion, 0.0044 mol) in a nitrogen atmosphere. After 30 min, 0.928 g (0.0043 mol) of 1,4-dibromobutane in 2 mL of DMF was added with rapid stirring and the reaction was maintained at room temperature for 20 h. The solvent was removed in vacuo (0.1 mm Hg) to afford crude **22**, which was purified on silica gel (hexane:ethyl acetate 3:2) to yield 0.301 g of **22** (82% yield) as a yellow semi-solid. ^{1}H NMR ($CDCl_3$): δ 6.93 (s, 6H, mesityl aromatic H-3 and H-5), 3.26 (t, 2H, H-1), 2.95–3.10 (m, 12H, H-4, H-6, H-8, H-10, H-12, ethyl CH_2), 2.55 (s, 18H, mesityl 2-CH_3 and 6-CH_3), 2.30 (s, 9H, mesityl 4-CH_3), 1.60–1.70 (m, 8H, H-2, H-3, H-7 and H-11), 0.95 (t, 3H, ethyl CH_3). Anal. ($C_{39}H_{58}N_3O_6S_3Br$) C, H, N.

1-(Ethoxy(methyl)phosphinyl)-5,9-bis[*N*-(2-mesitylene)-sulfonyl]-12-{*N*-ethyl-*N*-[(2-mesitylene)sulfonyl]}amino-5,9-diazadodecane (23). A mixture of **22** (0.248 g, 0.0003 mol) and 1.310 g of diethyl methylphosphonite (0.0096 mol) was stirred at 125 °C for 3 h under a nitrogen atmosphere. The volatile products and reactants were removed in vacuo and the residue was purified by silica gel chromatography (hexane:ethyl acetate:methanol 5:5:1) to yield 0.240 g of **23** (94% yield). ^{1}H NMR ($CDCl_3$): δ 6.93 (s, 6H, mesityl aromatic H-3 and H-5), 4.11 (m, 2H, ethoxy CH_2), 2.95–3.10 (m, 12H, H-4, H-6, H-8, H-10, H-12, ethyl

CH_2), 2.54 (s, 18H, mesityl 2-CH_3 and 6-CH_3), 2.30 (s, 9H, mesityl 4-CH_3), 1.65 (m, 8H, H-2, H-3, H-7 and H-11), 1.36–1.51 (m, 5H, H-1 and methyl CH_3), 0.93 (t, 3H, ethyl CH_3); IR cm^{-1} (neat): 2987 (aliphatic), 1273, 1203 (P=O), 1148, 1038, 908 (P—O—C). Anal. ($C_{42}H_{66}N_3O_8S_3P$), C, H, N.

1-(Hydroxy(methyl)phosphinyl)-12-(*N*-ethyl)amino-5,9-diazadodecane trihydrobromide (5) and trihydrochloride (5a). A 0.100 g portion of **23** (0.00012 mol) in 1 mL of ethyl acetate was added to a solution of 1.021 g of phenol (0.01065 mol) in 10 mL of 30% HBr in acetic acid and the mixture was heated to reflux. After 0.5 h, the mixture was concentrated in vacuo and 30 mL of water and 20 mL of ethyl acetate were added. The aqueous layer was collected and washed with two additional 20 mL portions of ethyl acetate. Removal of the water in vacuo afforded the crude product, which was crystallized from aqueous ethanol to afford 0.044 g of **5** (71% yield). For analysis, the trihydrochloride salt **5a** was obtained by ion exchange chromatography on Dowex 1X8–200 as a white solid. ^{1}H NMR (D_2O): δ 3.04–3.14 (m, 12H, H-4, H-6, H-8, H-10, H-12, ethyl CH_2), 2.04 (m, 4H, H-7 and H-11), 1.72 (m, 2H, H-3), 1.52 (m, 4H, H-1 and H-2), 1.25 (t, 3H, ethyl CH_3), 1.18 (d, 3H, methyl CH_3); ^{31}P NMR (D_2O): δ 43.75. Anal. ($C_{13}H_{35}N_3O_2PCl_3$) C, H, N.

Enzyme studies

Recombinant human SSAT was expressed in *Escherichia coli* using the plasmid pINSAT2 and purified to homogeneity as previously described.[27] Assays were carried out in a total volume of 0.1 mL of 50 mM Tris-HCl, pH 7.8, 2.5 mM dithiothreitol and 0.1 mM EDTA containing 3 ng of SSAT, 50 μg of bovine serum albumin, 15 μM of [^{14}C]acetyl CoA (53 μCi/μmol) and various concentrations of spermidine and the potential inhibitors. After incubation at 30 °C for 10 min, the reaction was stopped by the addition of 0.02 mL of 1.0 M hydroxylamine and the formation of [^{14}C]acetylspermidine was determined using cellulose phosphate disks as previously described.[28]

Cell culture studies

The NCI H157 nonsmall cell lung carcinoma line was maintained in culture as previously described.[29] This line was re-fed with fresh medium every 3 days to maintain log phase growth. Cells were exposed to the polyamine analogue N^1,N^{12}-bis(ethyl)spermine and the potential SSAT inhibitor as indicated in the Biological Evaluation section. Effects on growth, polyamine pools and SSAT activity were then determined as previously published.[5] Intracellular polyamine concentrations were measured by the precolumn dansylation, reversed-phase HPLC method of Kabra et al.[26] using perchloric acid cell extracts.[11] The SSAT activity of these cell lysates was determined by a previously published method.[11]

Acknowledgements

This work was supported by NIH Grants RO1 CA63552 (PMW), RO1 GM26290 (AEP) and RO1 CA51085 (RAC). The excellent technical assistance of Ms Emelina Franco is greatly appreciated.

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(*Received in U.S.A.* 4 *December* 1995; *accepted* 23 *February* 1996)