N-Phosphoryl Derivatives of Bisantrene. Antitumor Prodrugs with Enhanced Solubility and Reduced Potential for Toxicity

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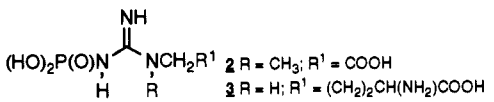
The selective phosphorylation of bisantrene (1) affords bis(phosphonoguanidinic acid) 6, a prodrug with enhanced aqueous solubility (as sodium salt 7) at physiological pH. Unlike 1, in a rat tail vein model, no precipitation was observed when bis(phosphonoguanidinic acid) 6 was injected. While in rats 6 hydrolyzed to monophosphonoguanidinic acid 9 with a half-life of *ca.* 12 min., complete hydrolysis to bisantrene required several hours. The corresponding monophosphonoguanidinic acid 9 was synthesized from bisantrene and also showed good solubility and antitumor activity. While the antitumor activities of 6 in mice were comparable to bisantrene against B-16 melanoma and P-388 and L-1210 leukemias, it was inactive *in vitro vs* several tumor cell types. Thus, its activity *in vivo* resulted from its ability to serve as a prodrug for bisantrene.

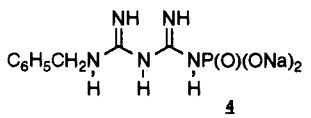
Introduction

Bisantrene (1)[1-3] is a synthetic antitumor agent that is clinically active against breast cancer,[4] leukemias,[5] and lymphomas.[6] While its mode of action is attributed to intercalation into DNA[7] and subsequent DNA cleavage,[8] it differs from adriamycin, mitoxantrone, and some other antitumor DNA intercalators in that it is essentially not cardiotoxic.[9] The drug, as the soluble dihydrochloride, is typically administered slowly into a central vein *via* a long-line catheter to minimize complications, *e.g.*, phlebitis, due to precipitation of either its salts or the free base at physiological pH.[10] Alternative formulations, *e.g.*, lactate salt[11] or emulsions,[12] were equally problematic. Thus, a search was made for a more easily administered, but slowly hydrolyzed, prodrug of bisantrene. During the course of these studies, the bis(phosphonoguanidinic acid) 6 was identified as a candidate for further antitumor evaluation.

Few examples of phosphonoguanidinic acid prodrug entities have been cited. Similarly, there are few citations on either the syntheses[13] and stability[14] of phosphonoguanidinic acids. The most notable are phosphocreatine (2) (creatine phosphate) and N^4-phosphoarginine (3) (arginine phosphate), which are mediators of energy storage in muscle,[15] and benfosformin (4),[16] an experimental prodrug for phenformin.



2 R = CH_3; R^1 = COOH
3 R = H; R^1 = $(CH_2)_2CH(NH_2)COOH$



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Chemistry

The chemical problems posed in the development of phosphorylated prodrugs of bisantrene include (a) the synthesis of suitably protected *N,N'*-bis(phosphoryl)-bisantrene derivatives and (b) their deprotection. With typical acylation, phosphorylation, or sulfonylation conditions employing tertiary amines, amidine bases, or aqueous bases, complex mixtures containing much unreacted bisantrene (1) (as free base or salt) were obtained. However in the presence of *N,O*-bis(trimethylsilyl)acetamide as a hydrogen chloride scavenger,[17] diphosphorylation of bisantrene with diethyl chlorophosphate or diphenyl chlorophosphate gave bis(diethoxyphosphoryl)-bisantrene (**5a**) (32%) and bis(diphenoxyphosphoryl)-bisantrene (**5b**) (54%), respectively. Subsequently, a convenient synthesis of **5a** was developed using diethyl cyanophosphonate (98%). Phosphorylation on the imidazoline nitrogen atoms of 5 rather than on the acyclic nitrogen atoms was evidenced by the nonequivalence of the chemical shifts in both the CMR and ^{1}H NMR signals for the $-NCH_2CH_2N-$ moieties and by comparison with bisantrene derivatives of known structure.[1,17]

The tetraethyl ester **5a** was chosen to allow the use of trimethylsilyl iodide (or bromide) for deblocking[18] to give bis(phosphonoguanidinic acid) 6 (Scheme I). This protection scheme avoided the more stringent conditions required for hydrogenolyses, aqueous hydrolyses with acid or base, or reduction with zinc that are typically used for removing other frequently used phosphate-blocking groups.[19] The anticipated problems with these other deblocking methods include poisoning of hydrogenation catalysts by bisantrene,[20] lability of the phosphorus–nitrogen bond to hydrolysis,[21] and reduction of the anthracene ring at the 9- and 10-positions by zinc.

Fortuitously, in one phosphorylation reaction with partially hydrated bisantrene, a separable mixture of bisphosphoryl product **5a** and monophosphoryl product 8 was obtained. Deblocking of 8 with trimethylsilyl iodide–triphenylphosphine afforded monophosphonoguanidinic acid 9, which also had good aqueous solubility at pH 7.4. In the absence of triphenylphosphine to scavenge iodoethane, the deblocked product 9, as the intermediate silyl ester, undergoes N-ethylation on the more basic substituent to give 10.

Bis(phosphonoguanidinic acid) 6 was also converted to the disodium salt 7 which in aqueous solution had a pH of 7.4, so *in vivo* the dianion is the predominant form. Thus, *in vivo* the polarity of 7 is opposite from that of 1, *i.e.*, anionic rather than cationic. It is interesting to note

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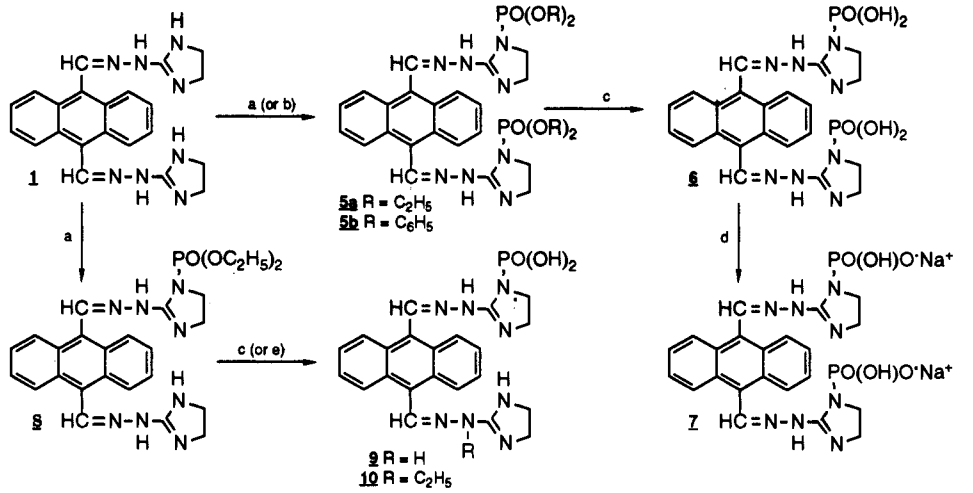
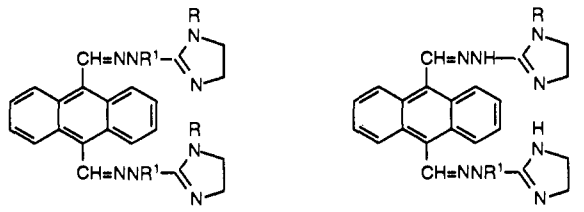
Scheme I[a]



[a] Key: (a) $(RO)_2POCl$, BTSA; (b) $(C_2H_5O)_2POCN$; (c) $(CH_3)_3SiI$, H_2O; (d) aqueous NaOH; (e) $(CH_3)_3SiCl$, $(C_6H_5)_3P$.

Table I. Antitumor Evaluation of *N*-Phosphorylbisantrene Derivatives



1, 5-6 (symmetrical series) 8-10 (unsymmetrical series)

			median % increase in life span (optimum dose, mg/kg)			
			P-388		B-16	
no.	R	R^1	leukemia[a]	"Cures"[b]	melanoma[c]	"Cures"[d]
1	H	H	137 (12.5)[e]	17/42	122 (6)[f]	2/10
5a	$PO(OC_2H_5)_2$	H	20 (100)		NT	
5b	$PO(OC_6H_5)_2$	H	60 (200)		NT	
6	$PO(OH)_2$	H	265 (25)		163 (25)[f]	
8	$PO(OC_2H_5)_2$	H	>164 (25)	3/6	>164 (25)	3/6
9	$PO(OH)_2$	H	345 (25)	4/6	120 (12.5)	3/12
10	$PO(OH)_2$	C_2H_5	132 (50)	2/6	114 (100)	3/12

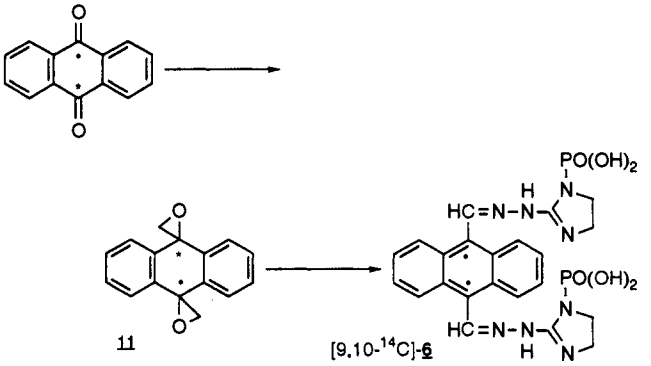
[a] BDF_1 or CDF_1 mice were injected ip with 10^6 ascitic leukemia cells and dosed ip on days 1, 5, and 9. [b] "Cures" = number of survivors/total at 30 days. When the cures were 50% or greater, the experiment was continued until a specific ILS could be calculated. [c] BDF_1 mice were implanted ip with a homogenate from 0.05 g of tumor and dosed ip on days 1–9. [d] "Cures" = number of survivors/total at 60 days. [e] Data are for 1·2HCl. [f] Against L-1210 leukemia the corresponding values were 100(25) *vs* concurrent 47(25) for bisantrene. NT = not tested.

the contrasting bulk stability profiles of these products. While bulk **6** was stable for at least 5 years at *ca.* 24 °C, both the disodium salt **7** and monophosphonoguanidinic acid **9** decomposed within 6 months. In contrast, the *N*-ethylmonophosphonoguanidinic acid **10** was stable for at least 1 year.

Biological Results

Bisantrene dihydrochloride **1** and its bis(phosphonoguanidinic acids) and monophosphonoguanidinic acids (**6** and **9**) had comparable antitumor activities in mice against P-388 leukemia and B-16 melanoma (Table I). Additionally, against L-1210 leukemia, **6** gave a maximum increase in life span of 100% at 25 mg/kg *vs* a concurrent 47% at 25 mg/kg for **1**. No precipitation was detected when [9,10-^{14}C]-**6** was injected into the tail vein of rats, and initial hydrolysis to [9,10-^{14}C]-**9** occurred with a half-life of 12 min. Further hydrolysis to bisantrene required several hours, as shown by HPLC assays of plasma samples. Surprisingly, these hydrolyses were not observed *in vitro* in plasma or whole blood. It is therefore likely that the hydrolyses were not only enzymatic, but that they proceeded in some cellular compartment(s). In contrast, derivatives **5a–b** were weakly active (Table I) *via* intraperitoneal injection.

Scheme II



The [9,10-^{14}C]bisantrene **1** was prepared *via* condensation of [9,10-^{14}C]anthracene-9,10-dione with dimethylsulfonium methylide to give the corresponding dioxirane **11**,[22] and subsequent transformations to [9,10-^{14}C]-**6** followed previously reported procedures (Scheme II).[1]

Conclusion

Bis(phosphonoguanidinic acid) **6** could be attractive as a second generation prodrug successor to bisantrene. It appears to have a greatly reduced potential for phlebitis, but its gradual hydrolysis to bisantrene could also reduce other toxicities. While preliminary animal test data suggest enhanced antitumor effectiveness, further evaluation might also show a broader spectrum of antitumor efficacies due to the reverse polarity and altered pharmacodynamics of this prodrug.

Experimental Section

All infrared (IR) (KBr) and ultraviolet (UV) spectral determinations were obtained on Nicolet Model 7199-FT or Hewlett-Packard 8450-A spectrophotometers, respectively. ^{1}H NMR spectra were obtained with Varian FT-80 or Nicolet NT-300 WB spectrometers. Mass spectra were obtained on a Finnigan MAT CH-7 spectrometer. Except for **9** and **10**, all compounds gave satisfactory elemental analyses. Antitumor testing followed the protocols of the National Cancer Institute.[23]

9,10-Anthracenedicarboxaldehyde Bis(2-imidazolin-2-ylhydrazone) (Bisantrene Base, 1). A solution of 60.0 g of 9,10-anthracenedicarboxaldehyde bis(2-imidazolin-2-ylhydrazone) dihydrochloride·2.5H_2O in 1400 mL of water was treated with a solution of sodium carbonate in 400 mL of water, with vigorous stirring. To facilitate filtration, the resulting fine suspension was allowed to stand for 5 h. The solid was then collected and washed with 0.003 M aqueous ammonia until the washings were chloride free to afford free base 1 (47.2 g) as a light orange solid. Anal. ($C_{22}H_{22}N_8$) C, H, N.

[9,10-Anthracenediylbis[methylidynehydrazo(4,5-dihydro-1*H*-imidazole-2,1-diyl)]]bis[phosphonic acid] Tetraethyl Ester (5a). Method A. To a stirred suspension of 7.97 g (20 mmol) of 1 in 400 mL of anhydrous methylene chloride, under argon, was added consecutively 8.14 g (40 mmol) of *N,O*-bis(trimethylsilyl)acetamide and 6.90 g (40 mmol) of diethyl chlorophosphate. After stirring for *ca.* 3 h, the solution was chromatographed through a 3.8-cm-wide column containing 200 g of neutral alumina (methylene chloride). The combined colored eluate was concentrated to 40 mL, and then 100 mL of toluene was gradually added to the boiling mixture, with swirling, as a crystalline solid separated and the volume boiled down to *ca.* 100 mL. The solid was washed consecutively with toluene and then methanol to afford 4.36 g (32%) of orange needles: mp 214–215 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.47 (t, 12H, CH_3), 3.57 (t, 4H, $PNCH_2CH_2N$), 3.98 (t, 4H, $PNCH_2CH_2N$), 4.37 (m, 8H, OCH_2), 5.90 (s, 2H, NH), 7.48 (m, 4H, arom), 8.55 (m, 4H, arom), 9.38 (s, 2H, CH=N); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_c 16.233 (d, $^3J_{CP}$ = 7.2 Hz, OCH_2CH_3), 41.403 (d, $^3J_{CP}$ = 7.5 Hz, C=NCH_2), 46.819 (d, $^2J_{CP}$ = 3.4 Hz, PONCH_2), 64.069 (d, $^2J_{CP}$ = 6.5 Hz, OCH_2CH_3), 125.726 (s, aromatic CH's), 125.967 (s, aromatic CH's), 129.495 (s, C-9 and C-10), 129.849 (s), 150.816 (s, HC=NNH) and 160.316 (d, $^2J_{CP}$ = 8.9 Hz, –NHC=N); FABMS (thioglycerol) *m/z* 671 (M + H). Anal. ($C_{30}H_{40}N_8O_6P_2$) C, H, N.

Method B. A mixture of 398 mg (1 mmol) of 1, 359 mg (2.2 mmol) of diethyl cyanophosphonate, and 5 mL of DMF was stirred for 5 h under argon (**Caution**: Hydrogen cyanide is formed). The resulting thick mixture was diluted with 15 mL of dry ether, and the solid was collected and washed with ether to give 655 mg of orange crystals, mp 215–216 °C, with identical TLC characteristics [silica gel, chloroform–methanol (8/1) (v/v)] to that of the product from method A, *i.e.*, a very strong spot at R_f 0.8 and a barely detectable spot (under UV light) at R_f 0.5. The latter spot may result from adventitious methanolysis of **5a** by the TLC system. (Note: After standing at 25 °C for several hours, a solution of **5a** in chloroform–methanol (3/1) (v/v), by TLC, showed three major spots and four minor spots, including a spot at R_f 0.5.)

[9,10-Anthracenediylbis[methylidynehydrazo(4,5-dihydro-1*H*-imidazole-2,1-diyl)]]bis[phosphonic acid] Tetraphenyl Ester (5b). This was prepared as in **5a** (method A) in 52% yield: mp 214–215 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.41 (t, 4H, $PNCH_2CH_2N$), 3.91 (t, 4H, $PNCH_2CH_2N$), 5.86 (s, 2H, NH), 7.15–7.60 (m, 20H, arom), 8.58 (m, 4H, arom), 9.50 (s, 2H, CH=N); FABMS (thioglycerol) *m/z* 863 (M + H). Anal. ($C_{46}H_{40}N_8O_6P_2$·0.4$C_6H_5CH_3$) C, H, N, P.

[9,10-Anthracenediylbis[methylidynehydrazo(4,5-dihydro-1*H*-imidazole-2,1-diyl)]]bis[phosphonic acid] (6). To a stirred, cold solution (0 °C) of 8.74 g (13.1 mmol) of **5a** in 150 mL of anhydrous methylene chloride, under argon, was added 13.0 g (65 mmol) of iodotrimethylsilane. After 30 min, the solution was evaporated *in vacuo* and the glassy residue suspended in 150 mL of acetone containing 5.2 mL of water to hydrolyze the intermediate silyl ester. The suspension was stirred for 16 h and the solid collected and washed with acetone to give 8.17 g of solid. This solid was recrystallized by dissolving in 200 mL of methanol containing 5.94 mL of triethylamine and then slow addition of 1.82 mL of 97% formic acid. The solid was collected by filtration and washed with ethanol to give 6.04 g (78%) of yellow solid which became yellowish-orange when dried, mp 235–238 °C. At pH 7.4 the solubility of **6** was 20 mg/mL, and HPLC data indicated <5% hydrolysis during 1 month at *ca.* 24 °C. Routine analysis of **6** showed the absence of free bisantrene. Recrystallization of **6** (methanol–triethylamine–formic acid) was not detrimental to its stability as determined by ^{1}H NMR and TLC (silica gel; 0.05 M pH 7.0 phosphate buffer, pH 7.0, R_f 0.4): ^{1}H NMR (D_2O–pyridine-d_5 (49/1) (v/v); 300 MHz) δ 3.81 (t, 4H, $PNCH_2CH_2N$), 4.08 (t, 4H, $PNCH_2CH_2N$), 7.54 (d, 4H, arom), 8.10 (d, 4H, arom), 8.44 (s, 2H, CH=N); ^{13}C NMR (D_2O–KOD; 75 MHz) δ_c 44.484 (d, $^2J_{CP}$ = 3.2 Hz, PONCH_2), 49.886 (C=NCH_2), 127.573 (aromatic CH's), 128.763 (C-9 and C-10), 129.560 (aromatic CH's), 131.183 (aromatic C), 150.525 (HC=N) and 158.843 ppm (d, $^2J_{CP}$ = 7.4 Hz, –NHC=N); FABMS (dithiothreitol/dithioerythritol (5/1)) *m/z* 557 (M + H); UV (0.1 N-pH 6.0 phosphate buffer) λ_{max} 261 nm (ϵ 78 100), 408 (ϵ 15 800). Anal. ($C_{22}H_{24}N_8O_6P_2$·2H_2O) C, H, N, P.

Disodium [9,10-Anthracenediylbis[methylidynehydrazo(4,5-dihydro-1*H*-imidazole-2,1-diyl)]]bis[phosphate] (7). A stirred suspension of 585 mg of **6** in 10 mL of water, monitored by a pH meter, was treated dropwise with 18.3 mL of 0.1 N sodium hydroxide to give a final pH of 7.4. Lyophilization of the resulting solution afforded 629 mg of reddish-orange solid **7**. It softened from 200 °C and blackened at *ca.* 300 °C. After standing at ambient temperature for 16 months, the solid was no longer soluble in water. Anal. ($C_{22}H_{22}N_8O_6P_2Na_2$·$H_2O$) C, H, N, P, Na.

[2-[[[10-[(4,5-Dihydro-1*H*-imidazol-2-yl)hydrazono]methyl]-9-anthracenyl]methylene]hydrazino]-4,5-dihydro-1*H*-imidazol-1-yl]phosphonic Acid Diethyl Ester (8), 1:1 Complex with Acetamide. The procedure was scaled up from the synthesis of **5a** except that 41.56 g (0.104 mol) of starting bisantrene, not specially dried, was used. Unreacted bisantrene (4.37 g) was recovered by filtration, and the filtrate was chromatographed over 1 kg of alumina (methylene chloride) to afford 12.50 g of **5a** from the first 2 L of eluate; the next 7 L of eluate gave 13.31 g of a mixture (by TLC) from which **5a** was mostly removed by trituration with 80 mL of methylene chloride. The solid was then washed with water to remove byproduct acetamide. A solution of the remaining 6.50 g of solid in 200 mL of methylene chloride was filtered through 30 g of silica gel and then concentrated to 30 mL. The solid which crystallized was washed sequentially with methylene chloride and then carbon tetrachloride to afford 4.50 g of yellow leaflets 8, mp 202–206 °C; TLC on silica gel ($CHCl_3$–MeOH (9/1)) gave R_f 0.3 (*vs* R_f 0.6 for **6**). An analytical sample was recrystallized from toluene–methylene chloride. Drying at 80 °C/0.1 mm/24 h did not remove an equimolar amount of cocrystallized acetamide: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.47 (t, 6H, OCH_2CH_3), 1.98 (s, 3H, CH_3-CON), 3.58 (t, 6H, NCH_2), 3.96 (t, 2H, NCH_2CH_2NP), 4.38 (m, 4H, OCH_2C), 5.81 (s, 1H, NH), 6.03 (s, 1H, NH), 7.48 (q, 4H, arom), 8.56 (m, 4H, arom), 9.21 (s, 1H, CH=N), 9.38 (s, 1H, CH=N); FABMS (dithiothreitol/dithioerythritol, 5/1) *m/z* 535 (M + H); UV (MeOH) λ_{max} 261 (ϵ 76 600), 414 (ϵ 16 700). Anal. ($C_{26}H_{31}N_8O_3P$·CH_3CONH_2) C, H, N, P.

[2-[[[10-[(4,5-Dihydro-1*H*-imidazol-2-yl)hydrazono]methyl]-9-anthracenyl]methylene]hydrazino]-4,5-dihydro-1*H*-imidazol-1-yl]phosphonic Acid·1.17 HI·1/3Acetone·0.83 H_2O (9). A solution of 1.07 g (2 mmol) of triphenylphosphine, and 90 mL of anhydrous methylene chloride, under argon, was treated with 1.0 g (0.71 mL, 5 mmol) of iodotrimethylsilane. After 30 min, the clear orange solution was evaporated to dryness and then reevaporated twice after addition of 50-mL aliquots of anhydrous methylene chloride. The residue was suspended in 50 mL of acetone and 1 mL of water was added, precipitating an orange gum which solidified on standing overnight. The solid was collected and washed with acetone, giving 1.2 g of an orange solid: ^{1}H NMR (Me_2SO-d_6, 300 MHz) δ 2.08 (acetone), 3.77 (m, 8H, NCH_2CH_2NP and NCH_2CH_2N), 7.66 (m, 4H, arom), 8.49 (m, 4H, arom), 8.79 (s, 2H), 8.92 (s, 1H), 9.34 and 9.36 (s × 2, 2H, CH=N), 12.58 (s, 1H, NC=N$^+$H–); FABMS *m/z* 479 (M + H); UV (MeOH) λ_{max} 258 (ϵ 76 100), 412 (ϵ 15 400).

Synthetic **9** was identical (by NMR and HPLC) to a metabolite isolated from rat plasma after intravenous injection of **6**. After storage for 1 year in the dark at 24 °C, **9** had become dark brown and was insoluble in 0.05 M pH 7 phosphate buffer.

[2-[[[10-[[(4,5-Dihydro-1*H*-imidazol-2-yl)ethylhydrazono]methyl]-9-anthracenyl]methylene]hydrazino]-4,5-dihydro-1*H*-imidazol-1-yl]phosphonic Acid·1.37 HI·0.2H_2O (10). A similar procedure for the synthesis of **9** was used except no triphenylphosphine was used. The crude, concentrated reaction mixture in 10 mL of methanol was filtered through 1 g of alumina. The filtrate was evaporated to partial crystallization, and acetone (20 mL) was added. After standing overnight, the solid was collected and washed with acetone, giving 1.057 g of orange solid:

mp 210–225 °C; ^{1}H NMR (Me_2SO-d_6, 300 MHz) δ 1.23 (t, CCH_3), 3.68 + 3.77 + 3.85 (m, >12H, $N^+CH_2CH_2$ + $N^+CH_2CH_2N$ + $PNCH_2CH_2N$ + H_2O), 7.70 (m, 4H, arom), 8.44 + 8.49 (m, 4H, arom), 8.78 (s, 2H), 9.04 (s, 1H), 9.34 (s, 1H, CH=N), 9.43 (s, 1H, CH=N), 12.54 (s, 1H, NC=N$^+$H or POH); FABMS (dithiothreitol–dithioerythritol (5/1)) m/z 507 (M + H); UV (MeOH) λ_{max} 257 (ϵ 76 300), 406 (ϵ 13 200).

Bis(phosphonoguanidinic Acid) *in Vivo* Hydrolysis Studies. *In vivo* hydrolysis of the bis(phosphonoguanidinic acid) **6** was investigated using a rat model system. Plasma samples were obtained from Sprague–Dawley rats which had received single 20 mg/kg intravenous doses of [9,10-^{14}C]-**6**. Plasma aliquots were added to solid-phase extraction cartridges (Bond-elut SCX, Analytichem International) which had been preconditioned with water. The cartridges were centrifuged to remove plasma components, while **6** and related components were retained. Drug-related material was recovered by elution of the cartridges with a mixture of methanol and ammonium hydroxide.

The eluate from the sample preparation procedure was evaporated and subsequently redissolved in 0.1 M pH 7.4 potassium phosphate buffer for HPLC analysis. Reversed-phase gradient HPLC analysis was used to quantitate **6** and its metabolites. Acetonitrile and 0.1 M pH 7.4 potassium phosphate buffer (containing 0.01 M tetrabutylammonium hydroxide) comprised the mobile phase, while an IBM C_{18} column (4.5 mm × 15 cm, 5 μm) was used with a Model IC radioactive flow detector (Radiomatic Instruments, Inc.) to measure all drug-related material.

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