Design, Synthesis, and Enzymatic Evaluation of Multisubstrate Analogue Inhibitors of *Escherichia coli* Thymidine Phosphorylase

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A series of acyclic phosphonate derivatives of thymine has been synthesized and tested as multisubstrate analogue inhibitors of *Escherichia coli* thymidine phosphorylase. The compounds synthesized include 1-(phosphonoalkyl)thymines with six to nine methylenes (**1**−**4**, respectively); 1-[(*Z*)-4-phosphonomethoxy-2-butenyl]thymine (**5**) and its butyl and 2,3-*cis*-dihydroxybutyl derivatives (**6** and **7**, respectively); 1-[(*Z*)-(4-(phosphonomethoxy)methoxy)-2-butenyl]thymine (**8**) and also its butyl and 2,3-*cis*-dihydroxybutyl analogues (**9** and **10**); and 1-[((*Z*)-4-(phosphonomethoxy)-2-butenoxy)methyl]thymine (**11**). Evaluation of these compounds against *E. coli* revealed significant enzymatic inhibition by **2**, **3**, **4**, **6**, and **8** at a concentration of 1000 μM, **3** and **4** being the most potent. Replacement of the thymine base in **3** by 6-amino-5-bromouracil and 7-deazaxanthine afforded compounds **12** and **13**, which showed a pronounced improvement of TPase inhibition, comparable to 7-deazaxanthine. When inorganic phosphate was used as a variable substrate, compounds **12** and **13** displayed competitive kinetics with respect to phosphate, indicating a direct interaction of these compounds with the phosphate binding site. Also compounds **12** and **13** were found to be competitive inhibitors of TPase against thymidine as a variable substrate. These results are consistent with the compounds being multisubstrate analogue inhibitors of *E. coli* TPase, and they represent the first example of such TPase inhibitors.

Introduction

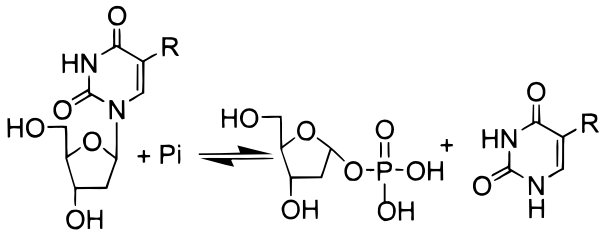
Thymidine phosphorylase (TPase; EC 2.4.2.4) is one of the key enzymes involved in salvage biosynthesis and catabolism of pyrimidine 2′-deoxynucleosides. This enzyme catalyzes their reversible phosphorolysis with formation of 2-deoxy-α-D-ribose-1-phosphate and the pyrimidine, as shown in Scheme 1.

Thymidine phosphorylase recognizes not only thymidine and 2′-deoxyuridine but also a variety of 5-substituted pyrimidine 2′-deoxynucleoside analogues that are endowed with potent antiviral (i.e., 5-(*E*)-(bromovinyl)-2′-deoxyuridine, 5-trifluoromethyl-2′-deoxyuridine, and 5-iodo-2′-deoxyuridine) and antitumor (i.e., 5-fluoro-2′-deoxyuridine) activities.[1] There has been a long-standing interest in the development of compounds inhibiting TPase, since such compounds could be expected to result in a considerable increase in the therapeutic effectiveness of the above-mentioned nucleosides.[1]

However, most interest in TPase in recent years came from the observation that TPase is identical to platelet-derived endothelial cell growth factor (PD-ECGF), an important factor involved in angiogenesis.[2−4] Moreover, it has been demonstrated that the enzymatic activity of TPase/PD-ECGF is crucial for its angiogenic effect.[5,6]

Histological analyses of a variety of human tumors have shown abnormally elevated TPase levels, and in many cases these levels have been correlated with an aggressive progression of the tumor.[7] TPase levels have also been correlated with microvessel density in breast,[8] colon,[9] and renal cell carcinomas,[10] indicating that TPase contributes to tumor vasculature. A very recent study suggests that TPase may not only be involved in angiogenesis but also in apoptosis.[11] Therefore, there is a need for TPase inhibitors to help clarify the precise role of TPase in the progression of a variety of tumors. Furthermore, such inhibitors could be used alone or in combination with other antitumoral agents, including TPase-sensitive nucleoside analogues, as therapeutic agents.

Scheme 1



Very few inhibitors of TPase have been reported, the most potent being pyrimidine bases such as 6-amino-5-bromouracil or 6-aminothymine[12] and, very recently, 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil.[11] Last year 7-deazaxanthine was reported by us as the first purine derivative able to inhibit TPase.[13,14]

The structure of human TPase has not yet been determined. To date, structure determinations are limited to those of two prokaryotic homologues: *Escherichia coli* TPase[15,16] and *Bacillus stearothermophilus*

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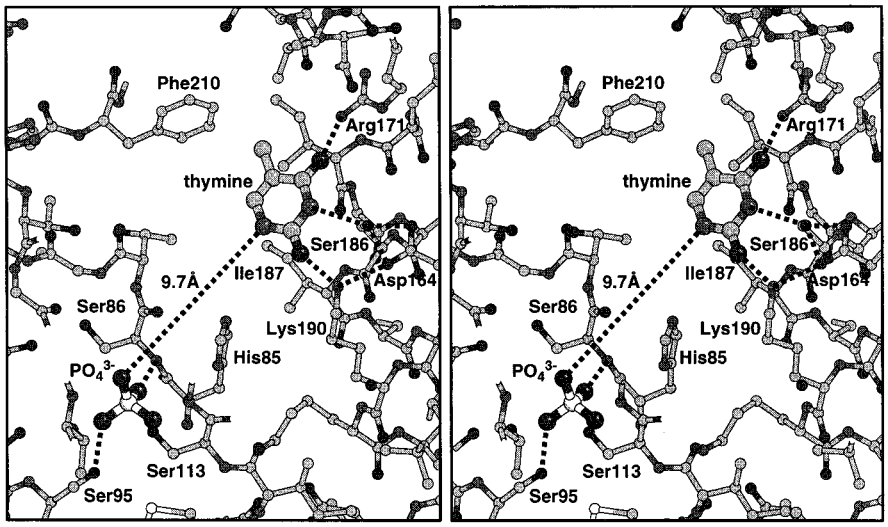


Figure 1. A stereo diagram showing the relantionship between the thymine and the phosphate binding sites based on the crystal structure of *E. coli* thymidine phosphorylase (Walter et al.[15]). Thymine and the phosphate ion are shown by thick ball-and-stick models, and the surrounding protein is represented by thinner ball-and-stick models. Nearby residues and stabilizing interactions are shown. Figure was drawn using BobScript.[39]

pyrimidine nucleoside phosphorylase.[17] These enzymes have 40% sequence identity with human TPase and with each other. However, the folds of the two bacterial enzymes are similar, and moreover, surrounding the catalytic site there is a particularly high degree of sequence and structural conservation. Of the residues near the catalytic site there is only one significant amino acid difference between human and bacterial TPases: residue 210 being either valine (human) or phenylalanine (bacterial). In other respects the catalytic sites of the bacterial enzymes would be expected to provide good models for human TPase. These bacterial enzyme structures reveal TPase as a dimer of identical subunits. Each subunit contains a large mixed α/β domain, where the phosphate ion (Pi) binds, and a smaller α-helical domain separated by a cleft which contains the nucleoside binding site. The two binding sites are separated by 8−10 Å.[15] It has been suggested that in the phosphorolytic reaction a domain movement must occur causing the cleft to close to an active conformation, bringing the Pi closer to the anomeric position of the nucleoside.[17] Therefore, there are two binding sites, one for the phosphate and one for the 2′-deoxynucleoside. The amino acids defining this second binding site (see Figure 1) explain the specifity of TPase for thymine and other 5-substituted uracil 2′-deoxynucleosides, but not 2′-deoxycytidine, based on the recognition pattern for the 2-CO, 3-NH, and 4-CO of the pyrimidine base.

In our search for TPase inhibitors, in the present paper it is described the design and synthesis of multisubstrate inhibitors, i.e., compounds that contain in their structure covalently linked elements of both substrates. In particular, these elements are a phosphate analogue, able to interact at the phosphate binding site, and a pyrimidine base, interacting at the nucleoside binding site. The goal was to obtain compounds able to "freeze" the conformation of the enzyme in an opened, inactive conformation. The approach of multisubstrate analogue inhibitors has led, among other reports, to potent inhibitors of purine nucleoside phosphorylase.[18] However, to the best of our knowledge, this strategy has not been previously applied to TPase.

The proposed inhibitors combined three structural elements: a phosphate analogue, a pyrimidine base, and an appropiate spacer (Figure 2). As a phosphate analogue, a phosphonate moiety has been chosen, which has a tetrahedral structure similar to that of phosphate but increased metabolic stability.[19] As spacers, polymethylene chains of six to nine units were initially used (compounds **1**−**4**). Although these could be considered as "too" flexible, they might provide an early test of the validity of the working hypothesis. Second, spacers of the same length were constructed by combining fragments (in particular, (*Z*)-2-butene-1,4-diol and oxymethylene units) that impose conformational restriction and/or can be easily transformed into other entities. The (*Z*)-2-butene-1,4-diol, present in compounds **5**, **8**, and **11**, can be easily transformed into the butyl (such as in **6** and **9**) and 2,3-*cis* dihydroxybutyl analogues (compounds **7** and **10**). The hydroxyl groups introduced into the spacers in **7** and **10** could give additional interactions with amino acids present in the channel between the two binding sites, such as His85 or Ser86. The incorporation of oxymethylene units in the spacers not only introduces a certain degree of conformational restriction but also, being contiguous to the phosphonate, can affect its pK_a value, making it closer to that of the phosphate.[20] For the pyrimidine base, thymine, the natural substrate, was initially chosen. The information obtained from the evaluation of the thymine-spacer-phosphonate series (**1**−**11**) was used for the synthesis of new series of compounds where thymine was replaced by 6-amino-5-bromouracil and 7-deazaxanthine (compounds **12** and **13**) and that are expected to show a greater inhibitory potency against TPase. The different synthetic strategies employed to obtain these structures and the evaluation of the compounds against *E. coli* TPase are described in detail in this paper.

Chemistry

The synthesis of the 1-(phosphonoalkyl)thymines (**1**−**4**) was performed in three steps starting from thymine (Scheme 2). Thus, reaction of the thymine sodium salt with the corresponding dibromoalkane afforded the N^1-

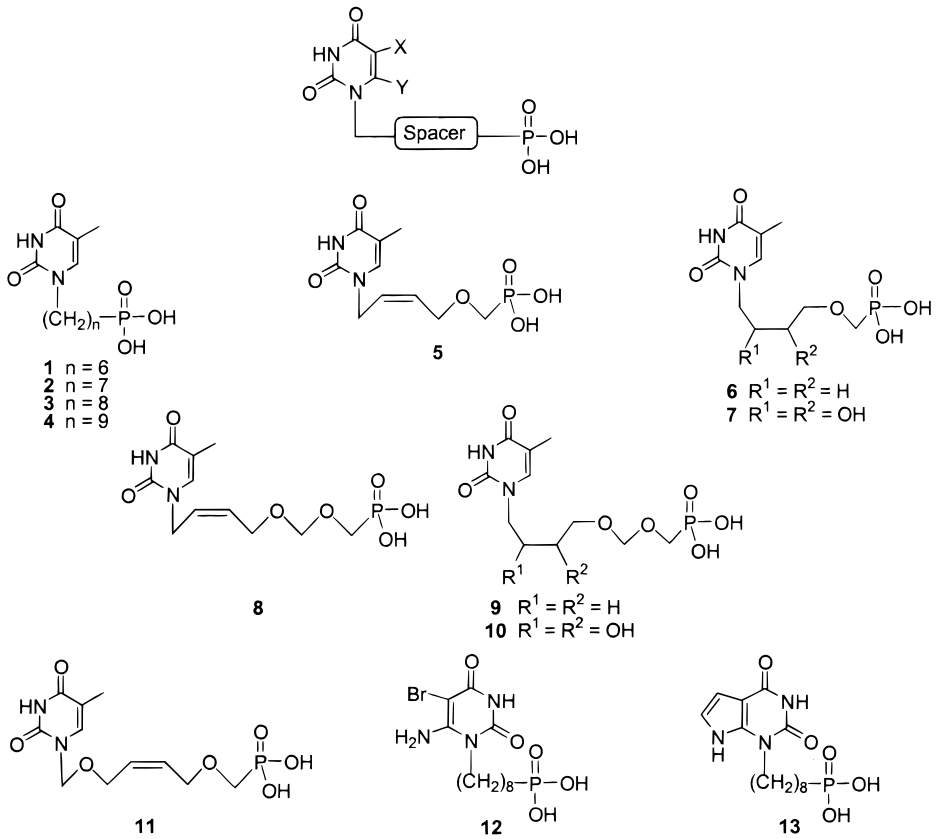
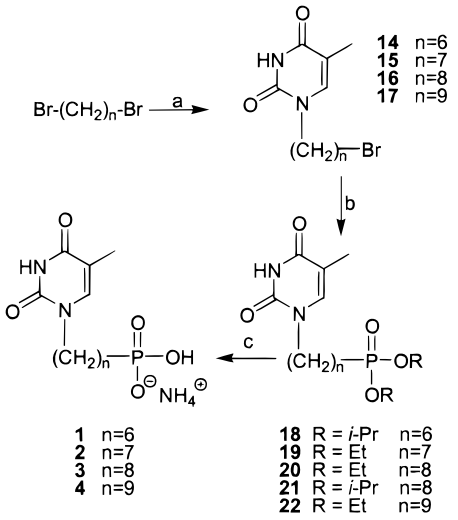


Figure 2. Chemical structures of the proposed multisubstrate analogue inhibitors.

Scheme 2[a]



[a] (a) Thymine, NaH, DMF (40−33%); (b) $P(OR)_3$ (86−58%); (c) 1. TMSBr, 2. H_2O (81−67%).

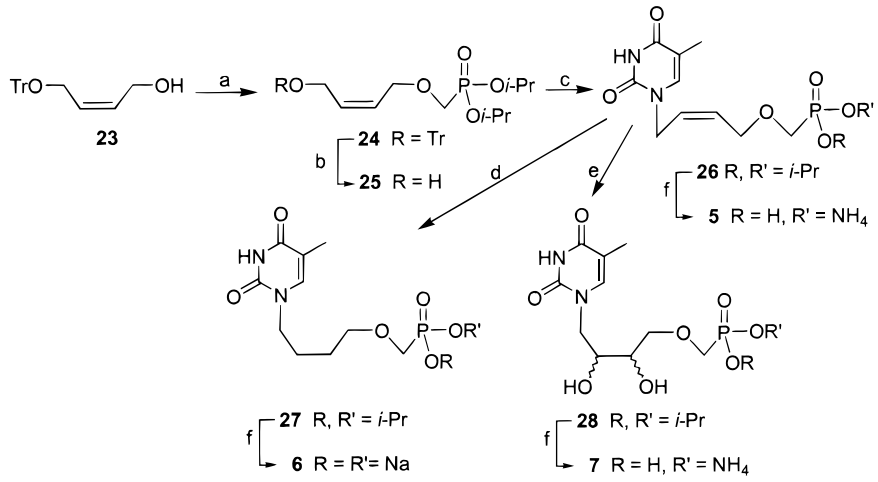
substituted products (**14**−**17**).[21,22] Although the yields are moderate and lower than those in a previously described procedure,[22] the present method provides the desired compounds in a "one pot" reaction, avoiding tedious silylation of thymine. Compounds **14**−**17** were heated with triisopropyl- or triethyl-phosphite according to the Arbuzow reaction to afford the corresponding alkylphosphonate derivatives **18**−**22** (58−85% yields). Deprotection of the phosphonate esters with TMSBr followed by hydrolysis and purification by ion exchange chromatography yielded the desired compounds **1**−**4**, isolated as their ammonium salts (67−81% yields).

The general strategy for the synthesis of compounds **5**−**11** (Figure 2) was the introduction of the oxymethylphosphonate moiety prior to the incorporation of the thymine base, to avoid possible alkylations on the N^3 position of thymine. The starting material, in all cases, was (*Z*)-2-butene-1,4-diol, conveniently monoprotected (**23** and **29** in Schemes 3 and 4).

Compounds **5**−**7**, with a six-membered spacer, were synthesized as described in Scheme 3. Reaction of **23**[23] with diisopropyl[(*p*-toluenesulfonyl)oxy]methanephosphonate[24] in the presence of NaH gave compound **24**, which was deprotected by treatment with 80% AcOH to yield the alcohol **25** (44% yield from **23**). Mitsunobu reaction[25] of this alcohol with N^3-benzoylthymine,[26] followed by in situ deprotection with methanolic $MeNH_2$, afforded the N^1-substituted derivative **26** in 62% yield. Reduction of the double bond in **26** by catalytic hydrogenation afforded the saturated derivative **27**. On the other hand, treatment of **26** with OsO_4 yielded the enantiomeric mixture of 1,2-*cis* diols **28**. Deprotection of the phosphonate esters in **26**, **27**, and **28** was performed by reaction with TMSBr, to afford compounds **5**,[27] **6**, and **7** (73, 61, and 50% yields, respectively).

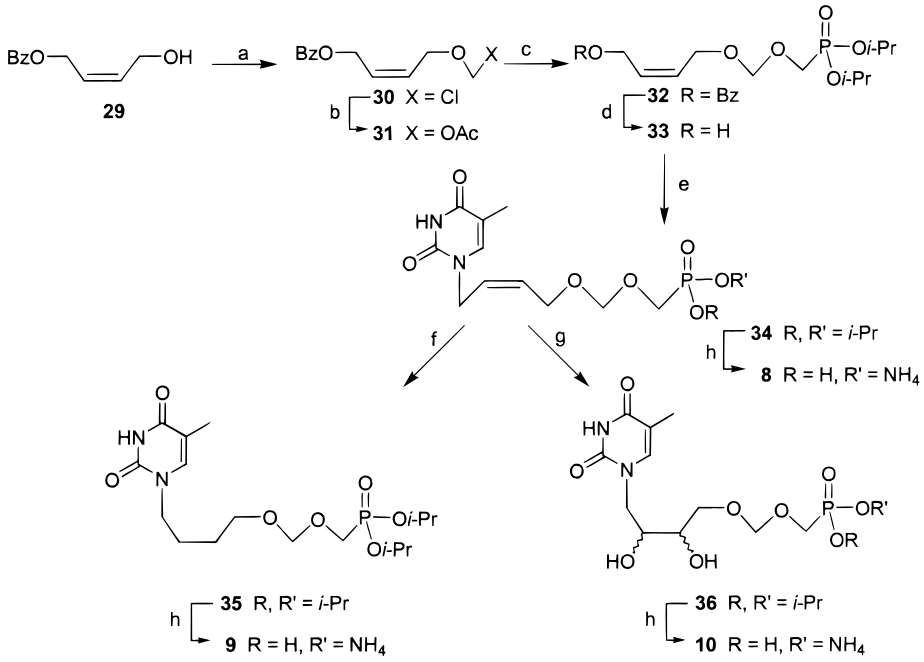
The synthesis of compounds **8**−**10**, with an eight-membered spacer, is shown in Scheme 4. Reaction of the *O*-benzoyl derivative **29**[28] with paraformaldehyde and HCl(g) afforded the chloromethyl ether **30**, which was transformed into the acetoxymethyl derivative **31** by treatment with sodium acetate in acetone. Condensation of **31** with $HOCH_2P(O)(OiPr)_2$[24] in the presence of TMSTf, followed by deprotection with methanolic $MeNH_2$, afforded the alcohol **33**. Mitsunobu reaction[25] of **33** with N^3-benzoylthymine,[26] followed by in situ

Scheme 3[a]



[a] (a) $(iPrO)_2P(O)CH_2OTs$, NaH, DMF (43%); (b) 80%-AcOH(aq) (96%); (c) 1. N^3-BzT, PPh_3, DIAD, THF, 2. $MeNH_2$, MeOH (62%); (d) H_2, 10%-Pd/C, EtOH (80%); (e) OsO_4, *N*-methylmorpholine-*N*-oxide, acetone−H_2O (90:1) (70%); (f) 1. TMSBr, DMF, 2. H_2O, **5** (73%), **6** (61%), **7** (50%).

Scheme 4[a]

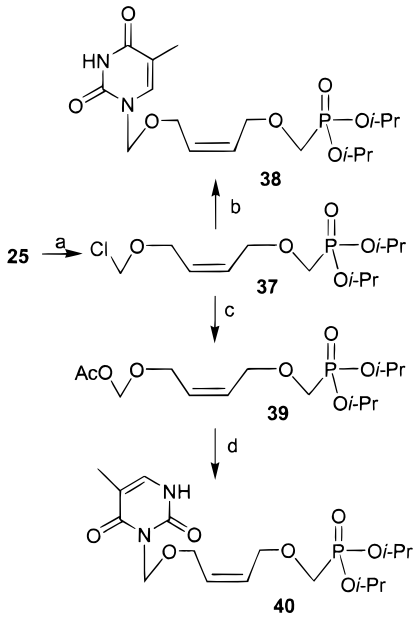


[a] (a) HCl(g), $(CH_2O)_n$, CH_2Cl_2; (b) AcONa, acetone; (c) $(iPrO)_2P(O)CH_2OH$, TMSTf, CH_3CN (91% from **29**); (d) $MeNH_2$, MeOH (68%); (e) 1. N^3-BzT, PPh_3, DIAD, THF, 2. $MeNH_2$, MeOH (76%); (f) H_2, Pd/C, EtOH (66%); (g) OsO_4, *N*-methylmorpholine-*N*-oxide, acetone−H_2O (90:1) (76%); (h) 1. TMSBr, 2,6-lutidine, CH_2Cl_2 or DMF 2. H_2O, **8** (70%), **9** (84%), **10** (46%).

deprotection with methanolic $MeNH_2$, afforded compound **34** (76% yield). Catalytic hydrogenation (Pd/C) of **34** afforded **35**. Treatment of **34** with OsO_4 gave the dihydroxylated derivative **36** in 76% yield. Deprotection of the phosphonate esters in **34**, **35**, and **36** with TMSBr, in the presence of 2,6-lutidine to avoid the breakthrough of the acetalic bond, afforded the target compounds **8**, **9**, and **10** in 71, 84, and 46% yields, respectively.

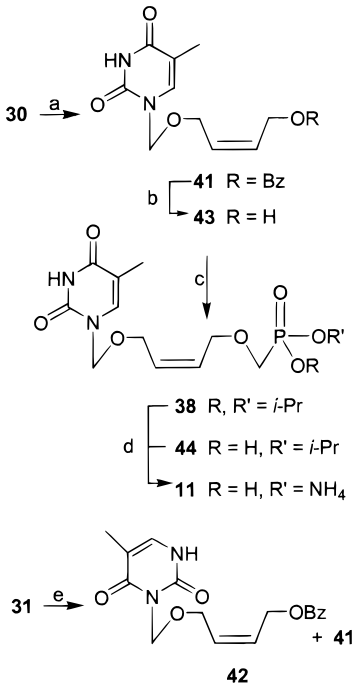
Concerning the synthesis of compound **11** (Figure 2), the initial strategy was based, as in the previous series, on the introduction of the phosphonate moiety prior to the pyrimidine base. Thus, the previously synthesized synthon **25** (Scheme 5) was transformed into the chloromethyl ether **37**, which by reaction with silylated thymine in the presence of catalytic amounts of BuN_4I afforded only traces of the desired compound **38** (<10% yield). Alternatively, the chloromethyl ether **37** was transformed into the acetoxymethyl ether **39** and then reacted with silylated thymine using $SnCl_4$ as catalyst. This method afforded a complex reaction mixture, from which the N^3-substituted thymine **40** was the only compound isolated. These results prompted us to employ a different strategy based on the sequential incorporation of the thymine base and then the phosphonate terminus (Scheme 6). Thus, reaction of the chloromethyl ether **30** with silylated thymine in the presence of BuN_4I afforded exclusively the N^1-substituted derivative **41** (75% yield). The reaction of the acetoxymethyl ether **31** with silylated thymine and $SnCl_4$ was also assayed. This second procedure afforded mixtures of N^1-(**41**) and N^3-

Scheme 5[a]



[a] (a) HCl(g), $(CH_2O)_n$, CH_2Cl_2; (b) silylated thymine, Bu_4NI, CH_2Cl_2, rt, 24 h; (c) AcONa, acetone; (d) silylated thymine, $SnCl_4$, 0 °C to rt, 1.5 h, CH_3CN.

Scheme 6[a]



[a] (a) Silylated thymine, Bu_4NI, CH_2Cl_2, rt, 24 h (72%); (b) $MeNH_2$, MeOH (98%); (c) 1. NaH, DMF 2. $(iPrO)_2P(O)CH_2OTs$, DMF **38** (25%) and **44** (31%); (d) 1. TMSBr, CH_2Cl_2, 2,6-lutidine; 2. H_2O (75%); (e) silylated thymine, $SnCl_4$, 0 °C to rt, 4 h, **42** (17%) + **41** (9%).

(**42**) substituted products, the latter predominating. Unequivocal assignment of the substitution position in **41** and **42** was performed based on ^1H and ^{13}C NMR correlation experiments (HSQC and HMBC) to establish directly bonded and long distance coupled proton and carbon atoms, respectively. In our opinion, it is rather surprising to obtain the N^3-derivative as the predominant isomer in the reaction of the acetoxymethyl ethers (**39** and **31**) with thymine in the presence of $SnCl_4$. It should be mentioned that there are very few reports of obtaining N^3-isomers of 5-substituted uracils, including thymine, in acyclonucleoside synthesis,[29,30] but in all these cases $SnCl_4$ was used as catalyst.

Removal of the benzoyl group in **41**, followed by treatment with NaH and then with $TsOCH_2P(O)(OiPr)_2$, afforded a mixture of the phosphonate derivative **38** and the monodeprotected derivative **44** together with N^3-alkylated compounds. Deprotection of **38** or **44** with TMSBr, in the presence of 2,6-lutidine, afforded the acycloderivative **11**.

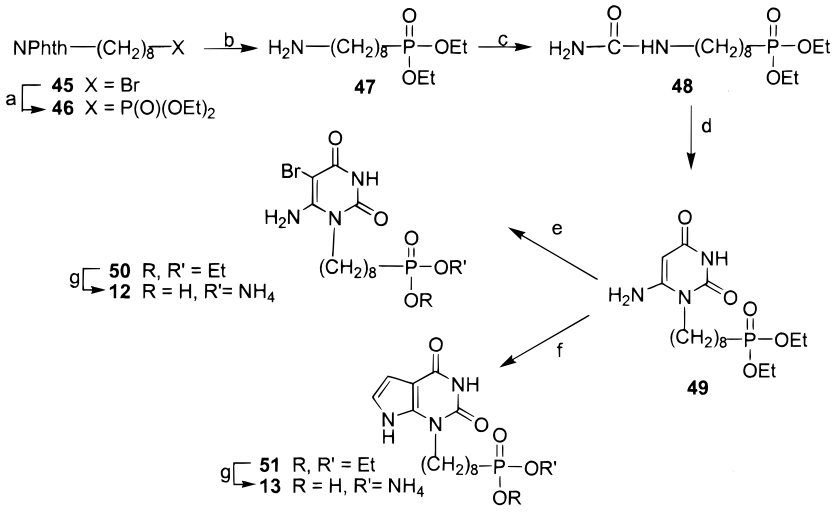
When compounds **1**−**11** were evaluated against the *E. coli* enzyme (see Biological Results section), several of them showed moderate inhibition of thymidine degradation, the most potent being compounds **3** and **4**. According to the working plan, the thymine in compound **3** was replaced by 6-amino-5-bromouracil (**12**) and 7-deazaxanthine (**13**), keeping the same anchoring point of the spacer on the pyrimidine base. Both compounds **12** and **13** could be obtained from a common intermediate of N^1-substituted 6-aminouracil. Direct alkylation of 6-aminouracil, as described for the thymine series, was discarded since this procedure is expected to afford mixtures of isomers. Even exclusive formation of the N^3-substituted derivative has been described when the base is silylated.[31] The method of choice to obtain N^1-monosubstituted 6-aminouracils is the condensation of monosubstituted ureas with ethyl cyanoacetate.[32,33] Therefore, the synthesis of **12** and **13** was accomplished as described in Scheme 7.

Reaction of *N*-(8-bromooctyl)phthalimide[34] (**45**) with triethyl phosphite, as described for similar analogues,[35] yielded the diethylalkylphosphonate **46**, which was deprotected at the amino terminus by reaction with NH_2NH_2 in MeOH to give **47** (90% yield). This amine, **47**, was transformed into the monoalkyl urea **48** by treatment with KNCO in acidic medium. The urea **48** was condensed with ethyl cyanoacetate under basic conditions to yield the N^1-substituted 6-aminouracil (**49**). Bromination of **49** with *N*-bromosuccinimide in the presence of AIBN in THF afforded the 5-bromo-6-aminouracil derivative **50** (72% yield). Alternatively, compound **49** was transformed into the 7-deazaxanthine derivative **51** by reaction with chloroacetaldehyde and sodium acetate.[36] Removal of the phosphonate esters of **50** and **51** was performed by treatment with TMSBr followed by hydrolysis to give **12** and **13** in 75 and 85% yields, respectively.

Biological Results and Discussion

Inhibition of Thymidine Phosphorylase. The phosphonate derivatives **1**−**13**, as well as their corresponding dialkyl ester, have been tested for their inhibitory effect on thymidine phosphorylase from *E. coli* (see Experimental Section). 6-Amino-5-bromouracil,[36] a well-established TPase inhibitor,[12] and 7-deazaxanthine,[37] recently described by us as a novel TPase inhibitor,[13] are included as reference inhibitors. Compounds **1**−**4** having a polymethylene spacer of 6−9 atoms between the N^1 position of thymine and the phosphonate moiety, were found to be inhibitory to *E. coli* TPase: the longer the spacer, the higher the inhibition (Table 1). Thus, compound **1**, containing six CH_2

Scheme 7[a]



[a] (a) $P(OEt)_3$ (96%); (b) $H_2N-NH_2 \cdot H_2O$, EtOH (90%); (c) HCl(aq), KOCN, H_2O (73%); (d) EtONa, NC-CH_2-COOEt, EtOH (66%); (e) NBS, AIBN, THF (72%); (f) Cl-CH_2-CHO, AcONa, EtOH (47%); (g) 1, TMSBr, CH_2Cl_2 2. H_2O, **12** (75%), **13** (85%).

Table 1. Inhibition of *E. coli* Thymidine Phosphorylase by Test Compounds as a Function of Incubation Time

		inhibition of TPase (%)[a]		
compd	concn (μM)	20 min	40 min	60 min
1	1000	11	5	2
2	1000	73	63	55
	100	17	13	8
3	1000	75	68	61
	100	29	22	16
4	1000	81	75	70
	100	35	27	18
5	1000	0	0	0
6	1000	27	17	12
	100	5	2	1
7	1000	0	0	0
8	1000	34	22	17
	100	0	0	0
9	1000	0	0	0
10	1000	0	0	0
11	1000	0	0	0
12	1000	94	91	89
	100	64	55	46
	20	26	19	13
13	1000	97	96	94
	100	83	77	72
	20	48	38	31
19	1000	0	0	0
20	1000	0	0	0
21	1000	0	0	0
22	1000	4	3	2
26	1000	0	0	0
27	1000	0	0	0
28	1000	0	0	0
34	1000	0	0	0
35	1000	0	0	0
36	1000	0	0	0
50	1000	21	16	10
	100	0	0	0
51	500	43	37	27
	100	0	0	0
6A5BU	100	nd	nd	71
	25	nd	nd	44
7DX	100	nd	nd	41
	25	nd	nd	7

[a] The inhibition data represent the mean of at least two or three independent experiments.

units between thymine and the phosphonate, was only marginally active at 1000 μM, and compound **2**, containing seven methylene units in the spacer, afforded 55 to 73% inhibition at 1000 μM and 8−17% inhibition at 100 μM, whereas **3** and **4**, containing eight and nine CH_2 units, respectively, inhibited TPase by 61 to 81% at 1000 μM and by 16 to 35% at 100 μM. It should be pointed out that their corresponding diethyl (**19**, **20**, **22**) or diisopropyl (**21**) ester derivatives were devoid of anti-TPase activity. Only **22** showed a marginal inhibitory effect at 1000 μM (2−4%), indicating the importance of a free phosphonic acid for anti-TPase activity.

Among the phosphonates carrying a (*Z*)-butene moiety in the spacer (i.e., **5**, **8**, and **11**), only compound **8** showed an inhibitory effect at the highest concentration tested (1000 μM: 17−34% inhibition). Interestingly, whereas **5** was devoid of anti-TPase activity, its butyl analogue **6** showed significant inhibition at 1000 μM (12−27%). The corresponding diisopropyl esters of **6** and **8** (viz. **27** and **34**) were completely inactive. Among the remaining thymine phosphonate derivatives synthesized (**7**, **9**, and **10**), no inhibition was found at 1000 μM, neither for their dialkyl ester derivatives.

Interestingly, replacement of the thymine base in compound **3** by 5-bromo-6-aminouracil or 7-deazaxanthine (compounds **12** and **13**, respectively) led to a considerable improvement of the inhibitory activity. Indeed, whereas **3** was inhibitory by 61−75% at 1000 μM and 16−29% at 100 μM, **12** was inhibitory by 89−94% at 1000 μM, 46−64% at 100 μM, and 13−26% at 20 μM. Compound **13** was even more inhibitory to TPase than **12** and, thus, showed an inhibition of TPase that was at least comparable, if not more pronounced, than previously noted for 7DX (Table 1). The isopropyl esters of **12** and **13** (viz. **50** and **51**) had still an inhibitory potential at 1000 μM (i.e., 10−21% and 27−43%, respectively); however, comparison of **50** and **51** with their deprotected analogues **12** and **13** stresses the importance of a free phosphonic acid for TPase inhibition by these series of compounds.

To clarify whether the inhibition of TPase was really due to a multisubstrate analogue inhibition (multisubstrate binding), kinetic experiments with the most potent compounds **12** and **13**, together with the previously described inhibitor 7DX, were carried out. By varying the concentrations of inorganic phosphate, a

competitive inhibition of **12** and **13** with respect to phosphate was revealed, while the free base 7DX proved noncompetitive with respect to phosphate. Whereas the K_m value of the enzyme for inorganic phosphate was 1420 μM, the K_i values of **12**, **13**, and 7DX for the enzyme against inorganic phosphate as the variable substrate were 48 μM, 39 μM, and 27.5 μM, respectively. Also, **12** and **13** were found to be competitive inhibitors of TPase against thymidine as the variable substrate (K_m value was 600 μM, K_i values were 123 μM and 68 μM, respectively). The enzyme kinetic data strongly suggest that, in contrast to 7DX, compounds **12** and **13** indeed must interact with both, the phosphate binding site and the nucleoside binding site of TPase, as originally designed.

Conclusion

A series of acyclic phosphonate derivatives of thymine with a spacer of six to nine atoms have been synthesized in order to evaluate their potential as multisubstrate analogue inhibitors of *E. coli* thymidine phosphorylase. Multisubstrate analogue inhibitors have yielded extremely potent inhibitors against other targets, such as purine nucleoside phosphorylase.[18] To the best of our knowledge, there are no previous reports on multisubstrate analogue inhibitors of TPase. Among the thymine derivatives synthesized in this paper, compounds **3** and **4**, having a polymethylene chain of eight and nine atoms between the thymine base and the phosphonic acid, showed marked inhibition of TPase. Replacement of thymine in **3** by 6-amino-5-bromouracil or 7-deazaxanthine (compounds **12** and **13**, respectively) resulted in a marked increase of the inhibitory potency, being comparable to that of 7DX. Kinetic experiments in the presence of variable concentrations of inorganic phosphate and variable concentrations of thymidine have shown that **12** and **13** compete with both substrates to inhibit TPase. These results strongly indicate that compounds **12** and **13** behave as multisubstrate analogue inhibitors of TPase. These new leads should be further explored to improve their inhibitory potency against TPase.

Experimental Section

Chemical Procedures. Melting points were obtained on a Reichert-Jung Kofler apparatus and are uncorrected. Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. Electrospray mass spectra were measured on a quadrupole mass spectrometer equipped with an electrospray source (Hewlett-Packard, LC/MS HP 1100). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini operating at 200 MHz (^{1}H) and 50 MHz (^{13}C), respectively, on a Varian INOVA 300 operating at 299 MHz (^{1}H) and 75 MHz (^{13}C), respectively, and Varian INOVA-400 operating at 400 MHz (^{1}H) and 100 MHz (^{13}C), respectively. Monodimensional ^{1}H and ^{13}C spectra were obtained using standard conditions. Two-dimensional inverse proton detected heteronuclear one-bond shift correlation spectra were obtained using the pulsed field gradient HSQC pulse sequence. Data were collected in a 2048 × 512 matrix with a spectral width of 3460 Hz in the proton domain and 22500 Hz in the carbon domain and were processed in a 2048 × 1024 matrix. The experiment was optimized for one bond heteronuclear coupling constant of 150 Hz. Two-dimensional inverse proton detected heteronuclear long-range shift correlation spectra were obtained using the pulsed field gradient HMBC pulse sequence. The HMBC experiment was acquired in the same conditions as those for the HSQC experiment and was optimized for long-range coupling constants of 7 Hz.

Analytical TLC was performed on silica gel 60 F_{254} (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or by charring with phosphomolibdic acid and/or ninhydrin. Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron (Kiesegel 60 PF $_{254}$ gipshaltig (Merck); layer thickness, 1 or 2 mm; flow rate, 4 or 8 mL/min, respectively). Flash column chromatography was performed with silica gel 60 (230−400 mesh) (Merck). Sephadex A-25 (HCO_3-form) was used for ion exchange chromatography. Analytical HPLC was performed on a Waters 484 system using a μBondapak C_{18} (3.9 × 300 mm: 10 μm), with UV detection at 254 nm and a flow rate of 1 mL/min. The systems used were the following: Mobile phases A: CH_3CN, B: H_2O (0.05% TFA). System I: 2% A (0−2 min), 2−20% A (2−4 min), 20% A (4−6 min), 20−95% A (6−16 min), 95−2% A (16−20 min). System II: 0% A (0−2 min), 0−10% A (2−4 min), 10% A (4−12 min), 10−95% A (12−25 min), 95−0% A (25−30 min). All retention times are quoted in minutes.

The deprotected phosphonates were noted to be hygroscopic and did not give useful microanalytical data but were found to be pure by high-field multinuclear NMR spectroscopy, mass spectrometry, and rigorous HPLC analysis.

All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions. Triethylamine and acetonitrile were dried by refluxing over calcium hydride. Tetrahydrofuran was dried by refluxing over sodium/benzophenone. Anhydrous *N*,*N*′-dimethylformamide was purchased from Aldrich.

Thymidine, thymine, and *E. coli* thymidine phosphorylase (TPase) (1030 units/mL) were obtained from Sigma Chemical Co., St. Louis, MO. The synthesis of 7-deazaxanthine was performed according to West et al.[37] 6-Amino-5-bromouracil (6A5BU) was synthesized according to E. F. Schroeder.[36]

General Procedure for the Preparation of 1-(Bromoalkyl)thymines. Sodium hydride (349 mg, 60% dispersion in mineral oil, 8.72 mmol) was added into a suspension of thymine (1.00 g, 7.93 mmol) in dry DMF (32 mL), and the mixture was heated at 80 °C for 1 h. After the mixture cooled to room temperature, the corresponding dibromoalkane (7.93 mmol) was added, and the resulting mixture was heated at 80 °C for 5 h whereupon it was evaporated. The crude was treated with CH_2Cl_2 (150 mL) and filtered. The filtrate was purified by column chromatography using EtOAc:hexane (1:2) as eluent.

1-(6-Bromohexyl)thymine (14). Yield: 40%. Mp (EtOAc): 113−114 °C (lit.[21] 109−110 °C; lit.[22] 112−113.5 °C).

1-(7-Bromoheptyl)thymine (15). Yield: 39%. Mp (EtOAc): 109−110 °C (lit.[21] 107−108 °C).

1-(8-Bromooctyl)thymine (16). Yield: 39%. Mp (EtOAc): 96−97 °C (lit.[21] 96−97 °C; lit.[22] 96−96.5 °C).

1-(9-Bromononyl)thymine (17). Yield: 33%. Mp (EtOAc): 104−105 °C (lit.[21] 104−105 °C).

General Procedure for the Preparation of 1-[(Dialkylphosphono)alkyl]thymines. A mixture of the corresponding 1-(bromoalkyl)thymine (5.00 mmol) (**14**−**17**) and trialkyl phosphite (35 mL) was heated at 130 °C for 24 h. After evaporation, the crude was purified by column chromatography to yield **18**−**22** as syrups.

1-[6-(Diisopropylphosphono)hexyl]thymine (18). The product was obtained from **14** (1.45 g) and triisopropylphosphite, followed by column chromatography (EtOAc: EtOH, 20:1). Yield 60%. ^{1}H NMR ($CDCl_3$) δ: 0.90−1.80 (m, 10H), 1.27 [d, J = 6.2 Hz, 12H, $CH(CH_3)_2$], 1.90 (s, 3H, 5-CH_3), 3.65 (t, J = 7.3 Hz, 2H, CH_2N), 4.40−4.80 [m, 2H, $CH(CH_3)_2$], 6.95 (s, 1H, H-6), 8.94 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 12.2 (5-CH_3), 22.4 (d, $J_{C,P}$ = 5.2 Hz, CH_2CH_2P), 24.0 [$CH(CH_3)_2$], 25.9, 28.8 (CH_2), 26.7 (d, $J_{C,P}$ = 141.2 Hz, CH_2P), 30.0 (d, $J_{C,P}$ = 16.7 Hz, $CH_2(CH_2)_2P$), 48.3 (CH_2N), 69.8 [d, $J_{C,P}$ = 6.6 Hz, $CH(CH_3)_2$], 110.5 (C-5), 140.3 (C-6), 150.9 (C-2), 164.4 (C-4). Anal. ($C_{17}H_{31}N_2O_5P$) C, H, N.

1-[7-(Diethylphosphono)heptyl]thymine (19). The product was obtained from **15** (1.52 g) and triethyl phosphite, followed by column chromatography (CH_2Cl_2:acetone, 2:1). Yield: 82%. ^{1}H NMR ($CDCl_3$) δ: 1.10−1.85 (m, 12H), 1.31 (t, J = 7.1 Hz, 6H, CH_2CH_3), 1.91 (s, 3H, 5-CH_3), 3.67 (t, J = 7.2 Hz, 2H, CH_2N), 3.90−4.30 (m, 4H, CH_2CH_3), 6.97 (s, 1H, H-6), 8.57 (br s, 1H, NH). Anal. ($C_{16}H_{29}N_2O_5P$) C, H, N.

1-[8-(Diethylphosphono)octyl]thymine (20). The product was obtained from **16** (1.59 g) and triethyl phosphite, followed by column chromatography (CH_2Cl_2:acetone, 2:1). Yield: 74%. ^{1}H NMR ($CDCl_3$) δ: 1.10−1.85 (m, 14H), 1.34 (t, J = 7.1 Hz, 6H, CH_2CH_3), 1.92 (s, 3H, 5-CH_3), 3.67 (t, J = 7.3 Hz, 2H, CH_2N), 4.0−4.30 (m, 4H, CH_2CH_3), 6.97 (s, 1H, H-6), 8.67 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.1 (5-CH_3), 16.2 (d, $J_{C,P}$ = 6.1 Hz, CH_2CH_3), 22.1 (d, $J_{C,P}$ = 5.3 Hz, CH_2CH_2P), 25.4 (d, $J_{C,P}$ = 141.1 Hz, CH_2P), 26.1, 28.8, 28.7 (CH_2), 30.2 [d, $J_{C,P}$ = 16.1 Hz, $CH_2(CH_2)_2P$], 48.2 (CH_2N), 61.2 (d, $J_{C,P}$ = 6.9 Hz, CH_2CH_3), 110.3 (C-5), 140.2 (C-6), 151.0 (C-2), 164.5 (C-4). Anal. ($C_{17}H_{31}N_2O_5P$) C, H, N.

1-[8-(Diisopropylphosphono)octyl]thymine (21). The product was obtained from **16** (1.59 g) and triisopropyl phosphite, followed by column chromatography (EtOAc:acetone, 2:1). Yield: 58%.^{1}H NMR ($CDCl_3$) δ: 1.00−1.90 (m, 14H), 1.30 [d, J = 6.2 Hz, 12H, $CH(CH_3)_2$], 1.92 (s, 3H, 5-CH_3), 3.67 (t, J = 7.3 Hz, 2H, CH_2N), 4.40−4.80 [m, 2H, $CH(CH_3)_2$], 6.97 (s, 1H, H-6), 8,60 (br s, 1H, NH). Anal. ($C_{19}H_{35}N_2O_5P$) C, H, N.

1-[9-(Diethylphosphono)nonyl]thymine (22). The product was obtained from **17** (1.66 g) and triethyl phosphite, followed by column chromatography (CH_2Cl_2:acetone, 2:1). Yield: 86%. ^{1}H NMR ($CDCl_3$) δ: 1.10−1.85 (m, 16H), 1.34 (t, J = 7.1 Hz, 6H, CH_2CH_3), 1.92 (s, 3H, 5-CH_3), 3.67 (t, J = 7.2 Hz, 2H, CH_2N), 3.90−4.30 (m, 4H, CH_2CH_3), 6.97 (s, 1H, H-6), 8.70 (br s, 1H, NH). Anal. ($C_{18}H_{33}N_2O_5P$) C, H, N.

General Procedure for the Deprotection of 1-[(Dialkylphosphono)alkyl]thymines. To a cooled solution of the corresponding 1-[(dialkylphosphono)alkyl]thymines (**18**−**22**) (1.00 mmol) in dry DMF or CH_2Cl_2 (5 mL) was added TMSBr (1.29 mL, 10.00 mmol). The mixture was stirred at room temperature for 18 h. Volatiles were removed, and the residue was stirred in concentrated NH_4OH (3 mL) for 30 min. The mixture was evaporated and applied onto an Amberlite XAD-2 column and eluted with H_2O and H_2O:MeOH (50:50). UV positive fractions were collected, evaporated, and purified by DEAD-Sephadex A25 (HCO_3^- form), eluting with a gradient H_2O−0.15 M NH_4HCO_3. Appropriate fractions were evaporated, coevaporated with H_2O, and lyophilized.

1-(6-Phosphonohexyl)thymine Ammonium Salt (1). Compound **18** (99 mg, 0.26 mmol) was deprotected and purified following the general procedure to afford 55 mg (68%) of **1** as a white lyophilate. UV (H_2O) λ_{max} = 273 (ϵ = 8700). MS (ES, negative mode): m/z 389 (M − NH_4)$^-$. ^{1}H NMR (D_2O) δ: 1.00−1.70 (m, 10H), 1.75 (s, 3H, 5-CH_3), 3.63 (t, J = 7.1 Hz, 2H, CH_2N), 7.37 (s, 1H, H-6). ^{13}C NMR (D_2O): 12.5 (5-CH_3), 24.2 (d, $J_{C,P}$ = 4.5 Hz, CH_2CH_2P), 26.4, 29.2 (CH_2), 29.0 (d, $J_{C,P}$ = 132.2 Hz, CH_2P), 31.0 (d, $J_{C,P}$ = 16.5 Hz, $CH_2CH_2CH_2P$), 50.0 (CH_2N), 111.8 (C-5), 144.7 (C-6), 153.7 (C-2), 168.3 (C-4). HPLC: system I, Rt = 14.50 min (100%); system II, Rt = 22.33 min (100%).

1-(7-Phosphonoheptyl)thymine Ammonium Salt (2). Starting from **19** (100 mg, 0.28 mmol) and following the general procedure, 65 mg (73%) of **2** was obtained as a white lyophilate. UV (H_2O) λ_{max} = 274 (ϵ = 8600). MS (ES, negative mode): m/z 303 (M − NH_4)$^-$. ^{1}H NMR (D_2O) δ: 1.10−1.75 (m, 12H), 1.83 (s, 3H, 5-CH_3), 3.70 (t, J = 7.1 Hz, 2H, CH_2N), 7.45 (s, 1H, H-6). ^{13}C NMR (D_2O): 12.5 (5-CH_3), 24.0 (d, $J_{C,P}$ = 4.5 Hz, CH_2CH_2P), 26.6, 29.1, 29.3 (CH_2), 28.7 (d, $J_{C,P}$ = 132.9 Hz, CH_2P), 31.2 (d, $J_{C,P}$ = 16.6 Hz, $CH_2CH_2CH_2P$), 49.9 (CH_2N), 111.8 (C-5), 144.6 (C-6), 153.6 (C-2), 168.3 (C-4). HPLC: system I, Rt = 13.06 min (100%); system II, Rt = 21.01 min (100%).

1-(8-Phosphonooctyl)thymine Ammonium Salt (3). Compound **20** (100 mg, 0.25 mmol) was deprotected and purified following the general procedure to afford 68 mg (81%) of **3** as a white lyophilate. UV (H_2O) λ_{max} = 274 (ϵ = 8700). MS (ES, negative mode): m/z 317 (M − NH_4)$^-$. ^{1}H NMR (D_2O) δ: 1.10−1.75 (m, 14H), 1.79 (s, 3H, 5-CH_3), 3.66 (t, J = 7.0 Hz, 2H, CH_2N), 7.41 (s, 1H, H-6). HPLC: system I, Rt = 14.40 min (100%); system II, Rt = 22.46 min (99.6%).

1-(9-Phosphononononyl)thymine Ammonium Salt (4). Deprotection of **22** (100 mg, 0.26 mmol) according to the general procedure afforded **4** (55 mg, 67%) as a white lyophilate. UV (H_2O) λ_{max} = 275 (ϵ = 9000). MS (ES, negative mode): m/z 331 (M − NH_4)$^-$. ^{1}H NMR (D_2O) δ: 1.10−1.75 (m, 16H), 1.84 (s, 3H, 5-CH_3), 3.71 (t, J = 7.0 Hz, 2H, CH_2N), 7.46 (s, 1H, H-6). ^{13}C NMR (D_2O): 12.5 (5-CH_3), 24.2 (d, $J_{C,P}$ = 3.9 Hz, CH_2CH_2P), 26.7, 29.2, 29.5, 29.6 (CH_2), 29.0 (d, $J_{C,P}$ = 131.4 Hz, CH_2P), 31.4 (d, $J_{C,P}$ = 16.0 Hz, $CH_2CH_2CH_2P$), 50.0 (CH_2N), 111.8 (C-5), 144.7 (C-6), 153.7 (C-2), 168.3 (C-4). HPLC: system I, Rt = 15.51 min (96.9%); system II, Rt = 23.48 min (97.5%).

(*Z*)-4-[(Diisopropylphosphono)methoxy]-1-triphenylmethoxy-2-butene (24). A solution of **23**[23] (4.50 g, 13.62 mmol) and diisopropyl[(*p*-toluensulfonyl)oxy]methanephosphonate[24] (7.50 g, 20.43 mmol) in dry DMF (81 mL) was cooled at −20 °C. Then, NaH (1.20 g, 60% dispersion in mineral oil, 30.00 mmol) was added, and the reaction was allowed to reach room temperature and stirred overnight. Volatiles were removed, and the residue was treated with EtOAc (100 mL) and brine (100 mL). The organic phase was dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue was purified by column chromatography (CH_2Cl_2:EtOAc, 20:1) to yield 2.98 g (43% yield) of **24** as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.32, 1.35 [2d, J = 6.0 Hz, 12H, $CH(CH_3)_2$], 3.63 (d, J = 9.2 Hz, 2H, CH_2P), 3.68 (d, J = 6.2 Hz, 2H, CH_2OTr), 4.00 (d, J = 6.4 Hz, 2H, CH_2OCH_2P), 4.60−4.90 [m, 2H, $CH(CH_3)_2$], 5.56−6.00 (m, 2H, CH=CH), 7.10−7.70 (m, 15H, arom.). Anal. ($C_{30}H_{37}O_5P$) C, H, N.

(*Z*)-4-[(Diisopropylphosphono)methoxy]-2-buten-1-ol (25). A solution of **24** (2.80 g, 5.50 mmol) in 80% AcOH (90 mL) was heated at 60 °C for 20 min. Solvents were removed, and the residue was purified by column chromatography (CH_2Cl_2:MeOH, 20:1) to yield 261 mg (96% yield) as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.30, 1.31 [2d, J = 6.2 Hz, 12H, $CH(CH_3)_2$], 3.34 (br s, 1H, OH), 3.70 (d, J = 8.2 Hz, 2H, CH_2P), 4.17, 4.20 (2d, J = 6.7, 6.5 Hz, 4H, CH_2O), 4.62−4.82 [m, 2H, $CH(CH_3)_2$], 5.54−5.92 (m, 2H, CH=CH). ^{13}C NMR ($CDCl_3$) δ: 23.8−24.1 [$CH(CH_3)_2$], 58.0 (CH_2OH), 64.0 (d, $J_{C,P}$ = 167.7 Hz, CH_2P), 67.9 (d, $J_{C,P}$ = 11.1 Hz, CH_2OCH_2P), 71.3 [d, $J_{C,P}$ = 7.1 Hz, $CH(CH_3)_2$], 126.3, 133.8 (CH=CH). Anal. ($C_{11}H_{23}O_5P$) C, H, N.

1-[(*Z*)-4-[(Diisopropylphosphono)methoxy]-2-butenyl]thymine (26). To a solution of **25** (400 mg, 1.50 mmol), Ph_3P (788 mg, 3.00 mmol), and N^3-benzoylthymine (692 mg, 3.00 mmol) in dry THF (15 mL) was slowly added a solution of DIAD (0.61 mL, 3.00 mmol) in dry THF (5 mL). The mixture was stirred at room temperature for 2 h. Volatiles were removed, and the residue was treated with $MeNH_2$ in MeOH (20 mL) overnight. Solvents were evaporated, and the residue was purified by column chromatography (CH_2Cl_2:acetone, 1:2) to yield 350 mg (62%) of **26** as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.33, 1.34 [2d, J = 6.2 Hz, 12H, $CH(CH_3)_2$], 1.92 (s, 3H, 5-CH_3), 3.77 (d, J = 8.2 Hz, 2H, CH_2P), 4.30 (d, J = 6.1 Hz, 2H, CH_2N), 4.44 (d, J = 6.8 Hz, 2H, CH_2OCH_2P), 4.60−4.90 [m, 2H, $CH(CH_3)_2$], 5.56−5.92 (m, 1H, CH=CH), 7.15 (s, 1H, H-6), 8.65 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.1 (5-CH_3), 23.8−24.1 [$CH(CH_3)_2$], 44.6 (CH_2N), 64.9 (d, $J_{C,P}$ = 168.5 Hz, CH_2P), 68.1 (d, $J_{C,P}$ = 10.9 Hz, CH_2OCH_2P), 71.1 [d, $J_{C,P}$ = 6.7 Hz, $CH(CH_3)_2$], 110.9 (C-5), 127.5, 129.9 (CH=CH), 140.0 (C-6), 151.0 (C-2), 164.4 (C-4). Anal. ($C_{16}H_{27}N_2O_6P$) C, H, N.

1-[(*Z*)-4-Phosphonomethoxy-2-butenyl]thymine Ammonium Salt (5). Compound **26** (100 mg, 0.27 mmol) was deprotected following the general procedure for deprotection of 1-[(dialkylphosphono)alkyl]thymines to yield 60 mg (73%) of **5**[27] as a white lyophilate. UV (H_2O) λ_{max} = 272 (ϵ = 9000). MS (ES, negative mode): m/z 289 (M − NH_4)$^-$. ^{1}H NMR (D_2O) δ: 1.75 (s, 3H, 5-CH_3), 3.42 (d, J = 8.7 Hz, 2H, CH_2P), 4.15 (d, J = 6.5 Hz, 2H, CH_2OCH_2P), 4.34 (d, J = 6.8 Hz, 2H, CH_2N), 5.50−5.80 (m, 2H, CH=CH), 7.36 (s, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ: 12.5 (5-CH_3), 46.7 (CH_2N), 67.5 (d, $J_{C,P}$ = 157.7

Hz, CH_2P), 69.0 (d, $J_{C,P}$ = 11.6 Hz, CH_2OCH_2P), 112.2 (C-5), 128.5, 131.1 (CH=CH), 144.0 (C-6), 153.5 (C-2), 168.3 (C-4). HPLC: system I, Rt = 9.01 min (98.7%); system II, Rt = 11.60 min (98.3%).

1-[4-[(Diisopropylphosphono)methoxy]butyl]thymine (27). A solution of **26** (190 mg, 0.51 mmol) in EtOH (15 mL) was hydrogenated at room temperature in the presence of 10% Pd/C (50 mg) at 30 psi for 4 h. The mixture was filtered, and the filtrate was purified in the chromatotron CCTLC (CH_2Cl_2:MeOH, 15:1) to give 150 mg (80% yield) of **27** as a syrup. ^{1}H NMR (acetone-d_6) δ: 1.28, 1.30 [2d, J = 6,2 Hz, 12H, CH(CH_3)$_2$], 1.45−1.90 (m, 4H, CH_2), 1.84 (s, 3H, 5-CH_3), 3.62, 3.76 (2t, J = 6.1, 7.0 Hz, 4H, CH_2O, CH_2N), 3.71 (d, J = 8.3 Hz, CH_2P), 4.60−4.80 [m, 2H, CH(CH_3)$_2$], 7.48 (s, 1H, H-6), 9.97 (br s, 1H, NH). Anal. ($C_{16}H_{29}N_2O_6P$) C, H, N.

1-[(4-Phosphonomethoxy)butyl]thymine Disodium Salt (6). Compound **27** (100 mg, 0.27 mmol) was deprotected following the general procedure for deprotection of 1-[(dialkylphosphono)alkyl]thymines and then transformed into the disodium salt by eluting through a Dowex 50WX4 (Na^+ form) to yield 50 mg (61%) of **6** as a white lyophilate. UV (H_2O) λ_{max} = 271 (ϵ = 8400). MS (ES, negative mode): m/z 291 (M + H − 2Na)$^-$. ^{1}H NMR (D_2O) δ: 1.40−1.65 (m, 4H, CH_2), 1.71 (s, 3H, 5-CH_3), 3.45 (d, J = 8.5 Hz, 2H, CH_2P), 3.46, 3.62 (2t, J = 7.3, 7.1 Hz, 4H, CH_2O, CH_2N), 7.35 (s, 1H, H-6). ^{13}C NMR (D_2O) δ: 12.5 (5-CH_3), 26.1, 26.6 (CH_2), 49.6 (CH_2N), 68.0 (d, J = 155.6 Hz, CH_2P), 73.6 (d, J = 10.6 Hz, CH_2O), 111.8 (C-5), 144.5 (C-6), 153.6 (C-2), 168.3 (C-4). HPLC: system I, Rt = 10.53 min (99.1%); system II, Rt = 11.90 min (98.8%).

(2*S*,3*S*)- and (2*R*,3*R*)-1-[2,3-Dihydroxy-4-[(diisopropylphosphono)methoxy]butyl]thymine (28). To a solution of **26** (136 mg, 0.363 mmol) in acetone:H_2O (90:10) (8 mL) were added *N*-methylmorpholine-*N*-oxide (0.075 mL of a 60% aqueous solution, 0.440 mmol) and OsO_4 (0.044 mL of a 4% aqueous solution, 0.007 mmol). The mixture was stirred at room temperature for 3 days. A small portion of $Na_2S_2O_5$ was added, and the mixture was filtered through Celite. The filtrate was evaporated and purified by column chromatography (CH_2Cl_2: acetone, 1:2) to yield 118 mg (79%) of **28** as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.32, 1.33 [2d, J = 6.2 Hz, 12H, CH(CH_3)$_2$], 1.89 (s, 3H, 5-CH_3), 3.40−3.52 (m, 1H, CHOH), 3.66 (dd, J = 4.6, 14.5 Hz, 1H, CHP), 3.65−4.04 (m, 5H, CHOH, CHP, CH_2-OCH_2P, CHN), 4.11 (dd, J = 4.4, 14.3 Hz, 1H, CHN), 4.13 (d, J = 6.5 Hz, 1H, OH), 4.62−4.84 [m, 2H, CH(CH_3)$_2$], 5.01 (d, J = 4.5 Hz, 1H, OH), 7.25 (s, 1H, H-6), 9.21 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.2 (5-CH_3), 23.8−24.1 [CH(CH_3)$_2$], 50.8 (CH_2N), 65,9 (d, $J_{C,P}$ = 166.7 Hz, CH_2P), 70.1, 70,6 (CHOH), 71.5 [d, $J_{C,P}$ = 6.4 Hz, CH(CH_3)$_2$], 74.0 (d, $J_{C,P}$ = 6.0 Hz, CH_2-OCH_2P), 110.0 (C-5), 142.8 (C-6), 152.5 (C-2), 164.6 (C-4). Anal. ($C_{16}H_{29}N_2O_8P$) C, H, N.

(2*S*,3*S*)- and (2*R*,3*R*)-1-[2,3-Dihydroxy-4-[(phosphonomethoxy)butyl]thymine Ammonium Salt (7). A solution of **28** (80 mg, 0.20 mmol) in dry DMF (3 mL) was treated with TMSBr (0.26 mL, 2.00 mmol) overnight and was coevaporated with H_2O. The residue was treated with a mixture of acetone: H_2O (1:6) at 5 °C for 24 h. The solid obtained was filtered and dissolved in NH_4OH to obtain the ammonium salt. The residue was finally purified by DEAD-Sephadex A25 (HCO_3^- form), eluting with a gradient of H_2O−0.15 M NH_4HCO_3. Appropriate fractions were evaporated, coevaporated with H_2O, and lyophilized to give 24 mg (50%) of **7** as a white lyophilate. UV (H_2O) λ_{max} = 272 (ϵ = 9600). MS (ES, negative mode): m/z 323 (M − NH_4)$^-$; ^{1}H NMR (D_2O) δ: 1.81 (s, 3H, 5-CH_3), 3.50−3.78 (m, 6H, CH_2P, CH_2OCH_2P, CHN, CHOH), 3.87 (ddd, J = 2.7, 7.5, 9.0 Hz, 1H, CHOH), 4.09 (dd, J = 2.7, 14.3 Hz, 1H, CHN), 7.40 (H-6). ^{13}C NMR (D_2O) δ: 12.5 (5-CH_3), 52.4 (CH_2N), 68.9 (d, $J_{C,P}$ = 168.0 Hz, CH_2P), 70.5, 72.5 (CHOH), 74.6 (d, $J_{C,P}$ = 11.1 Hz, CH_2OCH_2P), 111.6 (C-5), 145.3 (C-6), 153.8 (C-2), 168.3 (C-4). HPLC: system I, Rt = 4.85 min (97.6%); system II, Rt = 5.93 min (100%).

(*Z*)-1-Benzoxy-4-chloromethoxy-2-butene (30). To a cooled solution of **29**[28] (2.46 g, 12.80 mmol) and paraformaldehyde (1.93 g, 64.20 mmol) in dry CH_2Cl_2 (39 mL) was bubbled HCl(g) for 2 h. The mixture was stored at 4 °C for an additional 6 h. Then, $MgSO_4$ was added, filtered, and evaporated. The resultant syrup was used as such in the next steps. ^{1}H NMR ($CDCl_3$) δ: 4.34 (d, J = 6.3 Hz, 2H, CH_2OCH_2Cl), 4.86 (d, J = 6.1 Hz, 2H, CH_2OBz), 5.45 (s, 2H, CH_2OCH_2Cl), 5.60−6.00 (m, 2H, CH=CH), 7.15−8.10 (m, 5H, arom.).

(*Z*)-1-Benzoxy-4-(acetoxy)methoxy-2-butene (31). To a solution of the crude **30** in acetone (31 mL) was added AcONa (3.57 g, 43.53 mmol). The mixture was stirred at room temperature for 18 h. It was filtered, evaporated, and used as such in the next steps. ^{1}H NMR ($CDCl_3$) δ: 2.10 (s, 3H, CH_3-CO), 4.35 (d, J = 5.3 Hz, 2H, CH_2OCH_2OAc), 4.90 (d, J = 5.3 Hz, 2H, CH_2OBz), 5.30 (s, 2H, CH_2OCH_2OAc), 5.60−6.00 (m, 2H, CH=CH), 7.30−8.20 (m, 5H, arom.).

(*Z*)-1-Benzoxy-4-[(diisopropylphosphono)methoxy)-methoxy]-2-butene (32). To a solution of the crude **31** and diisopropyl hydroxymethylphosphonate (7.55 g, 38.52 mmol) in dry CH_3CN (46 mL) at 0 °C, TMSTf (3.5 mL, 3.77 mmol) was added. The mixture was allowed to reach room temperature and stirred for 4 h. Then, it was poured onto a cooled $NaHCO_3$ solution and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts were dried on Na_2SO_4, filtered, and evaporated. The residue was purified by column chromatography (EtOAc:hexane, 1:1) to yield 4.66 g (91%) of **32** as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.33, 1.34 [2d, J = 6.1 Hz, 12H, CH(CH_3)$_2$], 3.81 (d, J = 9.1 Hz, 2H, CH_2P), 4.28 (d, J = 5.1 Hz, 2H, CH_2OCH_2O), 4.76 (s, 2H, OCH_2O), 4.60−4.88 [m, 2H, CH(CH_3)$_2$], 4.90 (d, J = 5.4 Hz, 2H, CH_2OBz), 5.70−6.00 (m, 2H, CH=CH), 7.30−8.20 (m, 5H, arom.). Anal. ($C_{19}H_{29}O_7P$) C, H, N.

(*Z*)-4-[((Diisopropylphosphono)methoxy)methoxy]-2-buten-1-ol (33). Compound **32** (960 mg, 2.40 mmol) was treated with $MeNH_2$ in MeOH (25 mL) overnight. Volatiles were removed, and the residue was purified by column chromatography (EtOAc) to yield 504 mg (68%) of **33** as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.33, 1.34 [2d, J = 6.1 Hz, 12H, CH(CH_3)$_2$], 2.90 (br s, 1H, OH), 3.78 (d, J = 9.5 Hz, 2H, CH_2P), 4.10−4.30 (m, 4H, CH_2OH and CH_2OCH_2O), 4.73 (s, 2H, OCH_2O), 4.60−4.90 [m, 2H, CH(CH_3)$_2$], 5.50−6.00 (m, 2H, CH=CH). ^{13}C NMR ($CDCl_3$) δ: 23.8−24.1 [CH(CH_3)$_2$], 58.0 (CH_2OH), 61.5 (d, $J_{C,P}$ = 172.2 Hz, CH_2P), 63.1 (CH_2OCH_2O), 71.2 [d, $J_{C,P}$ = 6.6 Hz, CH(CH_3)$_2$], 95.2 (d, $J_{C,P}$ = 12.5 Hz, OCH_2O), 126.8, 133.1 (CH=CH). Anal. ($C_{12}H_{25}O_6P$) C, H, N.

1-[(*Z*)-4-[((Diisopropylphosphono)methoxy)methoxy]-2-butenyl]thymine (34). Compound **33** (504 mg, 1.70 mmol) was reacted with N^3-benzoylthymine, as described for the synthesis of **26**. After column chromatography (CH_2Cl_2: acetone, 2:1), 526 mg (76% yield) of **34** was obtained as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.30, 1.31 [2d, J = 6.2 Hz, 12H, CH(CH_3)$_2$], 1.90 (s, 3H, 5-CH_3), 3.78 (d, J = 9.3 Hz, 2H, CH_2P), 4.24 (d, J = 6.4 Hz, 2H, CH_2N), 4.40 (d, J = 5.9 Hz, 2H, CH_2OCH_2O), 4.72 (s, 2H, OCH_2O), 4.60−4.90 [m, 2H, CH(CH_3)$_2$], 5.50−6.00 (m, 2H, CH=CH), 7.08 (s, 1H, H-6), 9.90 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.2 (5-CH_3), 23.8−24.1 [CH(CH_3)$_2$], 44.5 (CH_2N), 61.6 (d, $J_{C,P}$ = 172.8 Hz, CH_2P), 62.4 (CH_2OCH_2O), 71.1 [d, $J_{C,P}$ = 6.5 Hz, CH(CH_3)$_2$], 95.1 (d, $J_{C,P}$ = 10.4 Hz, OCH_2O), 111.0 (C-5), 127.4, 130.1 (CH=CH), 139.9 (C-6), 150.7 (C-2), 164.2 (C-4). Anal. ($C_{17}H_{29}N_2O_7P$) C, H, N.

1-[(*Z*)-4-[(Phosphonomethoxy)methoxy]-2-butenyl]-thymine Ammonium Salt (8). To a solution of **34** (65 mg, 0.16 mmol) in dry CH_2Cl_2 (2 mL) and 2,6-lutidine (0.28 mL, 2.45 mmol), cooled at 0 °C, was added TMSBr (0.21 mL, 1.60 mmol). After 18 h, solvents were removed. The residue was treated with H_2O (20 mL) and extracted with CH_2Cl_2 (2 × 5 mL). The aqueous phase was treated with NH_4OH (3 mL) for 1 h and then evaporated. The residue was purified by ion exchange chromatography DEAD-Sephadex A25 (HCO_3^- form), eluting with a gradient of H_2O−0.15 M NH_4HCO_3. Appropriate fractions were evaporated, coevaporated with H_2O, and lyophilized to give 38 mg (70%) of **8** as a white lyophilate. UV (H_2O) λ_{max} = 272 (ϵ = 8700). MS (ES negative mode): m/z 319 (M − NH_4)$^-$. ^{1}H NMR (D_2O) δ: 1.68 (s, 3H, 5-CH_3), 3.52 (d, J = 9.1 Hz, 2H, CH_2P), 4.14, 4.28 (2d, J = 6.6, 6.8 Hz, 4H, CH_2N, CH_2-OCH_2O), 4.62 (s, 2H, OCH_2O), 5.46−5.78 (m, 2H, CH=CH), 7.29 (H-6). ^{13}C NMR ($CDCl_3$) δ: 12.5 (5-CH_3), 46.5 (CH_2N),

63.9 (CH_2OCH_2O), 64.7 (d, $J_{C,P}$ = 158.1 Hz, CH_2P), 96.5 (d, $J_{C,P}$ = 12.6 Hz, OCH_2O), 112.1 (C-5), 128.7, 130.6 (CH=CH), 143.9 (C-6), 153.4 (C-2), 168.2 (C-4). HPLC: system I, Rt = 11.15 min (97.8%); system II, Rt = 12.48 min (97.3%).

1-[4-[((Diisopropylphosphono)methoxy)methoxy]butyl]-thymine (35). Compound **34** (210 mg, 0.52 mmol) was hydrogenated as described for **27**, to yield after column chromatography (EtOAc:MeOH 10:1) 150 mg (66%) of **35** as a syrup that was used in the next step.

1-[4-[(Phosphonomethoxy)methoxy]butyl]thymine Ammonium Salt (9). A solution of **35** (85 mg, 0.21 mmol) and 2,6-lutidine (0.36 mL, 3.15 mmol) in dry CH_2Cl_2 was reacted with TMSBr (0.27 mL, 2.10 mmol) as described for the synthesis of **8**. After purification, 60 mg (84%) of **9** was obtained as a white lyophilate. UV (H_2O) λ_{max} = 273 (ϵ = 9400). MS (ES, negative mode): *m/z* 321 $(M - NH_4)^-$. ^{1}H NMR (D_2O) δ: 1.40−1.58 (m, 4H, CH_2), 1.66 (s, 3H, 5-CH_3), 3.42 (d, J = 9.1 Hz, 2H, OCH_2P), 3.45, 3.57 (2t, J = 6.1, 7.0 Hz, 4H, CH_2N, CH_2OCH_2O), 4.54 (s, 2H, OCH_2O), 7.30 (s, 1H, H-6). ^{13}C NMR (D_2O) δ: 12.5 (5-CH_3), 26.2, 26.8 (CH_2), 49.5 (CH_2N), 65.1 (d, $J_{C,P}$ = 156.2 Hz, CH_2P), 68.7 (CH_2OCH_2P), 97.0 (d, $J_{C,P}$ = 12.0 Hz, OCH_2O), 111.8 (C-5), 144.3 (C-6), 153.5 (C-2), 168.2 (C-4). HPLC: system I, Rt = 11.11 min (100%); system II, Rt = 12.53 min (99.9%).

(2*S*,3*S*)- and (2*R*,3*R*)-1-[2,3-Dihydroxy-4-[((diisopropylphosphono)methoxy)methoxy]butyl]thymine (36). Starting from **34** (200 mg, 0.49 mmol), a procedure analogous to that described for the synthesis of **28** was followed. After column chromatography (CH_2Cl_2:acetone, 1:2), 167 mg (76% yield) of **36** was obtained as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.32, 1.33 [2d, J = 6.2 Hz, 12H, $CH(CH_3)_2$], 1.90 (s, 3H, 5-CH_3), 3.30−3.60 (m, 1H, C*H*OH), 3.71 (dd, J = 3.2, 9.9 Hz, 1H, C*H*OCH_2O), 3.86 (d, J = 8.8 Hz, 2H, CH_2P), 3.84−4.04 (m, 2H, C*H*OH, CHN), 4.10 (dd, J = 3.1, 10.0 Hz, 1H, C*H*OCH_2O), 4.14 (dd, J = 3.9, 15.3 Hz, 1H, CHN), 4.32 (d, J = 5.6 Hz, 1H, OH), 4.62−4.82 [m, 2H, C*H*$(CH_3)_2$], 4.73 (s, 2H, OCH_2O), 5.29 (d, J = 3.5 Hz, 1H, OH), 7.29 (s, 1H, C-6), 9.00 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.2 (5-CH_3), 23.8−24.1 [$CH(CH_3)_2$], 51.1 (CH_2N), 62.4 (d, $J_{C,P}$ = 173.2 Hz, CH_2P), 68.8 (CH_2OCH_2O), 69.7, 70.7 (CHOH), 71.4, 71.8 [2d, $J_{C,P}$ = 6.9 Hz, $CH(CH_3)_2$], 96.6 (d, $J_{C,P}$ = 9.9 Hz, OCH_2O), 110.0 (C-5), 142.9 (C-6), 152.6 (C-2), 164.5 (C-4). Anal. ($C_{17}H_{31}N_2O_9P$) C, H, N.

(2*S*,3*S*)- and (2*R*,3*R*)-1-[2,3-Dihydroxy-4-[(phosphonomethoxy)methoxy]butyl]thymine Ammonium Salt (10). Starting from **36** (80 mg, 0.18 mmol) and following an analogous procedure to that described for the synthesis of **7**, compound **10** was isolated as a white lyophilate (22 mg, 46% yield). UV (H_2O) λ_{max} = 272 (ϵ = 9000). MS (ES, negative mode): *m/z* 353 $(M - NH_4)^-$. ^{1}H NMR (D_2O) δ: 1.81 (s, 3H, 5-CH_3), 3.62 (d, J = 9.2 Hz, 2H, CH_2P), 3.45−3.90 (m, 5H, CHN, CH_2OCH_2O, C*H*OH x 2), 4.10 (dd, J = 2.0, 14.4 Hz, 1H, CHN), 4.72 (s, 2H, OCH_2O), 7.39 (s, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ: 12.3 (5-CH_3), 52.2 (CH_2N), 64.5 (d, $J_{C,P}$ = 157.0 Hz, CH_2P), 69.8 (CH_2OCH_2O), 70.4, 72.4 (CHOH), 97.3 (d, $J_{C,P}$ = 11.4 Hz, OCH_2O), 111.3 (C-5), 145.1 (C-6), 153.5 (C-2), 168.1 (C-4). HPLC: system I, Rt = 4.88 min (98.8%); system II, Rt = 6.78 min (100%).

1-[((*Z*)-4-Benzoxy-2-butenoxy)methyl]thymine (41). Method A. A suspension of thymine (394 mg, 3.12 mmol) and $(NH_4)_2SO_4$ (13 mg) in hexamethyldisilazane (3 mL) was refluxed overnight. The resulting solution was evaporated and coevaporated with dry toluene. To the resulting syrup were added a solution of **30** (freshly prepared from 500 mg of **29**) in dry CH_2Cl_2 (5 mL) and Bu_4NI (192 mg, 0.52 mmol). The mixture was stirred at room temperature for 24 h. Then, CH_2Cl_2 (20 mL) and cooled saturated solution of $NaHCO_3$ (10 mL) were added. The aqueous phase was further extracted with CH_2Cl_2 (2 × 10 mL). The combined organic extracts were dried on anhydrous Na_2SO_4, filtered, and evaporated. The residue obtained was purified by column chromatography (EtOAc: hexane, 1:1) to yield 615 mg (72%) of **41**. Mp: 80−81 °C (EtOAc: diethyl ether). ^{1}H NMR ($CDCl_3$) δ: 1.93 (s, 3H, 5-CH_3), 4.30 (d, J = 5.2 Hz, 2H, CH_2OCH_2N), 4.88 (d, J = 5.8 Hz, 2H, CH_2-OBz), 5.16 (s, 2H, NCH_2O), 5.60−6.00 (m, 2H, CH=CH), 7.16 (s, 1H, H-6), 7.30−8.10 (m, 5H, arom.), 8.67 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.1 (5-CH_3), 60.4 (CH_2OBz), 64.8 (CH_2-OCH_2N), 75.8 (NCH_2O), 111.6 (C-5), 127.5, 129.3 (CH=), 128.2 (2C, arom.), 129.4 (2C, arom.), 129.8 (arom.), 132.8 (arom.), 138.9 (C-6), 151.3 (C-2), 164.3 (C-4), 166.0 (CO). Anal. ($C_{17}H_{18}N_2O_5$) C, H, N.

Method B. Thymine was silylated as described in method A. To the syrup containing silylated thymine was added a solution of **31** (freshly prepared from 500 mg (2.60 mmol) of **29**) in dry CH_2Cl_2 (10 mL). The resulting solution was cooled to 0 °C, and then $SnCl_4$ (0.15 mL, 1.30 mmol) was added. The mixture was stirred for 4 h at room temperature. Then it was worked up as in method A. Purification by column chromatography (EtOAc:hexane, 1:1) afforded 77 mg (9%) of **41** from the fastest moving fractions; the slowest moving fractions afforded 146 mg (17%) of the N^3 isomer (**42**). Data for **42**: ^{1}H NMR ($CDCl_3$) δ: 1.90 (s, 3H, 5-CH_3), 4.34 (d, J = 4.0 Hz, 2H, CH_2OCH_2N), 4.90 (d, J = 4.2 Hz, 2H, CH_2OBz), 5.44 (s, 2H, NCH_2O), 5.70−6.00 (m, 2H, CH=CH), 7.09 (s, 1H, H-6), 7.30−8.10 (m, 5H, arom.), 10.27 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.9 (5-CH_3), 60.9 (CH_2OBz), 66.0 (CH_2OCH_2N), 69.9 (NCH_2O), 110.4 (C-5), 127.0, 130.4 (CH=), 128.4 (2C, arom.), 129.6 (2C, arom.), 130.0 (arom.), 133.0 (arom.), 135.2 (C-6), 153.2 (C-2), 164.0 (C-4), 166.4 (CO).

1-[((*Z*)-4-Hydroxy-2-butenoxy)methyl]thymine (43). Compound **41** (413 mg, 1.25 mmol) was treated with $MeNH_2$ in MeOH (15 mL) overnight. Volatiles were removed, and the residue was purified by column chromatography (EtOAc) to yield 279 mg (98% yield) of **43** as a solid. Mp: 88−89 °C (AcOEt: diethyl ether 1:1) (lit.[38] mp 79−81 °C). ^{1}H NMR ($CDCl_3$) δ: 1,95 (s, 3H, 5-CH_3), 2.70 (br s, 1H, OH), 4.14 (br t, J = 5.4 Hz, 2H, CH_2OH), 4.20 (d, J = 6.7 Hz, 2H, CH_2OCH_2N), 5.15 (s, 2H, NCH_2O), 5.50−5.94 (m, 2H, CH=CH), 7.16 (s, 1H, H-6), 9.20 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.1 (5-CH_3), 57.7 (CH_2-OH), 64.6 (CH_2OCH_2N), 75.3 (NCH_2O), 111.9 (C-5), 126.4, 133.4 (CH=CH), 139.0 (C-6), 151.8 (C-2), 164.3 (C-4). Anal. ($C_{10}H_{14}N_2O_4$) C, H, N.

1-[((*Z*)-4-(Diisopropylphosphono)methoxy)-2-butenoxy)-methyl]thymine (38). To a solution of **43** (230 mg, 1.02 mmol) in dry DMF (5 mL) at −30 °C and under Ar atmosphere was added NaH (122 mg of 60% dispersion in mineral oil, 3.06 mmol). The mixture was allowed to reach room temperature and stirred for 1 h. Then it was cooled again to −30 °C, and diisoproypl[(*p*-toluensulfonyl)oxy] methanephosphonate (427 mg, 1.22 mmol) was added. The reaction was allowed to reach room temperature and was stirred for 20 h. Then, it was neutralized with AcOH and evaporated, and the residue was purified by column chromatography. Elution with CH_2Cl_2: MeOH 25:1 afforded **38** (100 mg, 25%) as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.31, 1.32 [2d, J = 6.2 Hz, 12H, $CH(CH_3)_2$], 1.92 (s, 3H, 5-CH_3), 3.70 (d, J = 9.0 Hz, 2H, CH_2P), 4.13, 4.17 (2d, J = 4.9, 5.0 Hz, 4H, CH_2O), 4.60−4.90 [m, 2H, C*H*$(CH_3)_2$], 5.13 (s, 2H, NCH_2O), 5.50−5.85 (m, 2H, CH=CH), 7.13 (s, 1H, H-6), 9.37 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 12.3 (5-CH_3), 23.8−24.1 [m, $CH(CH_3)_2$], 64.8 (d, $J_{C,P}$ = 169.5 Hz, CH_2P), 65.2 (CH_2-OCH_2N), 68.4 (d, $J_{C,P}$ = 13.8 Hz, CH_2OCH_2P), 71.1 [d, $J_{C,P}$ = 6.9 Hz, $CH(CH_3)_2$], 76.1 (NCH_2O), 111.6 (C-5), 128.7, 129.2 (CH=CH), 138.9 (C-6), 151.2 (C-2), 164.1 (C-4). Anal. ($C_{17}H_{29}$-N_2O_7P) C, H, N.

Then elution with iPrOH−NH_4OH:H_2O (8:1:1) gave the monoprotected derivative **44** (116 mg, 31%). ^{1}H NMR (DMSO-d_6) δ: 1.10 [d, J = 5.0 Hz, 6H, $CH(CH_3)_2$], 1.75 (s, 3H, 5-CH_3), 3.38 (d, J = 8.6 Hz, 2H, CH_2P), 3.90−4.20 (m, 4H, CH_2O), 4.20−4.60 [m, 1H, C*H*$(CH_3)_2$], 5.03 (s, 2H, NCH_2O), 5.40−5.80 (m, 2H, CH=CH), 7.57 (s, 1H, H-6), 8.40 (br s, 1H, NH).

1-[((*Z*)-4-Phosphonomethoxy)-2-butenoxy)methyl]thymine Ammonium Salt (11). A solution of **38** (80 mg, 0.20 mmol) and 2,6-lutidine (0.35 mL, 3.06 mmol) in dry CH_2Cl_2 (3 mL) reacted with TMSBr (0.26 mL, 2.00 mmol) as described for compound **8**. A white lyophilate (44 mg, 75% yield) was obtained. UV (H_2O) λ_{max} = 265 (ϵ = 8400). MS (ES, negative mode): *m/z* 319 $(M - NH_4)^-$. ^{1}H NMR (D_2O) δ: 1.68 (s, 3H, 5-CH_3), 3.39 (d, J = 8.3 Hz, 2H, CH_2P), 3.95 (d, J = 5.1 Hz, 2H, CH_2O), 4.02 (d, J = 5.3 Hz, 2H, CH_2O), 4.99 (s, 2H,

NCH_2O), 5.40−5.70 (m, 2H, CH=CH), 7.34 (s, 1H, H-6). ^{13}C NMR (D_2O) δ: 12.3 (5-CH_3), 65.8 (CH_2OCH_2N), 67.6 (d, $J_{C,P}$ = 162.3 Hz, CH_2P), 69.1 (d, $J_{C,P}$ = 12.1 Hz, CH_2OCH_2P), 78.1 (NCH_2O), 112.5 (C-5), 129.7, 131.2 (CH=CH), 143.0 (C-6), 153.5 (C-2), 168.1 (C-4). HPLC: system I, Rt = 10.65 min (96.6%); system II, Rt = 12.15 min (96.1%).

***N*-[8-(Diethylphosphono)octyl]phthalimide (46).** A solution of **45**[34] (3.00 g, 8.87 mmol) in triethyl phosphite (30 mL) was heated at 130 °C for 12 h. Solvent was removed, and the residue was purified by column chromatography (CH_2Cl_2:acetone, 5:1) to yield 3.37 g (96%) of **46** as a syrup. ^1H NMR ($CDCl_3$) δ: 1.31 (t, J = 7.1 Hz, 6H, CH_2CH_3), 1.20−1.80 (m, 14H), 3.66 (t, J = 7.2 Hz, 2H, CH_2N), 3.96−4.20 (m, 4H, CH_2CH_3), 7.65−7.75 (m, 2H, arom.), 7.80−7.90 (m, 2H, arom.). ^{13}C NMR ($CDCl_3$) δ: 16.3 (d, $J_{C,P}$ = 5.4 Hz, CH_2CH_3), 22.2 (d, $J_{C,P}$ = 5.4 Hz, CH_2CH_2P), 25.5 (d, $J_{C,P}$ = 140.3 Hz, CH_2P), 26.6, 28.4, 28.8 (CH_2), 30.3 (d, $J_{C,P}$ = 16.9 Hz, $CH_2(CH_2)_2P$), 37.8 (CH_2N), 61.2 (d, $J_{C,P}$ = 6.1 Hz, CH_2CH_3), 123.0, 132.0, 133.7 (arom.), 168.3 (CO). Anal. ($C_{20}H_{30}NO_5P$) C, H, N.

8-(Diethylphosphono)octylamine (47). To a solution of **46** (455 mg, 1.15 mmol) in EtOH (40 mL) was added $H_2N-NH_2 \cdot H_2O$ (0.57 mL, 11.50 mmol), and the mixture was stirred at room temperature for 18 h. It was filtered, and the filtrate was evaporated. The residue was purified by column chromatography (CH_2Cl_2:acetone:NH_4OH, 5:1:0.6) to afford 275 mg (90% yield) of **47** as a syrup. ^1H NMR ($CDCl_3$) δ: 1.31 (t, J = 7.0 Hz, 6H, CH_2CH_3), 1.10−1.85 (m, 14H), 2.14 (br s, 2H, NH_2), 2.68 (t, J = 6.8 Hz, 2H, CH_2N), 3.90−4.20 (m, 4H, CH_2CH_3). ^{13}C NMR ($CDCl_3$) δ: 16.1 (d, $J_{C,P}$ = 5.4 Hz, CH_2CH_3), 22.1 (d, $J_{C,P}$ = 4.6 Hz, CH_2CH_2P), 25.3 (d, $J_{C,P}$ = 140.3 Hz, CH_2P), 26.5, 28.7, 28.8 (CH_2), 30.2 (d, $J_{C,P}$ = 16.9 Hz, $CH_2(CH_2)_2P$), 32.7 (CH_2CH_2N), 41.5 (CH_2N), 61.2 (d, $J_{C,P}$ = 6,1 Hz, CH_2CH_3).

***N*-[8-(Diethylphosphono)octyl]urea (48).** To a stirred solution of **47** (577 mg, 2.18 mmol) in H_2O (3.5 mL) at 10 °C was added concentrated HCl (0.22 mL, 2.18 mmol). After a few minutes, KOCN (353 mg, 4.36 mmol) was added, and the mixture was heated at 70 °C overnight. Then, it was cooled and extracted with CH_2Cl_2 (15 mL). The organic phase was dried on anhydrous Na_2SO_4, filtered, and evaporated. The obtained syrup containing **48** (487 mg) was used as such in the next step. ^1H NMR ($CDCl_3$) δ: 1.31 (t, J = 7.0 Hz, 6H, CH_2CH_3), 1.10−1.90 (m, 14H), 3.15 (q, J = 6.5 Hz, 2H, CH_2N), 3.70−4.30 (m, 4H, CH_2CH_3), 4.63 (br s, 2H, NH_2), 5.13 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 16.3 (d, $J_{C,P}$ = 5.4 Hz, CH_2CH_3), 22.1 (d, $J_{C,P}$ = 4.6 Hz, CH_2CH_2P), 25.3 (d, $J_{C,P}$ = 139.6 Hz, CH_2P), 26.5, 28.7, 29.9 (CH_2), 30.1 [d, $J_{C,P}$ = 18.4 Hz, $CH_2(CH_2)_2P$], 40.0 (CH_2N), 61.4 (d, $J_{C,P}$ = 6.1 Hz, CH_2CH_3), 159.7 (CO).

6-Amino-1-[8-(diethylphosphono)octyl]uracil (49). To a 1.7 M solution of NaEtO in EtOH (0.45 mL) were added ethylcyanoacetate (0.07 mL, 0.65 mmol) and the urea **48** (200 mg, 0.65 mmol). The mixture was refluxed for 20 h. Then, it was cooled and neutralized with 1 M HCl, diluted with EtOH (10 mL), and filtered. The filtrate was purified in the Chromatotron CCTLC (CH_2Cl_2:MeOH, 10:1). Compound **49** (160 mg, 66% yield) was obtained as a syrup. ^1H NMR (methanol-d_4) δ: 1.51 (t, J = 7.0 Hz, 6H, CH_2CH_3), 1.40−2.10 (m, 14H), 4.03 (t, J = 7.3 Hz, 2H, CH_2N), 4.14−4.40 (m, 4H, CH_2CH_3). ^{13}C NMR (methanol-d_4) δ: 17.0 (d, $J_{C,P}$ = 5.4 Hz, CH_2CH_3), 23.6 (d, $J_{C,P}$ = 4.6 Hz, CH_2CH_2P), 26.0 (d, $J_{C,P}$ = 139.6 Hz, CH_2P), 27.7, 29.0, 30.3, 30.4 (CH_2), 31.6 [d, $J_{C,P}$ = 16.1 Hz, $CH_2(CH_2)_2P$], 43.0 (CH_2N), 63.4 (d, $J_{C,P}$ = 6.9 Hz, CH_2CH_3), 76.9 (C-5), 153.3 (C-2), 158.9 (C-6), 166.6 (C-4). Anal. ($C_{16}H_{30}N_3O_5P$) C, H, N.

6-Amino-5-bromo-1-[8-(diethylphosphono)octyl]uracil (50). To a suspension of **49** (120 mg, 0.320 mmol) in dry THF (1.5 mL) were added *N*-bromosuccinimide (57 mg, 0.320 mmol) and AIBN (0.6 mg, 0.003 mmol). The mixture was heated at 60 °C for 90 min. Then, it was diluted with EtOH (10 mL) and filtered through a Celite path. The filtrate was evaporated and purified in the Chromatotron CCTLC (CH_2Cl_2:MeOH, 20:1) to yield 104 mg (72%) of **50** as a syrup. ^1H NMR ($CDCl_3$) δ: 1.20−1.45 (m, 8H), 1.32 (t, J = 7.1 Hz, 6H, CH_2CH_3), 1.45−1.90 (m, 6H), 3.97 (t, J = 6.5 Hz, 2H, CH_2N), 3.90−4.20 (m, 4H, CH_2CH_3), 6.15 (br s, 2H, 6-NH_2), 9.47 (br s, 1H, NH-3). ^{13}C NMR ($CDCl_3$) δ: 16.4 (d, $J_{C,P}$ = 6.1 Hz, CH_2CH_3), 22.2 (d, $J_{C,P}$ = 5.4 Hz, CH_2CH_2P), 25.2 (d, $J_{C,P}$ = 139.6 Hz, CH_2P), 26.3, 27.7, 28.8, 28.9 (CH_2), 30.2 [d, $J_{C,P}$ = 16.1 Hz, $CH_2(CH_2)_2P$], 43.6 (CH_2N), 61.6 (d, $J_{C,P}$ = 6.9 Hz, CH_2CH_3), 72.7 (C-5), 150.1, 152.5 (C-2, C-6), 159.4 (C-4). Anal. ($C_{16}H_{29}BrN_3O_5P$) C, H, N.

6-Amino-5-bromo-1-(8-phosphonooctyl)uracil Ammonium Salt (12). A solution of **50** (70 mg, 0.15 mmol) in dry CH_2Cl_2 (1.5 mL) was reacted with TMSBr (0.20 mL, 1.52 mmol) in an analogous way to that described for compound **8**. After purification, 47 mg (75% yield) of compound **12** was obtained as a white lyophilate. UV (H_2O) λ_{max} = 278 (ϵ = 12000). MS (ES, negative mode): *m*/*z* 396 and 398 [$(M - NH_4)^-$ and $[(M + 2 - NH_4)^-]$. ^1H NMR (D_2O) δ: 1.40−2.10 (m, 14H), 4.21 (t, J = 7.1 Hz, 2H, CH_2N). ^{13}C NMR (D_2O) δ: 27.7 (d, $J_{C,P}$ = 4.6 Hz, CH_2CH_2P), 30.2, 31.2, 32.7, 32.9 (CH_2), 32.5 (d, $J_{C,P}$ = 132.8 Hz, CH_2P), 34.7 [d, $J_{C,P}$ = 16.8 Hz, $CH_2(CH_2)_2P$], 48.3 (CH_2N), 72.7 (C-5), 155.9, 158.5 (C-2, C-6), 165.8 (C-4). HPLC: system I, Rt = 13.63 min (100%); system II, Rt = 22.15 min (100%).

1-[8-(Diethylphosphono)octyl]-2,4-dioxopyrrolo[2,3-*d*]pyrimidine (51). To a stirred suspension of **49** (140 mg, 0.38 mmol) in EtOH (2 mL) was added AcONa (31 mg, 0.38 mmol), and the mixture was heated at 70 °C. Then, chloroacetaldehyde (0.073 mL of a 50% aqueous solution, 0.57 mmol) and AcONa (31 mg, 0.38 mmol) were added, and heating at 70 °C was maintained for 3 h. Then, it was cooled and filtered through a Celite path, and the filtrate evaporated. The residue was purified in the Chromatotron CCTLC (AcOEt:MeOH, 10:1) to yield 71 mg (47%) of **51** as a syrup. ^1H NMR ($CDCl_3$) δ: 1.00−2.00 (m, 14H), 1.33 (t, J = 7.1 Hz, 6H, CH_2CH_3), 3.80−4.25 (m, 6H, CH_2N, CH_2CH_3), 6.48 (dd, 1H, J = 1.9, 3.3 Hz, 1H, H-6), 6.62 (dd, J = 2.3, 3.3 Hz, 1H, H-5), 8.86 (br s, 1H, NH), 11.33 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 16.3 (d, $J_{C,P}$ = 6.1 Hz, CH_2CH_3), 22.0 (d, $J_{C,P}$ = 4.6 Hz, CH_2CH_2P), 25.0 (d, $J_{C,P}$ = 140.3 Hz, CH_2P), 26.2, 27.9, 28.6, 28.8 (CH_2), 30.1 [d, $J_{C,P}$ = 16.1 Hz, $CH_2(CH_2)_2P$], 43.4 (CH_2N), 61.6 (d, $J_{C,P}$ = 6.9 Hz, CH_2CH_3), 99.9 (C-4a), 104.1 (C-5), 117.3 (C-6), 140.3 (C-7a), 150.8 (C-2), 160.2 (C-4). Anal. ($C_{18}H_{30}N_3O_5P$) C, H, N.

1-(8-Phosphonooctyl)-2,4-dioxopyrrolo[2,3-*d*]pyrimidine Ammonium Salt (13). A solution of **51** (100 mg, 0.25 mmol) and 2,6-lutidine (0.51 mL, 3.76 mmol) in dry CH_2Cl_2 (2.5 mL) was reacted with TMSBr (0.20 mL, 1.52 mmol) as described for compound **8**. Compound **13** was obtained as a white lyophilate (77 mg, 85% yield). UV (H_2O) λ_{max} = 275 (ϵ = 7600), 243 (ϵ = 6500). MS (ES, negative mode): *m*/*z* 342 ($M - NH_4)^-$. ^1H NMR (D_2O) δ: 1.00−1.70 (m, 14H), 3.83 (t, J = 6.7 Hz, CH_2N), 6.37, 6.71 (2d, J = 3.5 Hz, 2H, H-6, H-5). ^{13}C NMR (D_2O) δ: 24.6 (d, $J_{C,P}$ = 5.0 Hz, CH_2CH_2P), 27.3, 28.6, 29.7, 29.8 (CH_2), 29.2 (d, $J_{C,P}$ = 163.1 Hz, CH_2P), 31.6 [d, $J_{C,P}$ = 15.6 Hz, $CH_2(CH_2)_2P$], 45.5 (CH_2N), 101.4 (C-4a), 105.1 (C-5), 120.5 (C-6), 142.5 (C-7a), 153.5 (C-2), 163.5 (C-4). HPLC: system I, Rt = 14.01 min (100%); system II, Rt = 21.98 min (100%).

TPase Enzyme Assay. The phosphorolysis of thymidine (dThd) by *E. coli* thymidine phosphorylase was measured by HPLC analysis. One milliliter of the incubation mixture contained 10 mM Tris·HCl (pH 7.6), 1 mM EDTA, 2 mM potassium phosphate, 150 mM NaCl, and 100 μM of thymidine in the presence or absence of 100 μM 2-deoxyribose-α-1-phosphate (α-dR-1-P) and 0.025 units of TPase. Incubations were performed at room temperature. At different time points (i.e., 0, 20, 40, and 60 min), 100 μL fractions were taken, transferred to an Eppendorf tube thermo block, and boiled at 95 °C for 5 min. Thereafter, the samples were rapidly cooled on ice, and thymidine was separated from thymine and quantified in the samples by HPLC analysis.

In the assays where the inhibitory effect of the compounds were evaluated, a variety of inhibitor concentrations, including 1 mM, 500 μM, 100 μM, 20 μM, and 0 μM (control) were added to the reaction mixture that contained 100 μM of dThd. Aliquots of 100 μL were withdrawn from the reaction mixture

at several time points, as described above, heated at 95 °C to inactivate the enzyme, and analyzed on HPLC.

In the kinetic assays, where the inhibitory effect of the test compounds was evaluated against TPase at varying inorganic phosphate, compounds **12**, **13**, and 7DX were tested at concentrations ranging between 200 and 10 μM, in the presence of 2, 5, 10, 25, 50, and 100 mM inorganic phosphate. The dThd concentration was kept fixed at 1000 μM. The reaction mixture was then incubated at room temperature for 20 min, after which an aliquot was withdrawn and analyzed by HPLC for dThd conversion to Thy. In the kinetic assays where the inhibitory effect of the test compounds was evaluated against TPase at varying dThd concentrations, compounds **12** and **13** were tested at concentrations ranging between 200 and 20 μM in the presence of 125, 250, 500, 750, and 1000 μM dThd. The inorganic phosphate concentration was kept constant at 25 mM. The analysis of the dThd-to-Thy conversion was performed as described above.

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