Activation of *p16* Gene Silenced by DNA Methylation in Cancer Cells by Phosphoramidate Derivatives of 2′-Deoxyzebularine

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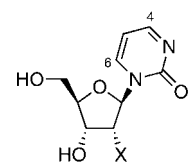
We report herein the application of the phosphoramidate ProTide technology to improve the metabolism of the DNA methytransferase inhibitor, zebularine (Z). Zebularine is a riboside that must undergo a complex metabolic transformation before reaching the critical 2′-deoxyzebularine 5′-triphosphate (dZTP). Because 2′-deoxyzebularine (dZ) is not phosphorylated and therefore inactive, the ProTide strategy was employed to bypass the lack of phosphorylation of dZ and the inefficient reduction of zebularine 5′-diphosphate by ribonucleotide-diphosphate reductase required for zebularine. Several compounds were identified as more potent inhibitors of DNA methylation and stronger inducers of *p16* tumor suppressor gene than zebularine. However, their activity was dependent on the administration of thymidine to overcome the potent inhibition of thymidylate synthase (TS) and deoxycytidine monophosphate (dCMP) deaminase by dZMP, which deprives cells of essential levels of thymidine. Intriguingly, the activity of the ProTides was cell line-dependent, and activation of *p16* was manifest only in Cf-Pac-1 pancreatic ductal adenocarcinoma cells.

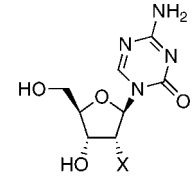
Introduction

The initiation and progression of cancer are driven by both genetic and epigenetic changes. Epigenetic changes, which are independent of the primary DNA sequence, have received a great deal of attention for their key role in cancer initiation and tumor progression.[1,2] In the epigenomics landscape, one of the most studied processes is DNA methylation, an event that controls the transcriptional silencing of a number of tumor suppressor genes. In cancer, such transcriptional repression is associated with the abnormal methylation of cytosines in CpG islands near the promoter regions of the genes and maintained through cellular division. Because these methylated CpG islands are incapable of initiating transcription unless the methylation signal is removed, therapeutic strategies to revert this process have been sought by the use of drugs that alter the transcriptional status of the affected tumor suppressor genes by inhibiting DNA methylation.[3,4] Therefore, silenced tumor suppressor genes present themselves as obvious targets for reactivation by DNA methylation inhibitors such as 5-azacytosine nucleosides (**2a**,**b**) and more recently zebularine (**1a**).[5,6]

Zebularine [1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] is a cytidine analogue that was initially developed as a cytidine deaminase (CDA)[a] inhibitor[7,8] and recently discovered to also inhibit DNA methylation.[5] The simple removal of the 4-amino group from cytidine increases the electrophilicity of the resulting 2-(1*H*)-pyrimidinone aglycon, which explains the ease of nucleophilic attacks at C4 or C6 of the 2-oxopyrimidine ring that are responsible for zebularine's activity as a potent inhibitor of both CDA and DNA methyltransferases, respectively.[9] Once incorporated into DNA, the 2-(1*H*)-pyrimidinone aglycon forms a covalent complex with DNA methyltransferases via nucleophilic attack at C6 from a key conserved cysteine residue (Cys81)[10] that results in the depletion of DNA methyltransferase 1 (DNMT1),[11,12] the reactivation of hypermethylated genes in yeast and solid tumor cells,[5] and antitumor activities in mouse xenografts[5] and radiation-induced T-cell lymphomas in mice.[13]



1a, X = OH
1b, X = H



2a, X = OH
2b, X = H

In vitro inhibition of bacterial M.*Hha*I DNA methyltransferase activity by double-stranded oligodeoxynucleotides containing either 2′-deoxy-5-azacytidine (**2b**) or 2′-deoxyzebularine (**1b**)

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[a] Abbreviations: CDA, cytidine deaminase; dCK, deoxycytidine kinase; dCMP, deoxycytidine monophosphate deaminase; DNMT1, DNA methyltransferase 1; dUMP, deoxyuridine monophosphate; dZMP, 2′-deoxyzebularine 5′-monophosphate; dZTP, 2′-deoxyzebularine 5′-triphosphate; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IMDM, Iscove's modified Dulbecco's medium; MAGEA1, melanoma antigen family A1; MOE SLog P, molecular operating environment SMART log *P*; Ms-SNuPE, methylation-sensitive single-nucleotide primer extension; NMI, *N*-methylimidazole; NMR, nuclear magnetic resonance; *P*, partition coefficient; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PD, population doubling; RNR, ribonucleotide-diphosphate reductase; RT-PCR, reverse transcriptase PCR, SAR, structure—activity relationship; SMART, Smiles arbitrary target specification; SMILES, simplified molecular input line entry specification; THF, tetrahydrofuran; TMP, thymidine monophosphate; TS, thymidylate synthase; Z, zebularine; ZMP, zebularine 5′-monophosphate; ZDP, zebularine 5′-diphosphate; ZTP, zebularine 5′-triphosphate; ZDP-Chol, zebularine-5′-diphosphocholine.

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in the hemimethylated recognition sequence [5′-GXGC-3′/3′-CGMG-5′ (M = 5-methylcytosine, X = 5-azacytosine or 2-(1*H*)-pyrimidinone)] confirmed that the two heterocyclic moieties have comparable reactivity.[14] In contrast, much higher doses of zebularine were required to achieve comparable levels of *p16* reactivation in T24 cells relative to the 5-azacytidine nucleosides, which suggested that the differences in potency were due to other factors.[5] Indeed, zebularine (**1a**) required doses 10−100-fold higher than 5-azacytidine (**2a**) and 2′-deoxy-5-azacytidine (**2b**), respectively, to induce comparable levels of *p16* expression.[5] On the other hand, the stability and reduced toxicity of zebularine allowed it to be administered continuously to cells resulting in marked *p16* expression.[5]

The incorporation of zebularine into DNA necessitates critical levels of 2′-deoxyzebularine 5′-triphosphate (dZTP) which is formed by a complex metabolic route that may explain its weaker potency.[15] A quantitative assessment of the phosphorylation and DNA incorporation of zebularine in T24 cells using 2-[^{14}C]-zebularine revealed that the drug is readily phosphorylated to the corresponding 5′-mono- (ZMP), 5′-di- (ZDP), and 5′-triphosphate (ZTP) in a dose- and time-dependent manner.[15] Two additional zebularine-containing metabolites were also observed and identified as diphosphocholine (ZDP-Chol) and diphosphoethanolamine adducts. Intracellular concentrations of ZTP and ZDP-Chol were comparable and greatly exceeded those of the other metabolites.[15]

When DNA and RNA levels of incorporation were compared, RNA incorporation surpassed DNA incorporation by at least 7-fold.[15] Thus, formation of zebularine riboside metabolites appears to be quite robust, but conversion of zebularine 5′-diphosphate (ZDP) to 2′-deoxyzebularine 5′-diphosphate (dZDP), which is catalyzed by ribonucleotide-diphosphate reductase (RNR), seems to be a rate-limiting step that could explain zebularine's weaker potency.[15] Unfortunately, the use of 2′-deoxyzebularine (**1b**) was unable to overcome this deficiency because it was completely ineffective, perhaps owing to lack of recognition by the activating enzyme, deoxycytidine kinase (dCK).[5,15] In a biochemical sense zebularine behaves like a cytidine analogue,[16] but its metabolic activation to form the dZTP required for DNA incorporation seems to be complex and inefficient.

In considering the limitations of zebularine described above and the inactivity of 2′-deoxyzebularine, we decided to investigate a prodrug strategy for dZMP that could simultaneously bypass both the dCK step (needed for 2′-deoxyzebularine) and the RNR step (involved in the case of zebularine at its 5′-diphosphate anabolite stage) as a means of increasing DNA incorporation and enhancing potency. Thus, the intracellular delivery of dZMP in a membrane-permeable "ProTide" form was explored by the well-known aryloxyphosphoramidate approach of McGuigan et al.[17,18] Through this technology the lipophilicity of the nucleotide is augmented, making the ProTide able to penetrate the interior of the cell by passive diffusion. Once inside the cell, the nucleotide monophosphate (dZMP) is expected to be released at effective intracellular levels. The McGuigan ProTide approach already has produced significant enhancements in the antiviral activity of various nucleosides against HIV and HCV viruses compared to the parent nucleoside analogues *in vitro*.[19,20] As far as we know, this work is the first attempt to utilize the ProTide technology for an anticancer agent.

Selection of ProTide Targets and Preliminary Activity

A rapid survey with different aryloxyphosphoramidates was explored with the idea of correlating some key structural parameters with increases in lipophilicity calculated according to the atom-based program MOE SLog P[21] as shown in Table 1. The objective was to quickly perform a limited structure−activity analysis to identify the most potent candidates for proof of concept. The biological response used to gauge our structure−activity analysis was the reexpression of the tumor suppressor gene *p16* in Cf-Pac-1 pancreatic tumor cells using real time PCR analysis.[11,22,23] For the purposes of our initial screen, the compounds in Table 1 were given a qualitative "yes" or "no" response to *p16* reactivation.

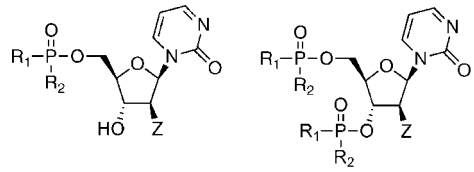
In the first and simplest ProTide (**3**) we combined a phenoxy group with the L-alanine methyl ester. The same two groups were also attached to a modified 2′-*β*-fluoro-2′-deoxyzebularine analogue (**4**) which was expected to function as a 2′-deoxy surrogate with increased lipophilicity. However, none of these compounds showed activity. A further increase in log *P* achieved with the *p*-chlorophenoxy group and the L-alanine ethyl ester in the next compound (**5a**) seems to suggest that a log *P* approaching 2 is a good threshold for activity as compound **5a** was indeed able to induce *p16* reactivation. Interestingly, the 3′,5′-bis[4-chlorophenoxy(ethoxy-L-alanyl)] phosphoramidate (**5b**, log *P* = 4.15) obtained as a byproduct also showed activity. This was unexpected on several grounds. First, the presence of the bulky group at the 3′ position might be anticipated to possibly impede enzyme processing of the ProTide. Second, if only the 5′-ProTide moiety were processed, as intended, the dZMP-3′-ProTide would not be expected to be active per se. Third, if both ProTide motifs were processed, the resulting 3′,5′-bisphosphate would not be expected to be the ideal pharmacophore. Thus, the data seem to imply that 3′,5′-bis-ProTides may have a surprising efficacy by a mechanism to be determined. The next two entries in Table 1 (compounds **6** and **7**) showed that contributions from the aryloxy and L-alanine ester sides are different and potency does not necessarily correlate with log *P*. In compound **6**, a rise in log *P* to 2.60, achieved by increasing the lipophilicity of the L-alanine moiety from an ethyl to a benzyl ester, while leaving the phenoxy group intact, resulted in an inactive compound. On the other hand, augmenting the log *P* from the aryloxy side with the naphthyloxy group and adding an extra methyl group on the ester side with the dimethylglycine ethyl ester to reach a similar log *P* of 2.70 produced a very potent compound (**7a**) which together with the two diastereoisomers of the 3′,5′-bis[naphthyloxy(ethyloxydimethylglycinyl)] phosphoramidate (**7b** and **7b′**, log *P* = 5.93) induced strong *p16* activation (*vide infra*). The next compound (**8**), which differs from **6** by the simple addition of fluorine, was also inactive despite having an adequate log *P* of 2.97.

Notwithstanding the fact that compound **6** failed to reactivate *p16*, the simple change of the L-alanine benzyl ester to the dimethylglycine benzyl ester was accompanied by a modest rise in log *P* to produce **9a**, which was active. Similarly to compound **5b**, the 3′,5′-bis[phenoxy(benzyloxydimethylglycinyl)] phosphoramidate **9b** (log *P* = 6.51) was active. The final compound of the initial series was compound **10**, which evolved from **9a** by replacing the phenyloxy moiety with the naphthyloxy group. This compound is the most lipophilic member of the 5′-ProTide series (log *P* = 4.15).

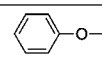
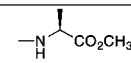
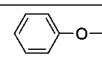
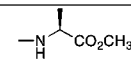
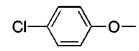
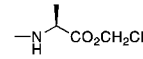
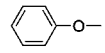
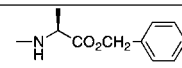
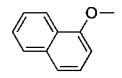
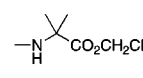
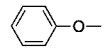
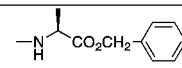
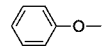
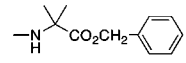
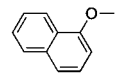
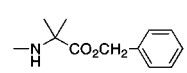
Chemistry

The synthesis of the phosphoramidates (**3**−**10**) was performed using either the THF/*N*-methylimidazole (NMI) or the *tert*-butylmagnesium chloride protocol (see Scheme 1 and Experimental Section). Because of the stereochemistry of the phosphorus center, each of the 5′-monophosphoramidates **3**, **4**, **5a**,

Table 1. Target Aryloxyphosphoramidates Synthesized for Initial SAR Analysis



5′-phosphoramidate 3′,5′-bisphosphoramidate

Compound[a]	Z	R_1	R_2	log P[b]	*p16* activation[c]
3	H			0.77	no
4	F			1.14	no
5a	H			1.81	yes
5b (bis)				4.15	yes
6	H			2.60	no
7a	H			2 .70	yes
7b (bis)				5.93	yes
7b' (bis)				5.93	yes
8	F			2.97	no
9a	H			3.00	yes
9b (bis)				6.51	yes
10	H			4.15	yes

[a] Compounds **5b**, **7b**, **7b′**, and **9b** are 3′,5′-bisphosphoramidates. [b] Calculated log *P* value. [c] *p16* activation in the presence of 100 mM thymidine.

Scheme 1. Synthesis of 2′-Deoxyzebularine Phosphoramidates

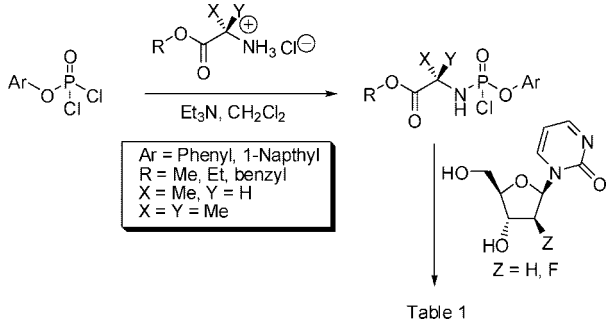


Table 1

6, **7a**, **8**, **9a**, and **10** was isolated as pairs of diastereoisomers and tested as such. Such isomerism was most evident from the presence of two closely spaced signals in the ^{31}P NMR in a ratio of roughly 1:1. Other signals were also duplicated in the proton and ^{13}C NMR spectra in each case. For the 3′,5′-bisphosphoramidates, four stereoisomers (eight ^{31}P NMR peaks) are possible, and multiple peaks were observed, consistent with mixtures being generated. In the case of **7** we achieved chromatographic separation of some of the 3′,5′-bisphosphoramidate isomers, and each fraction, **7b** and **7b′**, gave only two ^{31}P NMR signals, this being consistent with each being a single diastereoisomer. As discussed later these compounds possessed different potencies in the activation of *p16*.

The four most potent compounds **7a**, **7b**, **7b′**, and **10** from Table 1 that were identified in the preliminary screen as strong promoters of *p16* reexpression were fully characterized by chemical and spectroscopic means (see Experimental Section) and used for additional biological studies.

Biological Studies

All ProTides were initially tested in T24 cells by treating continuously with the compounds for 8 days. No induction of the *p16* gene was observed at 1, 10, and 100 μM concentrations, indicating that the compounds were unable to inhibit DNA methylation in T24 cells (data not shown). Similarly, all compounds failed to induce the *p16* gene in Cf-Pac-1 pancreatic cancer cells (data not shown).

When the compounds were tested with Cf-Pac-1 cells supplemented with 100 μM thymidine, activities began to

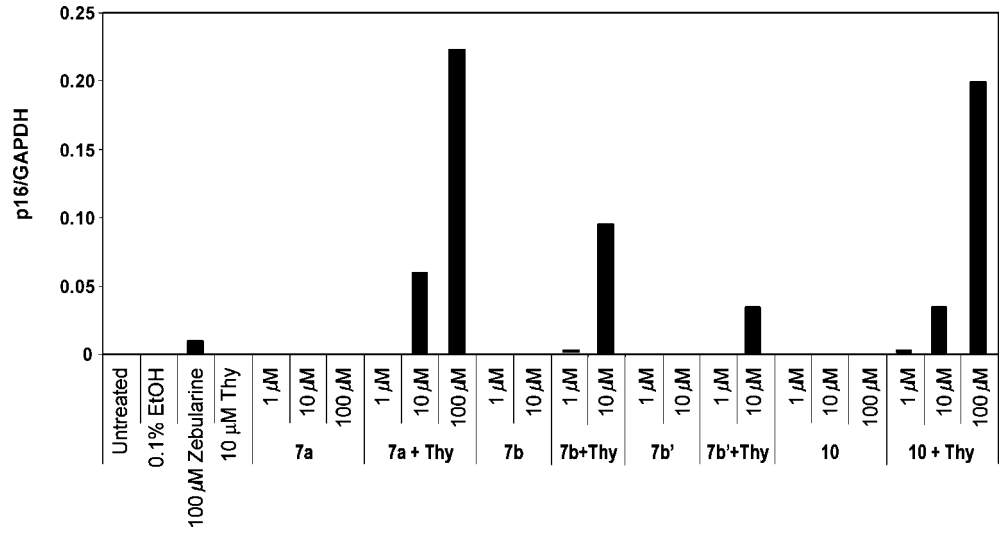


Figure 1. Effect of 2′-deoxyzebularine ProTides **7a**, **7b**, **7b′**, and **10** on *p16* expression in Cf-Pac-1 pancreatic cancer cells. Cf-Pac-1 cells were treated with ProTides continuously for 8 days. RNA was collected, and the levels of *p16* expression were measured by real time RT-PCR. *GADPH* was used as a reference gene. All compounds induce a strong *p16* expression in the presence of 100 μM thymidine.

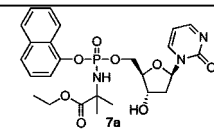
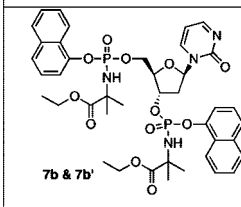
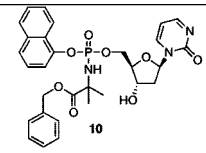
emerge (Table 1). The rationale for including thymidine in the treatment was to maintain a pool of thymine monophosphate (TMP) to allow DNA synthesis to progress without cell cycle arrest. 2′-Deoxyzebularine monophosphate (dZMP) is also known to inhibit two critical enzymes: (1) thymidylate synthase (TS),[24] the enzyme responsible for converting dUMP into TMP via the salvage pathway, and (2) dCMP deaminase,[25] which prevents formation of dUMP, the substrate for TS to make TMP. By inhibiting TS and dCMP deaminase, dZMP could slow DNA synthesis, which is essential for inhibition of DNA methylation. We therefore supplemented the cells with 100 μM thymidine to allow the cell cycle to progress without interference from the inhibition of TS and dCMP deaminase. Strong induction of *p16* expression was observed in Cf-Pac-1 cells in the presence of 100 μM thymidine for compounds **7a** and **10** at 100 μM (Figure 1). Importantly, these compounds induced measurable *p16* expression still at 10 μM and displayed a significantly stronger response than zebularine at an equivalent 100 μM concentration. However, every active compound in Cf-Pac cells failed to induce *p16* expression in T24 and HCT15 cancer cells even in the presence of thymidine (data not shown). Zebularine alone, in contrast, was effective in T24 cells and HCT15 cells.

Of the two 3′,5′-bis[naphthyloxy(ethyloxydimethylglycine)] phosphoramidate diastereoisomers **7b** was the most potent compound of the entire series showing stronger induction of *p16* at 10 μM compared to zebularine at 100 μM (Figure 1). Also, this diastereoisomer was ca. 3-fold more potent than **7b′**, indicating that the enzymatic release of dZMP was different and perhaps dependent on the stereochemistry of the phosphorus center.

Since thymidine is also known to block DNA synthesis at millimolar concentrations (1−2 mM),[26] we then tested to see if lower concentrations of thymidine were effective in facilitating the demethylating activity of the ProTides. However, the induction of the *p16* gene was observed only at 100 μM thymidine and not at lower concentrations, indicating that these cells must be supplemented with a 1:1 ratio of thymidine to ProTide. Replacement of 100 μM thymidine with 100 μM uridine did not aid the ProTides in promoting the induction of *p16*.

The activity of compounds **7a** and **10**, as well as diastereoisomers **7b** and **7b′**, correlated well with increases in doubling time in cell culture (Table 2). However, **7b** and **7b′** caused severe toxicity at 100 μM while **7a** and **10** were less toxic at the same concentrations (data not shown).

Table 2. Percent Increase in Doubling Time of Cf-Pac-1 Pancreatic Carcinoma Cells Treated with dZMP Prodrugs Continuously for 8 Days in the Presence of 100 μM Thymidine

Compound	Concentration	Doubling Time (% increase)
Untreated control	—	0
Ethanol	0.1%	23
Zebularine	100 μM	103
Thymidine	100 μM	0
 7a	1 μM	58
	10 μM	81
 7b & 7b'	1 μM	13
	10 μM	116
	1 μM	64
	10 μM	33
 10	10 μM	36
	100 μM	23

To confirm that these phosphoramidates were indeed hypomethylating the DNA in these cells, we analyzed the DNA methylation status of Cf-Pac-1 cells after treatment with **7a**, **10**, **7b**, and **7b′** at three different loci by Ms-SNuPE.[27] The regions analyzed were the CpG-rich promoter region of *p16*, the CpG-poor promoter region of *MAGEA1*, and a repeat sequence known as *D4Z4*. All three loci are hypermethylated

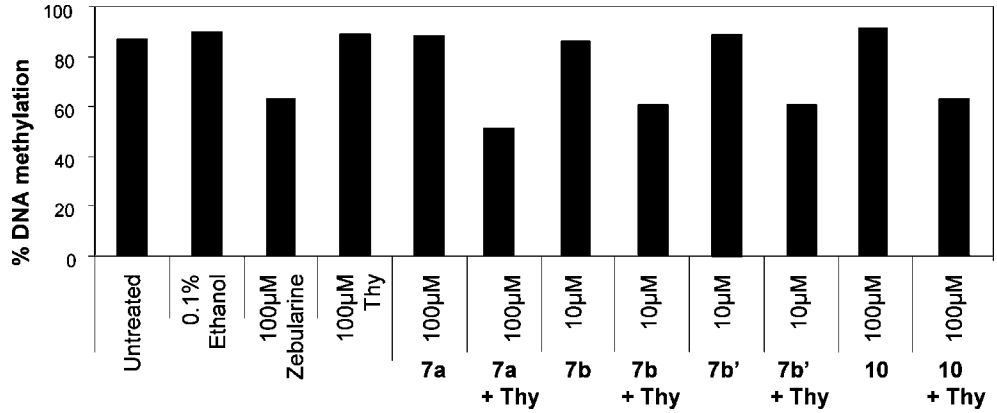


Figure 2. DNA methylation analysis of *D4DZ* in Cf-Pac-1 pancreatic cancer cells treated with 2′-deoxyzebularine ProTides **7a**, **7b**, **7b′**, and **10**. Cf-Pac-1 cells were treated with ProTides continuously for 8 days, and DNA was collected. DNA methylation status of the *D4DZ* repeats expressed in percent methylation was analyzed by quantitative Ms-SNuPE. Treatment with these compounds caused demethylation at the *D4DZ* locus.

and have previously been shown to lose DNA methylation after zebularine treatment.[11] The results of DNA methylation analyses at all three regions were similar: the dZMP ProTides caused a decrease in the methylation level in the presence of thymidine. The pattern is maintained whether the region is a CpG-rich, CpG-poor, or repetitive element containing regions. Figure 2 summarizes the DNA methylation status at *D4Z4*; all four compounds tested showed demethylation. As before, these drugs worked best in Cf-Pac-1 pancreatic cancer cells and marginally in HCT15 colon cancer cells (data not shown).

Discussion

Zebularine is an effective inhibitor of DNA methylation capable of inducing the expression of methylation-silenced genes upon treatment. It has many properties that are advantageous as a chemotherapeutic agent, including stability in aqueous solution as well as low toxicity in animals and cultured cells.[5,11,28] However, zebularine must undergo several metabolic steps before DNA incorporation,[15] which subsequently weakens the demethylating effect of the drug. A number of aryloxy-phosphoramidate nucleotide prodrugs (ProTides) of 2′-deoxy-zebularine (dZMP) were tested in search of a demethylating agent more potent and efficient than zebularine. The ProTides that were effective displayed good activity in Cf-Pac-1 pancreatic cancer cells only in the presence of 100 μM thymidine; they were marginally active in HCT15 colon cancer cells and inactive in T24 bladder cancer cells, even in the presence of thymidine. Such cell line specificity of the phosphoramidates may be due to the availability and differential activities of enzymes involved in the release of the dZMP moiety.

The unexpected finding of this investigation was that the phosphoramidates were not able to induce *p16* expression in the absence of thymidine. Thymidine was used to prevent the inhibition of TS[24] and dCMP deaminase[25] by dZMP, which would deplete TMP levels. Since zebularine depends on the synthesis of new DNA to inhibit methylation and the inhibition of TS and dCMP would block DNA synthesis due to the shortage of TMP, it was imperative to supplement the cells with an outside source of thymidine.

Although these compounds demonstrated conceptually that the treatment with dZMP ProTides inhibits DNA methylation efficiently, the required administration of thymidine might particularly limit their use in a potential clinical setting. Nevertheless, it might be feasible to use this approach in the future.

In summary, some dZMP ProTides were capable of inducing greater expression of the *p16* gene than zebularine. Although they are very cell line-specific and require the presence of thymidine, these drugs provide proof of concept that two enzymes, dCK and RNR, can be efficiently circumvented by these ProTides, which represent a promising start for the development of future prodrug strategies for 2′-deoxyzebularine.

Experimental Section

Materials and Methods. Cell Lines and Drug Treatment. T24 bladder carcinoma cells and HCT116 colon carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). Cf-Pac-1 pancreatic carcinoma cells also from American Type Culture Collection were cultured in IMDM medium (Gibco/Life Technologies Inc., Palo Alto, CA) supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). All cells were cultured in a humidified incubator at 37 °C in 5% CO_2. Zebularine and 5-aza-CdR (Sigma-Aldrich, St. Louis, MO) were dissolved in PBS. Zebularine prodrugs were dissolved in PBS or ethanol, depending on solubility, and stored at −80 °C. Cells were seeded (3×10^5 cells/10 cm dish) and treated with various compounds after 24 h. The medium was changed every 2−3 days after the initial treatment and supplemented with a fresh dose of drug.

Determination of Doubling Time. The cell number/dish was counted with a Z1 Coulter particle counter (Beckman Coulter Corp., Hialeh, FL) every 2−3 days. Untreated cells were analyzed under similar conditions as a control. The average cell number from two plates was determined, and the mean cell numbers were plotted to define the cell population doubling times, where population doubling PD = log (number of cells harvested/number of cells seeded)/log 2. Initial drug treatment was started 24 h after seeding.

Nucleic Acid Isolation. RNA was collected from T24 and HCT116 cells with the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. DNA was collected using the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Quantitative RT-PCR. Total RNA (5 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers (Invitrogen, Carlsbad, CA). The reverse transcription was carried out in a total volume of 50 μL as previously described.[23] The quantitation of mRNA levels was carried out by a real time fluorescence detection method as described previously.[11,22] All samples were normalized to the reference gene, *GAPDH*. The primer and probe sequences are as follows: for *p16*, sense 5′-CTG CCC AAC GCA CCG A-3′, probe 5′ 6-FAM-TGG ATC GGC CTC CGA CCG TAA CT BHQ-1 3′, and antisense 5′-CGC TGC CCA TCA TCA TGA C-3′; for *SPANXA1*, sense 5′-TGT GAT TCC AAC GAG GCC A-3′, probe 5′ 6-FAM-CGA GAT GAT GCC GGA GAC CCC A BHQ-1 3′,

and antisense 5′-GCG GGT CTG AGT CCC CA-3′; for *MAGEA1*, sense 5′-GAA CCT GAC CCA GGC TCT GTG-3′, probe 5′ 6-FAM-CAA GGT TTT CAG GGG ACA GGC CAA C BHQ-1 3′, and antisense 5′-CCA CAG GCA GAT CTT CTC CTT C′-3′; for *MAGEB2*, sense 5′-CGG CAG TCA AGC CAT CAT G-3′, probe 5′ 6-FAM-TCG TGG TCA GAA GAG TAA GCT CCG TGC BHQ-1 3′, and antisense 5′-GCG GGT CTG AGT CCC CA-3′; for *GAGE*, sense 5′-GCT GAT AGC CAG GAA CAG GG-3′, probe 5′ 6-FAM-CAC CCA CAG ACT GGG TGT GAG TGT GA BHQ-1 3′, and antisense 5′-CCT GCC CAT CAG GAC CAT C-3′; for *XAGEA1*, sense 5′-TCC CCA GAC GGG ACC AG-3′, probe 5′ 5-FAM-AGA GGG ACG GCA TGA GCG ACA CAC BHQ-1 3′, and antisense 5′-CTG GCT GTG TGG TTC TGT GTT T-3′; for *GAPDH*, sense 5′-TGA AGG TCG GAG TCA ACG G-3′, probe 5′ 6-FAM -TTT GGT CGT ATT GGG CGC CTG G BHQ-1 3′, and antisense 5′-AGA GTT AAA AGC AGC CCT GGT G-3′. The conditions for real time RT-PCR are 94 °C for 9 min followed by 45 cycles at 94 °C for 15 s and 60 °C for 1 min.

Quantitation of DNA Methylation. Genomic DNA (4 μg) was treated with sodium bisulfite as previously described.[11] Methylation analysis was performed using the methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) assay for the *p16* 5′ region as previously described.[27] The PCR primers used are as follows: for *p16*, sense 5′-TTT GAG GGA TAG GGT-3′ and antisense 5′-TCT AAT AAC CAA CCA ACC CCT CC-3′; for *MAGEA1*, sense 5′-GTT TAT TTT TAT TTT TAT TTA GGT AGG A-3 and antisense 5′-TTA CCT CCT CAC AAA ACC TAA A-3′; for *MAGEB2*, sense 5′-TTG AGG GAG GTG GGG GTA TTG T-3′ and antisense 5′-CTT CAA TTT ACA CTC AAA ATC CTC ACC T-3′; for *D4Z4*, sense 5′-GGG TTG AGG GTT GGG TTT AT-3′ and antisense 5′-AAC TTA CAC CCT TCC CTA CA-3′. An initial denaturation at 94 °C for 3 min was followed by 94 °C for 45 s, annealing for 45 s, and 72 °C for 45 s for 40 cycles. The annealing temperatures for each locus are as follows: for *p16*, 65 °C, for *MAGEA1*, 53 °C, for *MAGEB2*, 62 °C, and for *D4Z4*, 58 °C. Primers used for Ms-SNuPE analysis are as follows: for *p16*, 5′-TTT TTT TGT TTG GAA AGA TAT-3′, 5′-TTT TAG GGG TGT TAT ATT-3′, and 5′-GTA GAG TTT AGT T-3′; for *MAGEA1*, 5′-AGG TTT TTA TTT TGA GGG A-3′, 5′-TGG GGT AGA GAG AAG-3′ and 5′-TTT TAT TTT TAT TTA GGT AGG ATT-3′; for *MAGEB2*, 5′-ATT GTT TGG AGG TTG G-3′, 5′-GAG GAT TTT TAG TGA AGA-3′, and 5′-GAT GTG GTT TAT TTT GAT TTT-3′; for *D4Z4*, 5′-TGA GGG TTG GGT TTA TAG T-3′, 5′-TAT ATT TTT AGG TTT AGT TTT GTA A-3′, 5′-GTG GTT TAG GGA GTG GG-3′, and 5′-GAA AGG TTG GTT ATG T-3′. Conditions for primer extension were 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 20 s.

Synthesis. General Procedures. All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions. Anhydrous tetrahydrofuran (THF) and dichloromethane were purchased from Aldrich and used directly. Column chromatography refers to flash column chromatography carried out using Merck silica gel 60 (40−60 μm) as stationary phase. Proton, carbon, and phosphorus nuclear magnetic resonance (^{1}H, ^{13}C, ^{31}P NMR) spectra were recorded on Bruker Avance spectrometers operating either at 500, 125, and 202 MHz or at 300, 75, and 121 MHz. For compounds **3** and **4** the spectrometer was a Varian Unity Inova instrument operating at 400, 100, and 161.9 MHz. The solvents used are indicated for each compound. All ^{13}C and ^{31}P spectra were recorded proton decoupled. Chemical shifts for ^{1}H and ^{13}C spectra are in parts per million downfield from tetramethylsilane. Coupling constants are referred to as *J* values. Signal splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), broad signal (br), doublet of doublet (dd), doublet of triplet (dt), or multiplet (m). Chemical shifts for ^{31}P spectra are in parts per million relative to an external phosphoric acid standard. Many proton and carbon NMR signals were split because of the presence of (phosphate) diastereoisomers in the samples. The mode of ionization for mass spectrometry for compounds **3** and **4** was fast atom bombardment (FAB) using MNOBA (*m*-nitrobenzyl alcohol) as matrix. Electrospray mass spectra were obtained using a Waters LCT time-of-flight mass spectrometer coupled to a Waters M600 HPLC pump. Samples were dissolved in methanol and injected into the solvent stream via a Rheodyne injector. The mobile phase used was methanol at a flow rate of 200 μL/min. The electrospray source was operated at a temperature of 130 °C with a desolvation temperature of 300 °C, a capillary voltage of 3 kV, and cone voltage of 30 V. Data were collected in the continuum mode over the mass range 100−2000 amu and processed using Masslynx 4.1 software. Accurate mass measurements were facilitated by the introduction of a single lockmass compound of known elemental composition into the source concurrently with sample. The purity of crude compounds **7a**, **7b**, **7b′**, and **10** was established by HPLC chromatography which ranged from 85% to 93%. Single peaks of pure compounds were collected dried and weighed accurately.

Standard Procedure A: Synthesis of Phosphorodichloridate. Phosphorus oxychloride (1.0 mmol) was added to a stirred solution of the appropriate phenol or naphthol (1.0 mmol) in dry ether. Then the solution was stirred at −78 °C, and anhydrous triethylamine (1.0 mmol) was added dropwise. After 1 h the reaction was left to rise to room temperature and stirred for about 3 h, monitoring the formation of the desired compound by ^{31}P NMR. The triethylamine hydrochloride salt was filtered off and the filtrate reduced to dryness to give a crude oil as product that was used without further purification for the next step.

Standard Procedure B: Synthesis of Phosphorochloridate. Anhydrous triethylamine (2.0 mol equiv) was added dropwise at −78 °C to a stirred solution of the appropriate phosphorodichloridate (1.0 mol equiv) and the appropriate amino acid hydrochloride salt (1.0 mol equiv) in anhydrous dichloromethane (15−30 mL). After 1 h the reaction was allowed to slowly warm to room temperature, and the formation of the desired compound was monitored by ^{31}P NMR. The solvent was removed under reduced pressure, and the crude residue was resuspended in anhydrous ether and filtered under nitrogen. The corresponding filtrate was reduced to dryness and purified by flash chromatography (ethyl acetate/hexane 7/3) to give the product as an oil.

Standard Procedure C: Synthesis of Phosphoramidate (^{t}BuMgCl Method). *tert*-Butylmagnesium chloride (1 M solution in THF, 1.2 mmol) was added to a stirring suspension of the appropriate nucleoside (1.0 mmol) in dry THF (10 mL) under argon atmosphere. The appropriate phosphorochloridate (1.2 mmol) dissolved in dry THF was added dropwise, and the reaction was left stirring overnight. Volatiles were evaporated, and the residue was purified by flash chromatography (CH_2Cl_2/CH_3OH) to give the desired product.

Standard Procedure D: Synthesis of Phosphoramidate (NMI Method). *N*-Methylimidazole (NMI, 5.0 mmol) was added to a stirring suspension of the appropriate nucleoside (1.0 mmol) in dry THF (10 mL) under argon atmosphere. The appropriate phosphorochloridate (3.0 mmol) dissolved in THF was added dropwise and the reaction left stirring overnight. Volatiles were evaporated, and the residue was dissolved in CH_2Cl_2 and washed with 0.5 M HCl. The organic layer was dried over $MgSO_4$, filtered, reduced to dryness, and purified by flash chromatography (CH_2Cl_2/CH_3OH).

Standard Procedure E: Synthesis of Phosphoramidate (Variant of the NMI Method). A stirred solution of the appropriate nucleoside (1.0 mmol) in anhydrous pyridine (5 mL) was treated with the appropriate phosphorochloridate (3.4 mmol) in dry THF (4 mL) under argon atmosphere. *N*-Methylimidazole (NMI, 6.3 mmol) was then added, and the reaction was stirred overnight. Additional amounts of phosphorochloridate (3.4 mmol) and NMI (6.3 mmol) were added, and stirring was continued for an additional 4 h. Volatiles were evaporated, and the residue was dissolved in CH_2Cl_2 and chromatographed twice by flash column chromatography (CH_2Cl_2/CH_3OH).

2′-Deoxyzebularine 5′-[Phenyl(methoxy-L-alaninyl)]phosphate (3). Prepared according to standard procedure E, using 2′-deoxyzebularine (0.09 g, 0.42 mmol), methylchlorophenylphosphoryl-L-alaninate (2 × 0.40 g, 2.88 mmol), and NMI (2 × 0.24 mL, 6 mmol). The crude was purified twice by column chromatography

(CH_2Cl_2/CH_3OH 93/7) and coevaporated with ether (2 × 5 mL) to give the pure product as a white foamy solid (57 mg, 29.7% yield). ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 3.23, 3.36. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.43 (3H, m, C*H*$_3$), 2.00−2.10 (1H, m, H-2′), 2.60−2.70 (1H, m, H-2′), 3.50−3.70 (4H, m, H-4′, CO_2CH_3), 3.90−4.05 (1H, m, C*H*), 4.10 (1H, m, H-3′), 4.25−4.40 (2H, m, H-5′), 6.10 (1H, m, H-5), 6.22 (1H, m, H-1′), 7.10−7.30 (5H, m, PhO), 8.14 (1H, m, H-6), 8.50 (1H, br s, H-4). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.8 (*CH*$_3$CH), 40.2 (C-2′), 49.3, 51.6 (*CH*CH_3), 64.7 (*CH*$_3$O), 68.7 (C-5′), 68.9 (C-3′), 86.6 (C-4′), 103.3, 109.1, 118.9, 119.1, 124.2, 128.8, 142.6, 149.4, 154.5, 164.6, 172.9, 173.1 (*CO*$_2$Me). FAB-MS *m/e*: 454.1 (MH^+, 15%); Anal. Calcd for $C_{19}H_{24}N_3O_8P$: C, 50.33; H, 5.34; N, 9.27. Found: C, 50.04; H, 5.59; N, 9.03.

2′-β-Fluoro-2′-deoxyzebularine 5′-[Phenyl(methoxy-L-alaninyl)]phosphate (4). Prepared according to standard procedure E, using 2′-fluoro-2′-deoxyzebularine (0.230 g, 1 mmol), methylchlorophenylphosphoryl-L-alaninate (2 × 0.95 g, 6.8 mmol), and NMI (2 × 0.5 mL, 12.54 mmol). The crude was purified twice by column chromatography (CH_2Cl_2/CH_3OH 93/7) and coevaporated with ether (2 × 5 mL) to give the pure product as a white foamy solid (160 mg, 34% yield). ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 3.46, 3.82. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.30−1.36 (3H, 2 d, J = 5.4 Hz, C*H*$_3$), 3.63 and 3.65 (3H, singlets, CO_2CH_3), 3.70−3.85 (1H, dt, J = 37.6, 10.0 Hz, H-2′), 3.91−4.04 (1H, m, C*H*), 4.22−4.40 (3H, m, H-5′, H-4′), 5.28 (1H, dm, J = 50.8 Hz, H-3′), 6.26 (1H, dt, J = 19.9, 2.9 Hz, H-1′), 6.33 (1H, br t, H-5), 7.05−7.30 (5H, m, PhO), 8.08−8.14 (1H, ddd, J = 12.7, 6.6, 1.9 Hz, H-6), 8.54 (1H, br s, H-4). ^{13}C NMR ($CHCl_3$, 100 MHz): δ 19.6, 19.7, (*CH*$_3$CH), 49.2 (*CH*CH_3), 51.52, 64.6, 64.8 (C-5′), 73.4, 73.6 (C4′), 83.0, 83.1, 83.2, 83.3 (C3′), 86.12 (t), 93.0 (d, J = 195 Hz, C-2′), 103.3 (C-1′), 119.0, 119.1, 119.2, 119.3, 124.1, 128.7, 128.8, 144.3, 149.4, 149.5, 149.6, 154.3, 165.3, 172.9 (C═O). FAB-MS *m/e*: 472.4 (MH^+, 70%). Anal. Calcd for $C_{19}H_{23}FN_3O_8P\cdot 0.5H_2O$: C, 47.50; H, 5.04; N, 8.75. Found: C, 47.54; H, 5.06; N, 8.57.

2′-Deoxyzebularine 5′-[4-chlorophenyl(ethoxy-L-alaninyl)]phosphate (5a). Prepared according to standard procedure D, from 2′-deoxyzebularine (0.19 g, 0.88 mmol), 4-chlorophenyl(ethoxy-L-alaninyl) phosphorochloridate (0.86 g, 2.64 mmol), NMI (0.36 g, 4.40 mmol, 0.35 mL), and dry THF (10 mL). The crude was purified by column chromatography (CH_2Cl_2/CH_3OH 95/5) to give the pure product as a white foamy solid (88 mg, 20.0% yield). ^{31}P NMR ($CDCl_3$, 121 MHz): δ 3.23, 3.32. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.11−1.20 (3H, m, OCH_2CH_3), 1.39−1.42 (3H, m, $CHCH_3$), 1.99−2.09 (1H, m, one of H-2′), 2.61−2.73 (1H, m, one of H-2′), 3.83−4.40 (9H, m, H-3′, H-4′, H-5′, OH-3′, *CH*NH, CH*NH*, O*CH*$_2$CH$_3$), 6.10−6.30 (2H, m, H-5, H-1′), 7.13−7.22 (4H, m, pCl-Ph), 8.09−8.19 (1H, m, H-4), 8.45−8.53 (1H, m, H-6). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.1 (*CH*$_3$CH$_2$O), 20.8 (*CH*$_3$CH), 41.2 (C-2′), 50.3, 50.4 (*CH*CH_3), 61.8 (CH_3CH_2O), 65.9 (C-5′), 69.8, 70.0 (C-3′), 85.4, 85.5 (C-4′), 87.7 (C-1′), 121.4, 121.5, 121.6, 129.8 (C-5, pCl-Ph), 130.5 (C-1, pCl-Ph), 143.6 (C-4), 149.0 (C-4, pCl-Ph), 155.5 (C-2), 165.7 (C-6), 173.6 (*CO*$_2$Et). HPLC (H_2O/CH_3CN from 70/30 to 0/100 in 10 min): retention time 4.61 min.

2′-Deoxyzebularine 3′,5′-Bis[4-chlorophenyl(ethoxy-L-alaninyl)]phosphate (5b). Isolated through column chromatography purification of **5a** crude reaction mixture and purified by preparative reverse-phase HPLC (40 mg, 9.1% yield) and obtained as a white, foamy solid. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 1.84, 1.89, 2.42, 2.47, 3.04, 3.09, 3.11. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.11−1.37 (12H, m, 2 $CHCH_3$ 2 OCH_2CH_3), 1.98−2.14 (1H, m, one of H-2′), 2.78−2.97 (1H, m, one of H-2′), 3.82−4.42 (9H, m, H-4′, H-5′, 2 *CH*NH, 2 CH*NH*, 2 O*CH*$_2$CH$_3$), 4.99−5.21 (1H, m, H-3′), 6.03−6.30 (2H, m, H-5, H-1′), 7.01−7.28 (8H, m, 2 pCl-Ph), 8.03−8.18 (1H, m, H-4), 8.42−8.51 (1H, m, H-6). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.1 (2 *CH*$_3$CH$_2$O), 20.6, 20.8, 20.9 (2 *CH*$_3$CH), 39.8, 39.9, 40.0 (C-2′), 50.2, 50.3, 50.4, 50.5 (2 *CH*CH_3), 61.8, 61.9 (2 CH_3CH_2O), 65.6, 65.7, 66.8, 66.9 (C-5′), 76.7, 77.0, 77.1 (C-3′), 84.2, 84.3 (C-4′), 87.3, 87.4 (C-1′), 121.3, 121.5, 121.6, 129.7, 129.8 (C-5, 2 pCl-Ph), 130.7 (C-1, pCl-Ph), 143.8, 144.0, 144.1 (C-4), 149.1, 149.2 (2 C-4, pCl-Ph), 155.3, 155.4 (C-2), 165.5, 165.6 (C-6), 173.4, 173.5, 173.7 (*CO*$_2$Et). HPLC (H_2O/CH_3CN from 70/30 to 0/100 in 10 min): retention time 7.21 min.

2′-Deoxyzebularine 5′-[Phenyl(benzoxy-L-alaninyl)]phosphate (6). Prepared according to standard procedure D, from 2′-deoxyzebularine (0.20 g, 0.94 mmol), phenyl(benzoxy-L-alaninyl) phosphorochloridate (1.00 g, 2.83 mmol), NMI (0.39 g, 4.70 mmol, 0.37 mL), and dry THF (10 mL). The crude was purified by column chromatography (CH_2Cl_2/CH_3OH 96/4) and preparative TLC (CH_2Cl_2/CH_3OH 96/4) to give the pure product as a white foamy solid (70 mg, 14.1% yield). ^{31}P NMR ($CDCl_3$, 121 MHz): δ 4.09, 4.28. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.42−1.45 (3H, m, $CHCH_3$), 1.99−2.12 (1H, m, one of H-2′), 2.69−2.78 (1H, m, one of H-2′), 4.03−4.48 (7H, m, H-3′, H-4′, H-5′, OH-3′, *CH*NH, CH*NH*), 5.14−5.22 (2H, m, Ph*CH*$_2$), 6.18−6.32 (2H, m, H-5, H-1′), 7.20−7.38 (10H, m, PhO, *Ph*CH$_2$), 8.21−8.25 (1H, m, H-4), 8.46−8.54 (1H, m, H-6). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 19.6, 19.7, 19.8 (CH_3), 40.2 (C-2′), 40.3, 40.4, 40.5 (*CH*CH_3), 64.6, 64.7, 64.8 (C-5′), 66.2, 66.3 (*CH*$_2$Ph), 68.8, 68.9 (C-3′), 84.4, 84.5, 84.6, 84.7 (C-4′), 86.6 (C-1′), 103.3 (C-5), 119.0, 119.1, 119.3, 124.2, 127.2, 127.5, 127.6, 127.7, 128.7, 128.8 (Ph), 134.2 ("ispo" CH_2Ph), 142.5, 142.6 (C-4), 149.4, 149.5 ("ipso" OPh), 164.6, 164.8 (C-6), 172.3, 172.4 (*C*OOCH$_2$Ph). HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): retention time 12.37 min.

2′-Deoxyzebularine 5′-[Naphthyl(ethoxydimethylglycinyl)]-phosphate (7a). Prepared adopting the standard procedure D using 2′-deoxyzebularine (0.13 g, 0.60 mmol) in dry THF (10 mL), NMI (0.24 mL, 3.0 mmol), naphthyl(ethoxydimethylglycinyl) phosphorochloridate (0.64 g, 1.8 mmol). The use of procedure D minimized the amount of 3′,5′-bisphosphoramidates, whereas procedure C failed to provide **7a**. The crude was purified by column chromatography (CH_2Cl_2/CH_3OH 96/4) to give the pure product as a white solid (35.0 mg, 11.0%). ^{31}P NMR (MeOD, 202 MHz): δ 2.93, 2.94. ^1H NMR (MeOD, 500 MHz): δ 1.12−1.15 (3H, m, CH_2CH_3) 1.42−1.45 (6H, m, C*H*$_3$), 1.58−1.67 (1H, m, H-2′), 2.30−2.36 (1H, m, H-2′), 4.03−4.37 (6H, m, H-3′, H-4′, H-5′, *CH*$_2$CH$_3$), 5.39−5.97 (1H, m, H-1′), 6.08−6.17 (1H, m, H-5), 7.26−8.07 (7H, m, Naph), 8.09−8.12 (1H, m, H-4), 8.31−8.37 (1H, m, H-6). ^{13}C NMR (MeOD, 125 MHz): δ 14.45 (*CH*$_3$CH$_2$), 27.56, 27.59, 27.99, 28.05, 28.11 (CH_3), 42.21, 42.29 (C-2′), 62.66 (CH_3CH_2), 67.54, 67.58, 67.63 (C-5′), 71.73, 71.87 (C-3′), 87.57, 87.63, 87.69 (C-4′), 89.39, 89.46 (C-1′), 106.06 (C-5), 116.29, 116.31, 116.76, 116.78, 122.83, 122.95, 126.00, 126.10, 126.53, 126.58, 127.48, 127.53, 127.89, 128.95 (Naph), 136.31 (ipso Naph), 145.56 (C-4), 157.04 (C-2), 167.03, 167.12 (C-6), 176.81 (C═O). MS (ES) *m/e*: 554.4 (MNa^+, 100%). Accurate mass: $C_{25}H_{30}N_3O_8NaP$ required 554.1688, found 554.1664. HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): retention time 11.92 min.

2′-Deoxyzebularine 3′,5′-Bis[naphthyl(ethoxydimethylglycinyl)]phosphate (7b and 7b′). Prepared adopting the standard procedure C, using 2′-deoxyzebularine (0.15 g, 0.70 mmol) in dry THF (10 mL), ^{t}BuMgCl (1 M solution in THF, 0.84 mL, 0.84 mmol), and naphthyl(ethoxydimethylglycinyl) phosphorochloridate (0.30 g, 0.84 mmol). The crude was purified by column chromatography (CH_2Cl_2/CH_3OH 96/4) to give the pure product as a white solid (fast eluting fraction = 24.4 mg, 4.1%; slow eluting fraction = 19.4 mg, 3.2%).

Fast Eluting Fraction (7b). ^{31}P NMR (MeOD, 202 MHz): δ 2.60, 2.93. ^1H NMR (MeOD, 500 MHz): δ 1.17−1.26 (6H, m, CH_2CH_3), 1.42−1.57 (12H, m, C*H*$_3$), 1.75−1.78 (1H, m, H-2′), 2.75−2.80 (1H, m, H-2′), 4.09−4.17 (4H, m, *CH*$_2$CH$_3$), 4.32−4.41 (3H, m, H-4′, H-5′), 5.22−5.24 (1H, m, H-3′), 5.95−5.98 (1H, m, H-1′), 6.06−6.08 (1H, m, H-5), 7.34−8.24 (15H, m, Naph, H-4), 8.39−8.40 (1H, m, H-6). ^{13}C NMR (MeOD, 125 MHz): δ 14.41, 14.47 (*CH*$_3$CH$_2$), 27.46, 27.55, 27.58, 27.96, 28.02, 28.13, 28.18 (CH_3), 40.70, 40.74 (C-2′), 62.58, 62.66 (CH_3CH_2), 66.81, 66.85 (C-5′), 77.81, 77.85 (C-3′), 86.00, 86.07, 86.10 (C-4′), 89.14 (C-1′), 106.08 (C-5), 116.95, 123.00, 123.07, 126.13, 126.20, 126.53, 126.61, 127.51, 127.58, 127.84, 127.93, 128.89, 128.94 (Naph), 136.25, 136.37 (ipso Ph), 145.32 (C-4), 156.78 (C-2), 167.12 (C-6), 176.70, 176.88 (C═O). MS (ES) *m/e*: 873.4 (MNa^+, 100%).

Accurate mass: $C_{41}H_{48}N_4O_{12}NaP_2$ required 873.2642, found 873.2661. HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): retention time 18.04 min.

Slow Eluting Fraction (7b′). ^{31}P NMR (MeOD, 202 MHz): δ 2.14, 2.69. ^{1}H NMR (MeOD, 500 MHz): δ 1.04−1.14 (6H, m, CH_2CH_3), 1.36−1.45 (12H, m, CH_3), 1.78 1.84 (1H, m, H-2′), 2.69−2.73 (1H, m, H-2′), 3.95−4.05 (4H, m, CH_2CH_3), 4.22−4.30 (3H, m, H-4′, H-5′), 5.09−5.11 (1H, m, H-3′, 5.92−5.96 (1H, m, H-1′), 6.19−6.22 (1H, m, H-5), 7.21−8.13 (15H, m, Naph, H-4), 8.39−8.40 (1H, m, H-6). ^{13}C NMR (MeOD, 125 MHz): δ 14.41, 14.46 (CH_3CH_2), 27.55, 27.58, 27.61, 27.94, 28.00, 28.07, 28.13 (CH_3), 40.83, 40.87 (C-2′), 62.61, 62.64 (CH_3CH_2), 67.31, 67.36 (C-5′), 78.81, 78.85 (C-3′), 86.37, 86.43, 86.47 (C-4′), 89.35, 89.45 (C-1′), 106.21 (C-5), 116.31, 116.33, 116.85, 116.88, 122.81, 123.03, 125.98, 126.14, 126.52, 127.51, 127.81, 127.84, 127.96, 128.01, 128.87, 128.91 (Naph), 136.27, 136.34 (ipso Ph), 145.55 (C-4), 156.99 (C-2), 167.40 (C-6), 176.72, 176.81 (C═O). MS (ES) *m/e*: 873.2 (MNa^+, 100%). Accurate mass: $C_{41}H_{48}N_4O_{12}NaP_2$ required 873.2642, found 873.2669. HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 20 min): retention time 17.75 min.

2′-Deoxy-2′-β-fluorozebularine 5′-[Phenyl(benzoxy-L-alaninyl)]phosphate (8). Prepared adopting the standard procedure D, using 2′-deoxy-2′-β-fluorozebularine (200 mg, 0.87 mmol), in dry THF (10 mL), NMI (345 μL, 4.35 mmol), phenyl(benzoxydimethylglycininyl) phosphorochloridate, 1 M in dry THF (2.61 mL, 2.61 mmol). The crude mixture was purified by flash chromatography (CH_2Cl_2/CH_3OH gradient elution from 98/2 to 95/5) and semipreparative TLC to give the pure product as a white foamy solid (110 mg, 23%). ^{31}P NMR (MeOD, 121 MHz): δ 5.00, 5.21. ^{1}H NMR (MeOD, 300 MHz): δ 1.30−1.43 (3H, m, CH_3CH), 3.96−4.40 (5H, m, H-3′, H-4′, H-5′, $CHCH_3$), 5.06−5.26 (3H, m, H-2′, CH_2Ph), 6.15−6.30 (1H, m, H-1′), 6.45−6.55 (1H, m, H-5), 7.13−7.43 (10H, m, Ph), 8.26−8.36 (1H, m, H-4), 8.56−8.65 (1H, m, H-6). ^{13}C NMR (MeOD, 75 MHz): δ 20.5, 20.6, 20.7 (CH_3CH), 52.0 ($CHCH_3$), 66.9, 67.0 (C-5′), 68.2 (CH_2Ph), 75.3, 75.4, 75.6, 75.7 (C-4′), 85.4, 85.5, 85.6 (C-3′), 88.6, 88.8 (C-5), 95.7 (d, J = 195 Hz, C-2′), 106.2 (C-1′), 121.6, 121.7, 121.8, 126.5, 129.5, 129.6, 129.8, 131.0, 131.1, 131.2, 137.5 (Ph), 147.0 (C-4), 152.3, 152.4 ("ipso" OPh), 157.1 (C-2), 168.2, 168.4 (C-6), 174.8, 174.9, 175.0, 175.1 (C═O). HPLC (H_2O/CH_3CN from 100/0 to 0/100 in 10 min): retention time 7.05 min.

2′-Deoxyzebularine 5′-[Phenyl(benzoxydimethylglycininyl)]-phosphate (9a). Prepared adopting the standard procedure D, using 2′-deoxyzebularine (135 mg, 0.63 mmol), in dry THF (10 mL), NMI (250 μL, 3.15 mmol), and phenyl(benzoxydimethylglycinyl) phosphorochloridate, 1 M in dry THF (1.89 mL, 1.89 mmol). As before, the use of procedure D minimized the amount of 3′,5′-bisphosphoramidates, whereas procedure C failed to provide **9a**. The crude mixture was purified with flash chromatography (CH_2Cl_2/CH_3OH gradient elution from 98/2 to 95/5), semipreparative TLC, and eventually HPLC (isocratic elution water/acetonitrile, 60/40) to give the pure product as a white foamy solid (25 mg, 7%). ^{31}P NMR (MeOD, 121 MHz): δ 2.52, 2.60. ^{1}H NMR (MeOD, 300 MHz): δ 1.49−1.55 (6H, m, $(CH_3)_2C$), 1.80−2.03 (1H, m, H-2′), 2.45−2.63 (1H, m, H-2′), 4.13−4.40 (4H, m, H-3′, H-4′, H-5′), 5.10−5.20 (2H, m, CH_2Ph), 6.10−6.20 (1H, m, H-1′), 6.40−6.53 (1H, m, H-5), 7.13−7.43 (10H, m, Ph), 8.30−8.40 (1H, m, H-4), 8.50−8.60 (1H, m, H-6). ^{13}C NMR (MeOD, 75 MHz): δ 27.5, 27.8, 27.9, 28.0 ($(CH_3)_2C$), 42.3 (C-2′), 58.2 ($(CH_3)_2C$), 66.9 (C-5′), 67.3, 67.4 (OCH_2Ph), 71.7 (C-3′), 87.5, 87.6, 87.7 (C-4′), 89.5, 89.6 (C-1′), 106.2 (C-5), 121.5, 121.6, 126.3, 128.0, 128.3, 129.3, 129.4, 129.5, 129.6, 129.9, 130.8, 132.4, 133.6, 137.4 (Ph), 145.9 (C-4), 152.1, 152.2, 152.3 ("ipso" O*Ph*), 157.2 (C-2), 167.2, (C-6), 176.5 (C═O). HPLC (H_2O/CH_3CN 60/40): retention time 3.21 min.

2′-Deoxyzebularine 3′,5′-Bis[phenyl(benzoxydimethylglycinyl)]phosphate (9b). Prepared adopting the standard procedure C, using 2′-deoxyzebularine (0.15 g, 0.70 mmol), in dry THF (10 mL), NMI (0.28 mL, 3.50 mmol), and phenyl(benzoxydimethylglycinyl) phosphorochloridate (0.77 g, 2.10 mmol). The crude was purified by column chromatography (CH_2Cl_2/CH_3OH 96/4) to give the pure product as a white solid (60 mg, 9.8%). ^{31}P NMR (MeOD, 202 MHz): δ 1.50, 1.59, 1.62, 1.88, 2.12, 2.26, 2.31, 2.37. ^{1}H NMR (MeOD, 500 MHz): δ 1.35−1.40 (12H, m, CH_3), 1.76−1.86 (1H, m, H-2′), 2.57−2.73 (1H, m, H-2′), 4.15−4.27 (3H, m, H-4′, H-5′), 5.00−5.08 (5H, m, H-3′, CH_2Ph), 5.92−6.02 (1H, m, H-1′), 6.26−6.37 (1H, m, H-5), 7.00−7.27 (20H, m, Ph), 8.16−8.21 (1H, m, H-4), 8.40−8.45 (1H, m, H-6). ^{13}C NMR (MeOD, 125 MHz): δ 27.43, 27.49, 27.52, 27.64, 27.81, 27.87, 27.90, 27.94, 27.96, 28.00, 28.08, 28.16 (CH_3), 40.77, 40.81, 40.89, 40.92 (C-2′), 66.79, 66.83, 67.15, 67.19, 67.28 (C-5′), 68.22, 68.24, 68.27, 68.30 (CH_2Ph), 78.36, 78.40, 78.56, 78.59, 78.67, 78.72 (C-3′), 85.95, 86.05, 86.12, 86.17, 86.33, 86.38 (C-4′), 89.21, 89.32, 89.43, 89.48 (C-1′), 106.27, 106.32 (C-5), 121.51, 121.55, 121.60, 121.62, 121.63, 121.66, 121.70, 121.75, 121.76, 121.80, 126.31, 126.35, 129.16, 129.19, 129.24, 129.29, 129.32, 129.34, 129.37, 129.64, 130.88 (Ph), 137.32, 137.38 (ipso Ph), 145.73, 145.84 (C-4), 152.07, 152.10, 152.13 (ipso OPh), 157.01, 157.03, 157.06 (C-2), 167.48, 167.52 (C-6), 176.48 (C═O). MS (ES) *m/e*: 1771.4 ($2M + Na^+$, 100%).

2′-Deoxyzebularine 5′-[Naphthyl(benzoxydimethylglycinyl)]-phosphate (10). Prepared adopting the standard procedure D, using 2′-deoxyzebularine (0.13 g, 0.60 mmol), in dry THF (10 mL), NMI (0.24 mL, 3.0 mmol), and naphthyl(ethoxydimethylglycinyl) phosphorochloridate (0.76 g, 1.8 mmol). The crude was purified by column chromatography (CH_2Cl_2/CH_3OH 96/4) to give the pure product as a white solid (7.0 mg, 1.7%). ^{31}P NMR (MeOD, 202 MHz): δ 2.92, 2.89. ^{1}H NMR (MeOD, 500 MHz): δ 1.56−1.59 (6H, m, CH_3), 1.62−1.72 (1H, m, H-2′), 2.38−2.44 (1H, m, H-2′), 4.13−4.37 (4H, m, H-3′, H-4′, H-5′), 5.13−5.20 (2H, m, CH_2Ph), 6.02−6.06 (1H, m, H-1′), 6.17−6.25 (1H, m, H-5), 7.29−8.19 (8H, m, H-4, Naph), 8.42−8.47 (1H, m, H-6). ^{13}C NMR (MeOD, 125 MHz): δ 27.51, 27.54, 27.99, 28.06, 28.13 (CH_3), 42.20, 42.28 (C-2′), 67.51, 67.56, 67.60 (C-5′), 68.32 (CH_2Ph), 71.69, 71.83 (C-3′), 87.52, 87.59, 87.65 (C-4′), 84.37, 89.43 (C-1′), 106.06 (C-5), 116.33, 116.80, 116.83, 122.82, 122.94, 125.99, 126.09, 126.50, 126.56, 127.46, 127.51, 127.87, 128.93, 129.33, 129.59 (Naph, Ph), 136.29 (ipso Ph), 137.31 (ipso Naph), 145.52 (C-4), 156.98 (C-2), 166.99, 167.08 (C-6), 176.56 (C═O). MS (ES) *m/e*: 616.3 (MNa^+, 100%). Accurate mass: $C_{30}H_{32}N_3O_8NaP$ required 616.1825, found 616.1818.

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