Design, Synthesis, and Biological Evaluation of Cyclic and Acyclic Nitrobenzylphosphoramide Mustards for *E. coli* Nitroreductase Activation

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In efforts to obtain anticancer prodrugs for antibody-directed or gene-directed enzyme prodrug therapy using *E. coli* nitroreductase, a series of nitrobenzylphosphoramide mustards were designed and synthesized incorporating a strategically placed nitro group in a position para to the benzylic carbon for reductive activation. All analogues were good substrates of *E. coli* nitroreductase with half-lives between 2.9 and 11.9 min at pH 7.0 and 37 °C. Isomers of the 4-nitrophenylcyclophosphamide analogues **3** and **5** with a benzylic oxygen para to the nitro group showed potent selective cytotoxicity in nitroreductase (NTR) expressing cells, while analogues **4** and **6** with a benzylic nitrogen para to the nitro group showed little selective cytotoxicity despite their good substrate activity. These results suggest that good substrate activity and the benzylic oxygen are both required for reductive activation of 4-nitrophenylcyclophosphamide analogues by *E. coli* nitroreductase. Isomers of analogue **3** showed 23000−29000× selective cytotoxicity toward NTR-expressing V79 cells with an IC_{50} as low as 27 nM. They are about as active as and 3−4× more selective than 5-aziridinyl-2,4-dinitrobenzamide (CB1954). The acyclic 4-nitrobenzylphosphoramide mustard ((±)-**7**) was found to be the most active and most selective compound for activation by NTR with 170000× selective cytotoxicity toward NTR-expressing V79 cells and an IC_{50} of 0.4 nM. Compound (±)-**7** also exhibited good bystander effect compared to 5-aziridinyl-2,4-dinitrobenzamide. The low IC_{50}, high selectivity, and good bystander effects of nitrobenzylphosphoramide mustards in NTR-expressing cells suggest that they could be used in combination with *E. coli* nitroreductase in enzyme prodrug therapy.

Introduction

Most anticancer agents in clinical use are often associated with debilitating side effects because of their lack of tumor selectivity. The side effects limit the maximum dose that can be given to effectively treat tumors. Recently, tumor-targeted prodrug therapy has been extensively studied to increase the selectivity of cytotoxic agents toward tumor cells.[1−3] In this strategy, the cytotoxic agent is given in its prodrug form, which is safe to normal cells but is selectively activated by certain biochemical mechanisms unique to tumor cells, resulting in localized cytotoxicity in tumor tissues. The mechanisms explored for this purpose include hypoxic reduction in solid tumors, activation by enzymes overexpressed in tumor tissues, and targeting of antigens or receptors specifically expressed on tumor cell surface.[1,2] Furthermore, activating enzymes can also be delivered to tumor cells through antibody and gene therapy approaches called antibody-directed enzyme prodrug therapy (ADEPT) or gene-directed enzyme prodrug therapy (GDEPT). In ADEPT and GDEPT, an exogenous enzyme is delivered site-specifically into tumor cells through chemical conjugation or genetic fusion to a tumor-specific antibody or by enzyme gene delivery systems. This is then followed by the administration of a prodrug, which is selectively activated by the delivered enzyme at tumor cells.

A number of enzyme/prodrug systems for ADEPT/GDEPT are in development and have been reviewed.[4] Among the enzymes under evaluation is the nfsB gene product of *Escherichia coli*, an oxygen-insensitive flavin mononucleotide-containing nitroreductase (NTR). This flavoprotein is capable of reducing certain aromatic nitro groups to the corresponding hydroxylamines in the presence of cofactor NADH or NADPH.[5,6] This reduction process represents a very large electronic change and can provide an efficient electronic "switch" that can be exploited to generate potent cytotoxins.[7] Four classes of prodrugs for activation by NTR have been described, including 5-aziridinyl-2,4-dinitrobenzamide (CB1954), dinitrobenzamide mustards (e.g., SN 23862), 4-nitrobenzylcarbamates, and nitroindolines.[7−9] Of the four classes, the first two are considered most promising when used in combination with NTR. 5-Aziridinyl-2,4-dinitrobenzamide, currently under phase II clinical trial in conjunction with the virally delivered NTR enzyme, has high selectivity (>1000-fold) in cell lines transfected with NTR and potent and long-lasting inhibition of nitroreductase-transfected tumors in mice. The related mustard SN 23862 has similar selectivity and good bystander effects in animal models.

We were interested in converting cyclophosphamide (**1**) into reductively activated prodrugs and previously reported analogues of nitrobenzene-fused cyclophosphamide (**2**) for activation by NTR.[10] While these compounds showed good substrate activity for NTR with half-lives between 7 and 24 min, only compound

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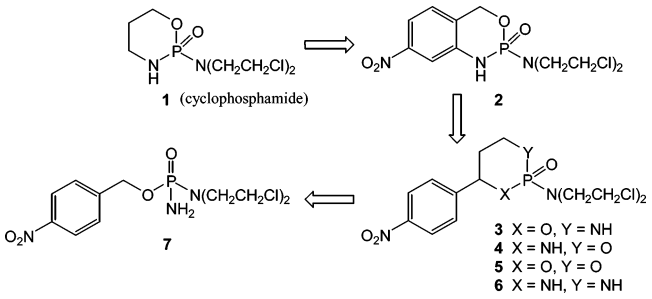
Chart 1. Development of 4-Nitrophenylphosphoramide Mustard Analogues for Bioreductive Activation



2 and the dioxa analogue with an oxygen at the benzylic position showed a modest >33- to 36-fold enhanced cytotoxicity toward NTR-expressing cells. Our efforts then focused on a series of related exocyclic cognates, 4-nitrophenyl-substituted cyclophosphamide analogues (**3**−**6**). Structure−activity relationship studies led to the conclusion that cytotoxicity was dependent on not only good substrate activity toward NTR but also the presence of a benzylic oxygen para to the nitro group. Compared to the most active compound **2** in the nitrobenzene-fused cyclophosphamide series, the exocyclic cognate **3** exhibited an over 100-fold increase in cytotoxicity and nearly 1000-fold increase in selectivity toward NTR-expressing cells. This observation prompted us to synthesize the acyclic 4-nitrobenzylphosphoramide mustard (**7**), which to our surprise turned out to be the most active and most selective compound for activation by NTR reported so far. Compound **7** showed 170000× selective cytotoxicity toward NTR-expressing V79 cells with an IC_{50} as low as 0.4 nM. Chart 1 shows the development process of 4-nitrobenzylphosphoramide mustard analogues for reductive activation by NTR enzyme. A communication highlighting the selectivity and potency of the most active compounds in each series appeared recently in this journal.[11] Here, we report in more detail the structure−activity relationship studies of 4-nitrophenyl-substituted cyclophosphamide analogues and 4-nitrobenzylphosphoramide mustard as well as their cytotoxic bystander effects in cell culture assays.

Results and Discussion

Design Principle. Cyclophosphamide (**1**) is one of the most successful anticancer agents developed over the past few decades.[12] Because of its activity against both cycling and noncycling cells, it is one of the few anticancer agents effective in the treatment of slow-growing solid tumors.[13,14] Cyclophosphamide is an anticancer prodrug that has to be activated by cytochrome P-450 enzyme in the liver to release the activated phosphoramide mustard and acrolein.[15−18] Phosphoramide mustard is the ultimate alkylating species that cross-links interstrand DNA.[19−21] Acrolein is a byproduct that is responsible for hemorrhagic cystitis, a life-threatening side effect associated with cyclophosphamide.[22]

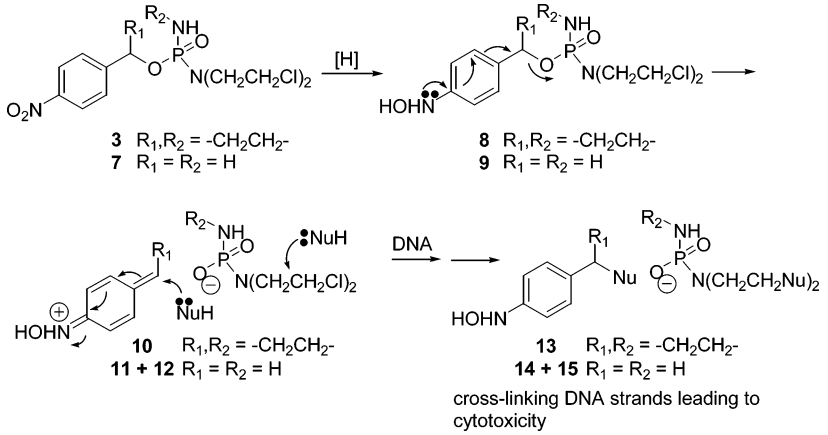
The interest in developing more selective cyclophosphamide-type anticancer agents led to the development of phosphoramidate prodrugs incorporating a variety of specific activation mechanisms, including acid-sensitive hexenopyranoside of aldophosphoramide,[23] hypoxia-selective nitroheterocyclic phosphoramides,[24,25] and indolequinone phosphoramidates for DT-diaphorase activation.[26] Our efforts have focused on the design of cyclophosphoramide analogues incorporating site-specific activation mechanisms by strategically placing a nitro group for bioreductive activation in order to move the site of activation from liver into the tumor tissues. The nitro group is a strong electron-withdrawing group (Hammett σ_p electronic parameter of 0.78) and reduces the oxidation potential of the phosphorinane ring system toward hepatic cytochrome P-450 oxidation. The electron-withdrawing nitro group is converted to an electron-donating hydroxylamino group ($\sigma_p = -0.34$) upon NTR reduction. This large difference in electronic effect ($\Delta\sigma_p = 1.12$) has been shown to cause cleavage of the benzylic C−O bond in 4-nitrobenzyl carbamates upon nitro reduction.[7,8,27,28] It is used here to effect the formation of highly cytotoxic phosphoramide mustard from compounds **3** and **7** as shown in Scheme 1. Upon nitro reduction by NTR, the resulting electron-donating hydroxylamino group relays its electrons to the para position and promotes the cleavage of benzylic C−O bond, leading to the subsequent activation of the phosphoramide mustard portion in **10** and **12**. The intermediates **10** and **11** also possess electrophilic centers that could potentially form cross-links with functionally important macromolecules, providing yet an additional mechanism for cytotoxicity. To confirm the requirement of the presence of benzylic oxygen in **3** and **7** in fragmentation and the subsequent activation of the phosphoramide mustard, the corresponding regioisomer **4** and the dioxa and diaza analogues **5** and **6** were also synthesized and evaluated for NTR activation. Although the analogues **4** and **6** are not expected to undergo cleavage upon NTR reduction to release the activated phosphoramidate, they were also designed to test whether the aromatic hydroxylamine contributes to cytotoxicity. It was proposed that the bioactivation of 5-aziridinyl-2,4-dinitrobenzamide upon NTR reduction went through further acylation of the 5-(aziridinyl)-4-hydroxylamino-2-nitrobenzamide by coenzyme A, resulting in the formation of lethal DNA−DNA interstrand cross-links in cells.[29]

Chemistry. The synthesis of **3**−**5** all started from 4-nitrobenzaldehyde (**16**). As shown in Scheme 2, the synthesis of the dioxa analogue **5** of 4-(4-nitrophenyl)cyclophosphamide was accomplished in four steps starting from 4-nitrobenzaldehyde. Grignard reaction of aldehyde **16** with vinylmagnesium bromide in THF at −78 to −50 °C gave the racemic allylic alcohol **17**. The low temperature was necessary to prevent the nitro group from being affected under the Grignard reaction conditions. Hydroboration of the allylic alcohol **17** followed by basic hydrogen peroxide oxidation afforded the racemic diol **18**, which reacted with bis(2-chloroethyl)phosphoramidic dichloride to give two chromatographically separable diastereomers of the target compound: *cis*-(±)-**5** and *trans*-(±)-**5**. The combined yield of the two isomers varied with the base used in the cyclization step; it increased to 68% from 14% when *n*-BuLi was used instead of TEA. This is most likely due to the higher nucleophilicity of hydroxyl group upon deprotonation by *n*-BuLi.

4-Nitrophenylcyclophosphamide diaza analogue **6** was synthesized from the 1,3-diol intermediate **18** as shown in Scheme 3. Compound **18** was converted to the corresponding diazide **19** using a PPh_3/DEAD-mediated Mitsunobu reaction.[30] Hydrazoic acid (HN_3) solution in benzene was prepared from NaN_3 and sulfuric acid according to a literature procedure.[31] The two azido groups were reduced to amino groups by 1,3-propanedithiol to afford 1,3-diamine **20**. Treatment with PPh_3-H_2O failed to reduce the secondary azido group to the amino group.[32] Compound **20** was cyclized with bis(2-chloroethyl)phosphoramidic dichloride in the presence of TEA to give the target product **6** as a pair of diastereomers (*cis*-(±)-**6** and *trans*-(±)-**6**) in a combined yield of 62%.

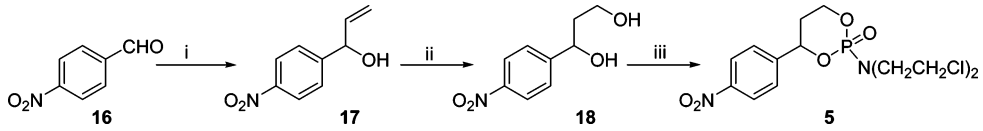
For the synthesis of 6-(4-nitrophenyl)cyclophosphamide (**3**) shown in Scheme 4, the Grignard reaction product allylic alcohol **17** was protected by the methoxymethyl (MOM) group prior to

Scheme 1. Proposed Mechanism of Activation of Nitrobenzylphosphoramide Mustards upon Bioreduction.



3 R_1,R_2 = -CH_2CH_2-
7 $R_1 = R_2$ = H
8 R_1,R_2 = -CH_2CH_2-
9 $R_1 = R_2$ = H
10 R_1,R_2 = -CH_2CH_2-
11 + 12 $R_1 = R_2$ = H
13 R_1,R_2 = -CH_2CH_2-
14 + 15 $R_1 = R_2$ = H

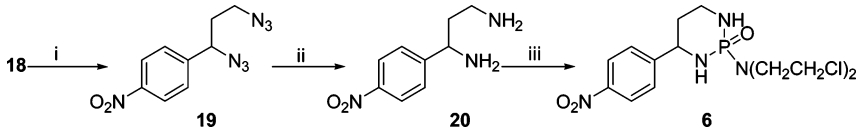
Scheme 2. Synthesis of 4-Nitrophenylcyclophosphamide Dioxa Analogue **5**[a]



16 **17** **18** **5**

[a] Reagents and conditions: (i) vinylmagnesium bromide, THF, −78 to −50 °C, 4 h, 95%; (ii) B_2H_6/THF, 0 °C, 20 h, followed by 3 N NaOH, 30% H_2O_2, 30 min, 82%; (iii) *n*-BuLi, bis(2-chloroethyl)phosphoramidic dichloride, THF, −78 °C, 6 h, *cis*-(±)-**5**, 28%, *trans*-(±)-**5**, 40%.

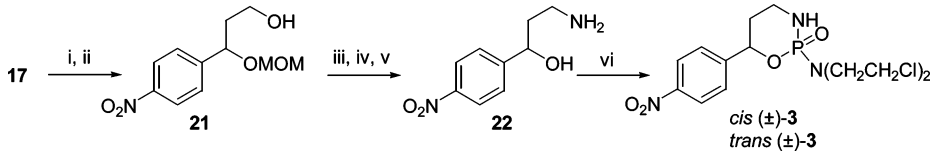
Scheme 3. Synthesis of 4-Nitrophenylcyclophosphamide Diaza Analogue **6**[a]



19 **20** **6**

[a] Reagents and conditions: (i) PPh_3, DEAD, HN_3, THF, room temp, 12 h, 73%; (ii) Et_3N, 1,3-propanedithiol, MeOH, room temp, 36 h, 87%; (iii) bis(2-chloroethyl)phosphoramidic dichloride, Et_3N, EtOAc, room temp, 40 h, *cis*-(±)-**6**, 28%, *trans*-(±)-**6**, 34%.

Scheme 4. Synthesis of 6-(4-Nitrophenyl)cyclophosphamide **3**[a]



21 **22** *cis* (±)-**3** *trans* (±)-**3**

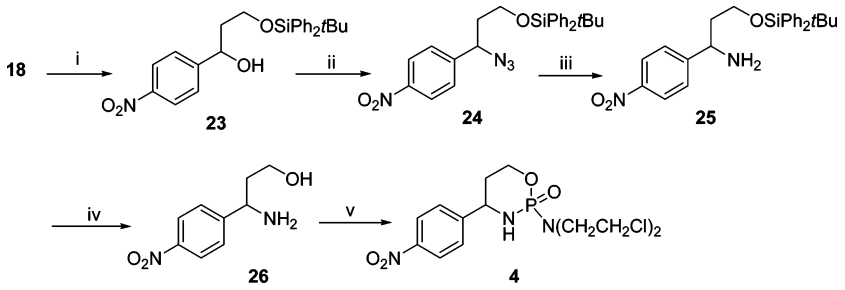
[a] Reagents and conditions: (i) MOMCl/DIEA, 0 °C to room temp, 24 h, 95%; (ii) B_2H_6/THF, 0 °C, 5 h, then 3 N NaOH, 30% H_2O_2, 30 min, 78%; (iii) MsCl/ Et_3N, then NaN_3, 91%; (iv) PPh_3, THF−H_2O, room temp, 72%; (v) CBB followed by HOAc, 76%; (vi) bis(2-chloroethyl)phosphoramidic dichloride, Et_3N, EtOAc, 48 h, *cis*-(±)-**3**, 34%, *trans*-(±)-**3**, 33%.

hydroboration to give compound **21**. A two-step sequence of activation and S_N2 displacement converted the hydroxyl group to azido. This was then followed by PPh_3-mediated reduction and deprotection to give the corresponding amino alcohol **22**. The deprotection of MOM was accomplished using a combination of catechol boron bromide (CBB) with acetic acid, a modified procedure developed specifically to avoid the problem of cyclization and formation of cyclic formylacetal encountered using other common MOM-deprotection conditions.[33] The amino alcohol **22** was cyclized upon treatment with bis(2-chloroethyl)phosphoramidic dichloride in the presence of TEA to give the target product **3** as a pair of diastereomers (*cis*-(±)-**3** and *trans*-(±)-**3**) in a combined yield of 67%.

Scheme 5 outlined the synthesis of 4-(4-nitrophenyl)cyclophosphamide (**4**). The primary hydroxyl group of **18** was selectively protected with TBDPS at low temperature to give the monoprotected compound **23**. The secondary hydroxyl group was converted to the azido group using the Mitsunobu reaction conditions. Other conditions including $(CF_3SO_2)_2O$/Pyr-NaN_3, MsCl/NEt_3−NaN_3, PPh_3/DEAD-$(PhO)_2PON_3$,[34] and DBU-$(PhO)_2PON_3$ failed to give the desired product. This difficulty could be attributed to facile elimination of the activated ester intermediate under these conditions. Reduction of the azido group in **24** using 1,3-propanedithiol gave the amino product **25**.[35] The TBDPS group was removed using TBAF to afford **26**, which was cyclized with bis(2-chloroethyl)phosphoramidic dichloride to give the desired product **4** as a pair of diastereomers (*cis*-(±)-**4** and *trans*-(±)-**4**) in a combined yield of 31%.

Because of the presence of two chiral centers, one at the 4-nitrophenyl-substituted carbon and the other at the phosphorus atom, analogues **3**−**6** exist as a pair of diastereomers that are referred to as the cis and trans (cis = *RS*/*SR*; trans = *RR*/*SS*) as shown in Chart 2. The relative configurations of cis and trans are defined on the basis of the relative orientations of the aryl substituent and the oxygen atom of the P═O bond.[36] Because of the greater equatorial preference of the bulky aryl group, cis isomers have both groups in equatorial positions and trans isomers have the aryl group in the equatorial position and the phosphoxide group in the axial position.[37] Assignment of cis and trans configurations was based on their chromatographic behavior, ^1H and ^{31}P NMR chemical shifts, and infrared spectral data (Table 1).[37] Compared to its trans diastereomer, the cis

Scheme 5. Synthesis of 4-(4-Nitrophenyl)cyclophosphamide **4**[a]



[a] Reagents and conditions: (i) *t*-BuPh$_2$SiCl, imidazole, DMF, −30 to −20 °C, 1.2 h, 95%; (ii) PPh$_3$, DEAD, HN$_3$/PhH, THF, 0 °C to room temp, 5 h, 87%; (iii) Et$_3$N, 1,3-propanedithiol, MeOH, room temp, 36 h, 70%, (iv) TBAF, THF, 1 h, 82%; (v) bis(2-chloroethyl)phosphoramidic dichloride, Et$_3$N, EtOAc, room temp, 48 h, *cis*-(±)-**4**, 14%, *trans*-(±)-**4**, 17%.

Chart 2. Diastereomers of Nitrophenyl-Substituted Cyclophosphamides

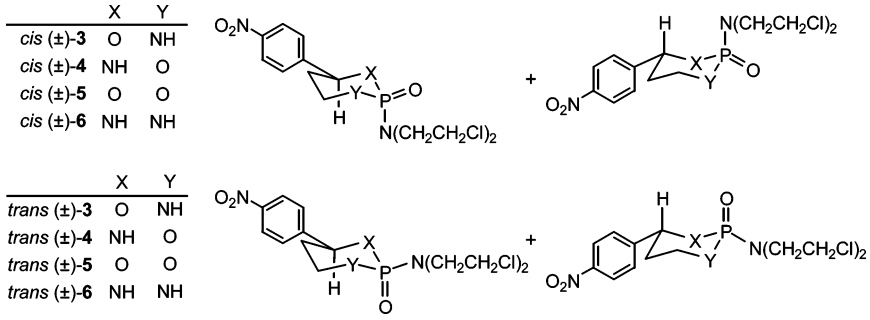


Table 1. Analytical Data of Diastereomeric 4-Nitrophenylcyclophosphamide Analogues

	compd	R_f (TLC)[a]	IR ($\nu_{P=O}$, cm^{-1})	NMR δ (ppm) ^{1}H[b]	NMR δ (ppm) ^{31}P
X = O	*cis*-(±)-**3**	0.45	1330	4.74	9.6
Y = NH	*trans*-(±)-**3**	0.10	1325	4.81	14.2
X = NH	*cis*-(±)-**4**	0.45	1350	5.45	11.2
Y = O	*trans*-(±)-**4**	0.12	1330	5.63	14.6
X = O	*cis*-(±)-**5**	0.26	1259	5.62	3.5
Y = O	*trans*-(±)-**5**	0.20	1246	5.79	7.4
X = NH	*cis*-(±)-**6**	0.50	1330	4.65	12.9
Y = NH	*trans*-(±)-**6**	0.45	1325	4.74	17.1

[a] TLC (on silica gel) developing solvents: hexanes/EtOAc (2:1) for **3**−**5**, CHCl$_3$/CH$_3$OH (20:1) for **6**. [b] The ^{1}H chemical shift refers to the proton on the carbon with the 4-nitrophenyl substituent.

isomer elutes more quickly on silica gel, has a higher P═O stretching frequency, a more downfield C-5 proton signal,and a more upfield phosphorus signal. The two isomers were easily separated by flash column chromatography on silica gel. The synthesis of 4-nitrobenzylphosphoramide mustard ((±)-**7**) was accomplished in a three-step, one-pot process as reported earlier.[11]

Chemical Stability. All four 4-nitrophenylcyclophosphamide analogues and 4-nitrobenzylphosphoramide mustard were incubated in 50 mM phosphate buffer, pH 7.4, at 37 °C. The stability of each compound was monitored by reversed-phase HPLC analysis of the incubation mixtures. The analogues were all stable under these conditions with no significant changes over a period of 72 h (<10%).

Substrate Activity for *E. coli* Nitroreductase. Isomers of **3**−**6** and compound **7** were evaluated as substrates of *E. coli* nitroreductase by incubating each compound (0.2 mM) in 10 mM phosphate buffer, pH 7.0, at 37 °C in the presence of 1 mM NADH as the cofactor. The reaction was initiated by the addition of 1.8 μg of *E. coli* nitroreductase. Aliquots were withdrawn at various time intervals, quenched with acetonitrile, and stored frozen prior to HPLC analysis. The half-lives were calculated on the basis of the disappearance of the substrates (Figure 1). 5-Aziridinyl-2,4-dinitrobenzamide, an excellent substrate of *E. coli* nitroreductase currently in phase II clinical trials,[4,6] was used as a control in our experiments. All compounds were found to be good substrates of *E. coli* nitroreductase with half-lives between 2.3 and 11.9 min, while the control 5-aziridinyl-2,4-dinitrobenzamide had a half-life of 5 min under the same assay conditions. The trans isomers were better substrates of *E. coli* nitroreductase than the corresponding cis isomers as indicated by their relatively shorter half-lives, suggesting that configuration might affect the substrate binding to and/or the catalytic activity of *E. coli* nitroreductase. Compound (±)-**7** turned out to be the best substrate with a half-life of 2.3 min.

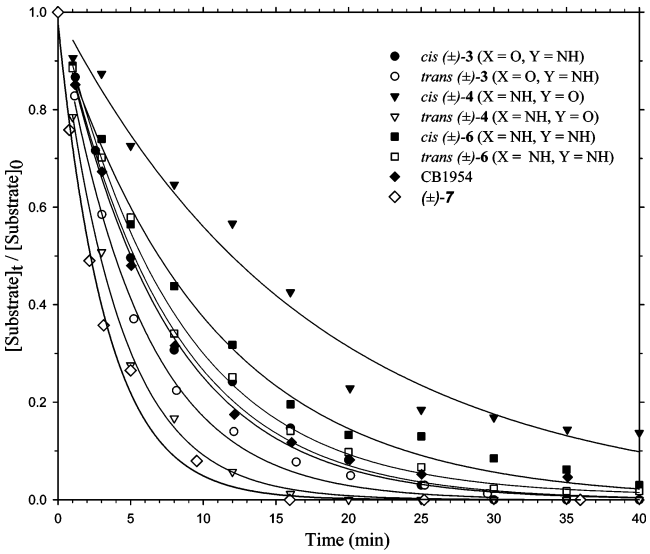


Figure 1. Disappearance of substrates **3**, **4**, **6**, **7**, and CB1954 during reduction by *E. coli* nitroreductase as monitored by HPLC. Each substrate (0.2 mM) was incubated with 1.8 μg of *E. coli* nitroreductase in 10 mM phosphate buffer, pH 7.0, in the presence of 1 mM of NADH at 37 °C in a total volume of 250 μL.

Table 2. *E. coli* Nitroreductase Activation of 4-Nitrophenyl-Substituted Cyclophosphamide Analogues in Chinese Hamster V79 Cells and Human Ovarian Cancer Cells

		V79 cells,[i] stable clones, 72 h of drug exposure[b]			V79 cells,[i] stable clones, 1 h of drug exposure[b]			SKOV3 cells,[i] virally infected, 18 h of drug exposure[b]			SKOV3 cells,[i] stable clones, 18 h of drug exposure[b]		
compd	NR assay $t_{1/2}$ (min)[a]	IC_{50},[c] NTR^-	IC_{50},[c] NTR^+	ratio[d]	IC_{50},[c] NTR^-	IC_{50},[c] NTR^+	ratio[d]	IC_{50},[c] NTR^-	IC_{50},[c] NTR^+	ratio[d]	IC_{50},[c] NTR^-	IC_{50},[c] NTR^+	ratio[d]
cis-(±)-**3**	5.2	832	0.029	29,000	>100	0.34	>290	680	4.7	150	>930	2.1[g]	>440
trans-(±)-**3**	3.9	608	0.027	23,000	>100	0.17	>590	>1000	5	>200	570	2.1[g]	270
cis-(±)-**4**	11.9	>100	45.3	>2.2	ND[e]	ND[e]	ND[e]	>1000	530	>1.9	>1000	530	>1.9
trans-(±)-**4**	2.9	>100	51.5	>1.9	ND[e]	ND[e]	ND[e]	>1000	220	>4.5	>1000	210	>4.8
cis-(±)-**5**	7.3	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	>1000	4.3[g]	>230
trans-(±)-**5**	4.8	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	505	2.5[g]	200
cis-(±)-**6**	6.4	56.8	4.6	12	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]
trans-(±)-**6**	4.2	>100	48.3	>2.1	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]
(±)-**7**	2.3	67	0.0004	170,000	>100	0.01	>10,000	>1000	1.2	>830	>890	0.16[g]	>5600
								>1000	6.2[f]	>160[f]			
5-aziridinyl-2,4-dinitrobenzamide	5.0	254	0.036	7,100	>100	0.31	>320	>1000	4.7	>210	>790	2.0[g]	>400
								625	52[f]	12[f]			
4-hydroperoxy-cyclophosphamide		3.1[h]	2.7[h]	1.1									

[a] Half-lives of reduction by *E. coli* nitroreductase were determined using 0.2 mM of substrate in 10 mM phosphate buffer (pH 7.0) in the presence of 1 mM NADH at 37 °C in a total volume of 250 μL. The reaction was initiated by the addition 1.8 μg of *E. coli* nitroreductase. Aliquots were withdrawn and analyzed by HPLC. [b] Cells were exposed to each test compound, and a standard cell viability assay was performed at the end of indicated incubation period. Initially, the maximum concentration used was 100 μM in the case of V79 cells and 1000 μM in the case of SKOV3 cells. When necessary, assays were repeated at higher drug concentrations to obtain accurate IC_{50} values. [c] IC_{50} values are the concentration in μM required to reduce cell number to 50% of control after the cells were exposed to the drug for the indicated time. The standard errors of all assays were within 10% of the mean between replicates at a given concentration and 10−23% for the fitted IC_{50} values. [d] Ratio of IC_{50} values (NTR^-/NTR^+) as an indication of activation by *E. coli* nitroreductase. [e] Not determined. [f] SKOV3 cells for these experiments were infected at a lower infection ratio of 10 pfu/cell, while the others in the same column were infected at a higher infection ratio of 100 pfu/cell. [g] SKOV3 cells stably expressing NTR (SKOV NTR), compared with nonexpressing cells (SKOV3). [h] 4-Hydroperoxycyclophosphamide was tested using sealed plates and HEPES medium. [i] Cell lines: V79 (Chinese hamster fibroblast) and SKOV3 (human ovarian carcinoma).

Antiproliferative Activity in Cell Culture. Compounds **3**−**7** were assayed for their cytotoxicity against cells expressing or not expressing *E. coli* nitroreductase. Three pairs of cell lines were used that were derived from V79 Chinese hamster cells and the SKOV3 human ovarian cancer cells. The NTR^+ V79 cells had been transfected with a bicistronic vector encoding for the *E. coli* nitroreductase and puromycin resistance protein as the selective marker, while the NTR^- V79 cells were transfected with vector only and were used as the controls. The SKOV3 human ovarian cancer cells were made to express *E. coli* nitroreductase in two ways. In some experiments, the SKOV3 cells were infected with an E1, E3-deleted replication-defective adenovirus vector vPS1233 expressing the wild type *E. coli* nitroreductase from the CMV promoter at infection ratios of 10 or 100 plaque forming units (pfu) per cell. Other experiments utilized SKOV3 cells that had been stably transduced with the retroviral vector rv.RD18.LNC-nr,[38] which confers resistance to neomycin/G418 and expresses nitroreductase from the CMV promoter. These SKOV NTR cells were compared with the parental, untransduced SKOV3 cells. The IC_{50} values and the ratios of IC_{50} (NTR^-/NTR^+) of **3**−**7** are presented in Table 2.

It is clear from the data presented in Table 2 that analogues with benzylic oxygen para to the nitro group were consistently much more cytotoxic to NTR^+ cells than to NTR^- cells while analogues with benzylic nitrogens were only marginally more cytotoxic to NTR^+ cells than to NTR^- cells. In stable clones of Chinese hamster V79 cells, *cis*-(±)-**3** and *trans*-(±)-**3** were 23000−29000× more cytotoxic toward NTR-expressing cells. This level of selectivity is about 3−4× that of 5-aziridinyl-2,4-dinitrobenzamide while IC_{50} values of *cis*-(±)-**3** and *trans*-(±)-**3** in NTR^+ V79 cells were comparable to that of 5-aziridinyl-2,4-dinitrobenzamide. In virally infected as well as in stably transfected clones of SKOV3 cells, *cis*-(±)-**3**, *trans*-(±)-**3**, and 5-aziridinyl-2,4-dinitrobenzamide showed similar activity and selectivity. The dioxa analogues *cis*-(±)-**5** and *trans*-(±)-**5** showed somewhat reduced activity and selectivity in stably transfected clones of SKOV3 cells compared to *cis*-(±)-**3**, *trans*-(±)-**3**, and 5-aziridinyl-2,4-dinitrobenzamide. Replacement of the benzylic oxygen with a nitrogen as in *cis*-(±)-**4**, *trans*-(±)-**4**, *cis*-(±)-**6**, and *trans*-(±)-**6** nearly completely eliminated the selectivity toward NTR^+ cells, suggesting that these analogues were not activated even though they were reduced by NTR as indicated in our enzyme assays. This apparently relates to the ability of $-NH-PO(NR_2)(OR')$ or $-NH-PO(NR_2)(NHR')$ as a much poorer leaving group than that of $-O-PO(NR_2)(NHR')$ and $-O-PO(NR_2)(OR')$. Without cleavage of the benzylic C−N bond and the negatively charged phosphate oxy anion under near-physiological pH, these analogues were not cytotoxic. These results indicate that nitroreductase reduction is an important first step and the conversion of the nitro to hydroxylamino group is not sufficient for enhanced cytotoxicity in nitroreductase-expressing cells. This is consistent with the mechanism proposed in Scheme 1 where cleavage of the benzylic C−O bond upon reduction is required for the activation of phosphoramide mustard.

The acyclic analogue, 4-nitrobenzylphosphoramide mustard ((±)-**7**), was originally synthesized as a control compound to explore the mechanism of activation of cyclic phosphoramide analogues and has turned out to be the most active compound in all our assays. The selectivity of compound (±)-**7** was a remarkable 170000× in NTR^+ V79 cells with an IC_{50} as low as 0.4 nM. Thus, compound (±)-**7** was about 100× more active and over 20× more selective toward NTR^+ V79 cells than 5-aziridinyl-2,4-dinitrobenzamide. 4-Hydroperoxycyclophosphamide, a preactivated cyclophosphamide analogue, was used as a control to exclude the possibility that V79 cells expressing *E. coli* nitroreductase was sensitized to phosphoramide mustard-type alkylating agents. Because it was known that 4-hydroperoxycyclophosphamide releases a volatile metabolite, the control experiment was performed using sealed plates as suggested in the literature.[39] The results of the control experiment shown in Table 2 clearly indicate that expression of *E. coli* nitroreductase has no effect on the antiproliferative activity

in V79 cells of the active metabolites released from the preactivated cyclophosphamide precursor. The extremely low IC_{50} for compound (±)-**7** in NTR^+ V79 cells suggests that intracellular activation and release of cytotoxic phosphoramide mustard are apparently more effective. Even for the 1 h drug exposure that was used to better mimic the in vivo situation, compound (±)-**7** still showed an IC_{50} of 10 nM, about 30× lower than that of 5-aziridinyl-2,4-dinitrobenzamide, suggesting that compound (±)-**7** was quickly activated and had long lasting cytotoxic effects in *E. coli* nitroreductase-expressing Chinese hamster V79 cells. The fast activation kinetics and the lasting cytotoxic effects upon activation could potentially overcome an important hurdle known in the development of the enzyme−prodrug therapy using *E. coli* nitroreductase.[4]

In clinical use, NTR is delivered using a viral vector. Therefore, we confirmed the level of cell sensitization to different prodrugs that could be achieved, using a replication-defective adenovirus to express NTR in the SKOV3 ovarian cancer cells. When SKOV3 cells were infected with 100 pfu/cell of adenovirus expressing NTR, compound (±)-**7** showed an IC_{50} that is about 4× lower than the IC_{50} of 5-aziridinyl-2,4-dinitrobenzamide and compound **3** isomers. When the level of NTR expression was made more limiting by infection with just 10 pfu/cell of the adenovirus, although both IC_{50} values were higher, the differential increased such that the IC_{50} for compound (±)-**7** was about 9× lower than that for 5-aziridinyl-2,4-dinitrobenzamide. In stably transduced SKOV3 cells, compound (±)-**7** had a submicromolar IC_{50} that is 6× lower than that for 5-aziridinyl-2,4-dinitrobenzamide. The higher IC_{50} values of all prodrugs in the SKOV3 cells compared to those in V79 cells could theoretically be due in part to differences in the level of NTR expression. Another contributory factor could be a greater DNA repair capability, or resistance to DNA damage-induced apoptosis, in the human ovarian tumor cells.

Furthermore, compound (±)-**7** was confirmed to be a much better substrate for NTR than 5-aziridinyl-2,4-dinitrobenzamide in enzyme kinetic assays.[11] Compound (±)-**7** has a K_m of 195 ± 14 μM and a k_{cat} of 14.03 ± 0.35 s^{-1}, while 5-aziridinyl-2,4-dinitrobenzamide has a K_m of 881 ± 42 μM and k_{cat} of 6.60 ± 0.11 s^{-1}. Since 5-aziridinyl-2,4-dinitrobenzamide is reduced to a 1:1 mixture of 2-hydroxylamino and 4-hydroxylamino products by NTR and the 4-hydroxylamino is known to be the major cytotoxic product,[40,41] the productive $^1/_2k_{cat}$ of 3.30 s^{-1} is used for comparison purposes. This gave a specificity constant of 71 900 M^{-1} s^{-1} (k_{cat}/K_m) for compound (±)-**7** and 3750 M^{-1} s^{-1} ($^1/_2k_{cat}/K_m$) for 5-aziridinyl-2,4-dinitrobenzamide. Thus, compound (±)-**7** is 19 times better as a substrate of NTR than 5-aziridinyl-2,4-dinitrobenzamide. This at least partially contributed to the better activity and superior selectivity in cell culture assays of compound (±)-**7** in comparison with 5-aziridinyl-2,4-dinitrobenzamide.

It should also be noted that compound (±)-**7** upon reductive activation releases phosphoramide mustard, which is the active metabolite of the clinical drug cylcophosphamide.[15−17] The excellent activity of compound (±)-**7** in nitroreductase-expressing cells was unexpected considering the fact that only a 2-fold increase in cytotoxicity was observed for 4-nitrobenzyl *N*,*N*,*N*′,*N*′-tetrakis(2-chloroethyl)phosporodiamidate toward cancer cells under hypoxic conditions.[25] This suggests that either these compounds were poor substrates of the human reductase(s) present or the expression of these reductase(s) was limited under the hypoxic assay conditions used. For cancer treatment, the extent of bystander effect will be critical to the success of any gene-directed enzyme prodrug therapy. The bystander cytotoxicity can be quantitated in terms of the percentage of activator (NTR^+) cells in a mixed population of NTR^+ and NTR^- cells to produce an IC_{50} midway between those in either NTR^+ or NTR^- cell type alone.[42] In this assay, compound (±)-**7** showed the best bystander effect with a TE_{50} value of 3.3%, slightly better than that of 5-aziridinyl-2,4-dinitrobenzamide (TE_{50} = 4.5%), while cyclic analogues, *cis*-(±)-**3**, *trans*-(±)-**3**, *cis*-(±)-**5**, and *trans*-(±)-**5**, exhibited poor bystander effects (data not shown). The excellent bystander effect and selectivity suggest that compound (±)-**7** is a better drug candidate than 5-aziridinyl-2,4-dinitrobenzamide for use in combination with nitroreductase in ADEPT or GDEPT.

Conclusions

In summary, we have developed a novel and superior class of nitroaryl phosphoramides as potential prodrugs for nitroreductase-mediated enzyme−prodrug therapy. All analogues were stable in phosphate buffer at pH 7.4 and 37 °C and were all good substrates of *E. coli* nitroreductase with half-lives between 2.9 and 11.9 min, but only the analogues with a benzylic oxygen para to the nitro group showed significant selective cytotoxicity in NTR-expressing cells. These results suggest that the good substrate activity and the benzylic oxygen are required for reductive activation of 4-nitrobenzylphosphoramide mustard analogues by *E. coli* nitroreductase. The low IC_{50} and the high selectivity of *cis*-(±)-**3**, *trans*-(±)-**3**, *cis*-(±)-**5**, *trans*-(±)-**5**, and (±)-**7** in *E. coli* nitroreductase-expressing cells indicated their potential to become a drug candidate in enzyme−prodrug therapy. These nitrobenzylphosphoramide mustards have low cytotoxicity before reduction and are converted to phosphoramide mustard or like reactive species upon bioreduction. In addition, compound (±)-**7** exhibited excellent bystander effects with a TE_{50} of 3.3% compared to 4.5% for 5-aziridinyl-2,4-dinitrobenzamide. The excellent biological activity of these compounds correlates well with their substrate activity for *E. coli* nitroreductase and is consistent with the expected high cytotoxicity of the reactive species released upon reduction. Work is in progress in our laboratories to further evaluate the biological activity of these analogues as potential prodrugs for nitroreductase activation.

Experimental Section

General Methods. Moisture-sensitive reactions were performed in flame-dried glassware under a positive pressure of nitrogen or argon. Air- and moisture-sensitive materials were transferred by syringe or cannula under an argon atmosphere. Except for redistillation prior to use, solvents were either ACS reagent grade or HPLC grade. Tetrahydrofuran was dried over sodium/benzophenone. Triethylamine, dichloromethane, and ethyl acetate were dried over calcium hydride. Methanol was distilled over sodium methoxide. Pyridine was dried over potassium hydroxide and distilled over calcium hydride. *N*,*N*-Dimethylformamide was dried over 4 Å molecular sieves at least for 1 week prior to use. Unless otherwise stated, all reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using 0.25 mm Whatman pre-coated silica gel plates. TLC plates were visualized using either 7% (w/w) ethanolic phosphomolybdic acid or 1% (w/w) aqueous potassium permanganate containing 1% (w/w) $NaHCO_3$. Flash column chromatography was performed using silica gel (Merck 230−400 mesh). Yield refers to chromatographically and spectroscopically (^{1}H NMR) homogeneous material, unless otherwise noted. All reagents were purchased at the commercial quality and used without further purification. *n*-BuLi was used freshly as purchased and was not titrated.

Melting points were determined on a Mel-Temp capillary apparatus and are uncorrected. Infrared spectra were recorded with

Perkin-Elmer model 1600 series FTIR spectrometer using polystyrene as an external standard. Infrared absorbance is reported in reciprocal centimeters (cm^{-1}) with broad signals denoted by br. ^1H NMR spectra were recorded on Varian Gemini 200, 300, or 400 MHz spectrometers as indicated at ambient temperature and calibrated using residual undeuterated solvents as the internal reference. ^{13}C NMR spectra were recorded at 50 MHz on a Varian Gemini 200 MHz spectrometer or 75 MHz on a Varian Gemini 300 MHz spectrometer. ^{31}P NMR spectra were recorded at 121 MHz on a Varian Gemini 300 MHz spectrometer or 162 MHz on a Varian Gemini 400 MHz spectrometer using 5% H_3PO_4 in D_2O as an external standard. Chemical shifts are reported in parts per million (δ) relative to $CDCl_3$ (δ 7.27 ppm for ^1H and 77.2 ppm for ^{13}C) or CD_3OD (δ 3.31 ppm for ^1H and 49.2 for ^{13}C). Coupling constants (J values) are given in hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High-resolution mass spectral (HRMS) data were obtained from the University of Kansas Mass Spectrometry Laboratory (Lawrence, KS). HPLC analysis was performed on an HP 1090 system equipped with a Phenomenex C_{18} column (5 μm, 4.6 mm × 250 mm) with gradient elution of 5−80% CH_3CN containing 0.1% TFA in 15 min at a flow rate of 1 mL/min and a detection wavelength at 220 nm.

1-(4-Nitrophenyl)prop-2-en-1-ol (17). To a solution of 4-nitrobenzaldehyde (855 mg, 5.66 mmol) in freshly distilled THF (20 mL) was added dropwise vinylmagnesium bromide solution (1 M in THF, 6.8 mL) at −78 °C. The mixture was stirred at −50 °C for 40 min, and the reaction was then quenched by saturated aqueous ammonium chloride (10 mL). After the addition of ethyl acetate (100 mL), the organic phase was washed with saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The crude product was purified by flash column chromatography (petroleum ether/ethyl acetate, 6:1 to 4:1) to afford the desired product **17** as a yellow solid (968 mg, 95%): mp (EtOAc) 54−55.5 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 8.15 (d, J = 8.1 Hz, 2H), 7.51 (d, J = 8.2 Hz, 2H), 6.02−5.90 (m, 1H), 5.40−5.20 (m, 3H), 2.80 (br, s, 1H); IR (KBr) 3300 (br), 1580, 1500, 1330, 1250, 1030, 920, 840, 730 cm^{-1}; ^{13}C NMR (50 MHz, $CDCl_3$) δ 149.9, 147.4, 139.3, 127.1, 123.8, 116.8, 74.6; MS (FAB, NBA) *m/z* (relative intensity) 180.1 (M + 1, 18.9), 162.0 (M − OH, 18.8); HRMS (FAB) *m/z* calcd for $C_9H_{10}NO_3$ (MH^+) 180.0661, found 180.0670.

1-(4-Nitrophenyl)propane-1,3-diol (18). To a solution of 1-(4-nitrophenyl)prop-2-en-1-ol (3.1 g, 17.3 mmol) in freshly distilled THF (150 mL) at 0 °C was added dropwise a solution of borane in THF (1 M, 18 mL). After the reaction solution was stirred at 0 °C overnight, 3 N NaOH (19 mL) was added, followed by the dropwise addition of 30% hydrogen peroxide (19 mL). The turbid solution was stirred at 0 °C for 30 min, and the remaining hydrogen peroxide was destroyed by the addition of a solution of sodium bisulfite. Ethyl acetate (150 mL) was then added, and the organic phase was washed with saturated aqueous $NaHCO_3$ and saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The crude product was purified by flash column chromatography (petroleum ether/ethyl acetate, 4:1 to 1:2) to afford the desired product **18** as a yellow oil (2.8 g, 82%): ^1H NMR (300 MHz, $CDCl_3$) δ 8.20 (d, J = 8 Hz, 2H), 7.55 (d, J = 8 Hz, 2H), 5.10 (t, J = 7 Hz, 1H), 3.90 (m, 2H), 3.65 (br s, 1H), 2.40 (br, s, 1H), 1.96 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 152.0, 147.3, 126.5, 123.8, 73.1, 61.1, 40.4; IR (KBr) 3400 (br), 1500, 1320 cm^{-1}; MS (FAB, NBA) *m/z* (relative intensity) 198.1 (MH^+, 11.0), 180.1 (MH^+ − OH, 13.6); HRMS (FAB) *m/z* calcd for $C_9H_{12}NO_4$ (MH^+) 198.0766, found 198.0788.

2-[Bis(2-chloroethyl)amino]-4-(4-nitrophenyl)-2*H*-1,3,2-dioxaphosphorinane 2-Oxide (5). To a stirred solution of 1-(4-nitrophenyl)propane-1,3-diol (186 mg, 0.944 mmol) in anhydrous THF (100 mL) at −78 °C under argon was added a solution of *n*-BuLi in hexane (2.5 M, 760 μL). After 15 min, a solution of bis(2-chloroethyl)phosphoramidic dichloride (252 mg, 0.944 mmol) in anhydrous THF (20 mL) was added. The reaction mixture was stirred at <−70 °C for 6 h and then at room temperature for 18 h. After removal of the white precipitate by filtration, the filtrate was washed with saturated NaCl, dried over Na_2SO_4, and evaporated to dryness. The crude product was purified by flash column chromatography (hexanes/ethyl acetate, 2:1 to 1:2) to afford the desired product as two diastereomers.

***cis*-(±)-5:** yellow oil (100 mg, 28%); ^1H NMR (200 MHz, $CDCl_3$) δ 8.26 (d, J = 8.4 Hz, 2H), 7.68 (d, J = 8.4 Hz, 2H), 5.68−5.57 (m, 1H), 4.57−4.46 (m, 2H), 3.70−3.63 (m, 4H), 3.53−3.40 (m, 4H), 2.62−2.2.51 (m, 1H), 2.29−2.18 (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 148.1, 146.5 (d, J = 4.2 Hz), 126.9, 124.1, 80.1 (d, J = 6.5 Hz), 66.6 (d, J = 5.7 Hz), 49.2 (d, J = 4.6 Hz), 41.9, 33.3 (d, J = 12.6 Hz); ^{31}P NMR (162 MHz, $CDCl_3$) δ 3.5 (s); IR (film) 1607.0, 1521.5, 1348.4, 1258.6, 1081.2 cm^{-1}; MS (FAB, NBA) *m/z* (relative intensity) 383.0 (MH^+, 3.6), 385.0 (MH^+ + 2, 1.6); HRMS (FAB) *m/z* calcd for $C_{13}H_{18}N_2O_5PCl_2$ (MH^+) 383.0330, found 383.0293.

***trans*-(±)-5:** yellow solid (145 mg, 40%); ^1H NMR (300 MHz, $CDCl_3$) δ 8.28 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H), 5.79 (dd, J = 10.9, 1.4 Hz, 1H), 4.84−4.71 (m, 1H), 4.54−4.33 (m, 1H), 3.76−3.46 (m, 8H), 2.28−1.95 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 148.1, 146.2 (d, J = 9.5 Hz), 126.3, 124.2, 77.9 (d, J = 5.0 Hz), 66.3 (d, J = 5.7 Hz), 49.3 (d, J = 5.0 Hz), 42.0, 33.8 (d, J = 4.2 Hz); ^{31}P NMR (162 MHz, $CDCl_3$) δ 7.4 (s); IR (KBr) 1606.8, 1522.1, 1348.6, 1246.0, 1102.6, 1057.0 cm^{-1}; MS (FAB, NBA) *m/z* (relative intensity) 382.9 (MH^+, 2.4), 385.0 (MH^++2, 0.7); HRMS (FAB) *m/z* calcd for $C_{13}H_{18}N_2O_5PCl_2$ (MH^+) 383.0330, found 383.0325.

1-(4-Nitrophenyl)propane-1,3-diazide (19). Hydrazoic acid solution (1.2 M in benzene) was prepared according to the reported procedure.[31] (Warning! Hydrazoic acid is a highly toxic and volatile compound. The following operations must be carried out under a well-ventilated hood.) To a solution of 1-(4-nitrophenyl)propane-1,3-diol (709 mg, 3.6 mmol) and triphenylphosphine (2.83 g, 10.8 mmol) in anhydrous THF (50 mL) was added, at room temperature, a hydrazoic acid solution (1.2 M benzene solution, 7.7 mL) and then a solution of diethyl azodicarboxylate (1.68 mL, 10.8 mmol) in THF (5 mL). The reaction solution was stirred at room temperature for 12 h and poured into ethyl acetate (100 mL) and brine (30 mL). The organic phase was washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, 5:1) to give the desired product **19** as an oil (653 mg, 73%): ^1H NMR (300 MHz, $CDCl_3$) δ 8.27 (d, J = 8.1 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 4.81−4.70 (m, 1H), 3.57−3.42 (m, 1H), 2.05−1.82 (m, 2H); IR (film) 2980, 2080, 1720, 1510, 1470, 1425, 1335, 1220, 1170, 1110, 1050, 980, 700, 680 cm^{-1}; MS (FAB, 3NBA) *m/z* (relative intensity) 248.1 (MH^+, 7.9), 219.2 (M − 28, 30.8), 177.1 (31.5).

1-(4-Nitrophenyl)propane-1,3-diamine (20). To a solution of 1-(4-nitrophenyl)propane-1,3-diazide (625 mg, 2.5 mmol) in anhydrous methanol was added propane-1,3-dithiol (1.0 mL, 10.1 mmol) and triethylamine (1.4 mL, 10.1 mmol). The reaction solution was stirred at room temperature for 36 h and filtered to remove the precipitate. The filtrate was evaporated in vacuo, and the residue was purified by flash column chromatography (chloroform/methanol, 5:1 to 2:1, the chloroform was saturated with ammonium hydroxide) to give the desired product **20** as a yellow oil (428 mg, 87%): ^1H NMR (300 MHz, $CDCl_3$) δ 8.21 (d, J = 9.0 Hz, 2H), 7.51 (d, J = 9.0 Hz, 2H), 4.19 (t, J = 6.8, 1H), 2.75 (t, J = 6.75 Hz, 2H), 1.81−1.76 (m, 2H), 1.40 (br s, 4H); ^{13}C NMR (50 MHz, $CDCl_3$, 5% CD_3OD) δ 153.3, 147.2, 127.2, 124.0, 53.9, 41.4, 38.8; IR (film) 3300, 2950, 1590, 1500, 1500, 1330, 840 cm^{-1}; MS (FAB, 3NBA) *m/z* (relative intensity) 196.1 (MH^+, 72.8), 165.1 (M − 30, 8.4), 151.1 (14.2); HRMS (FAB) *m/z* calcd for $C_9H_{14}N_3O_2$ (MH^+) 196.1086, found 196.1124.

2-[Bis(2-chloroethyl)amino]-4-(4-nitrophenyl)-2*H*-1,3,2-diazaphosphorinane 2-Oxide (6). To a solution of 1-(4-nitrophenyl)-propane-1,3-diamine (50 mg, 0.26 mmol) in anhydrous ethyl acetate (40 mL) at 0 °C was added a solution of bis(2-chloroethyl)-phosphoramidic dichloride (77.7 mg, 0.3 mmol) and triethylamine (86 μL, 0.6 mmol). The reaction solution was stirred at room temperature for 48 h and filtered to remove the precipitate. The

filtrate was washed with saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by flash column chromatography (chloroform/methanol, 30:1 to 24:1) to give the desired product as two diastereoisomers.

***cis*-(±)-6:** yellow solid (27 mg, 28%); mp ($CHCl_3$−MeOH) 119−120 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (d, J = 9.0 Hz, 2H), 7.69 (d, J = 8.7 Hz, 2H), 4.70−4.60 (m, 1H), 3.69 (t, J = 6.3 Hz, 4H), 3.60−3.20 (m, 8H), 2.15−2.00 (m, 1H), 1.90−1.80 (m 1H); ^{13}C NMR (50 MHz, $CDCl_3$, 5% CD_3OD) δ 151.3 (d, J = 3.8 Hz), 127.2, 123.9, 57.3 (d, J = 2.3 Hz), 48.5 (d, J = 4.6 Hz), 42.2 (d, J = 1.6 Hz), 40.1, 34.8 (d, J = 8.8 Hz); ^{31}P NMR (121 MHz, $CDCl_3$) δ 12.9 (s); IR (KBr) 3140, 2940, 2900, 2830, 1580, 1495, 1440, 1330, 1190, 1155, 1100, 960, 890, 710 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 381.1 (MH^+, 72.4), 383.1 (MH^+ + 2, 43.8), 385.1 (MH^+ + 4, 9.4); HRMS (FAB^+) *m*/*z* calcd for $C_{13}H_{20}N_4O_3PCl_2$ (MH^+) 381.0650, found 381.0626; HRMS (FAB^+) *m*/*z* calcd for $C_{13}H_{20}N_4O_3P^{35}Cl^{37}Cl$ (MH^+ + 2) 383.0621, found 383.0630.

***trans*-(±)-6:** yellow solid (33 mg, 34%); mp ($CHCl_3$−MeOH) 148.5−149.2 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (d, J = 8.7 Hz, 2H), 7.54 (d, J = 8.8 Hz, 2H), 4.78−4.70 (m, 1H), 3.70−3.50 (m, 12H), 2.10−1.75 (m, 2H), 1.90−1.80 (m 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 151.1 (d, J = 11.8 Hz), 147.6, 127.0, 124.2, 56.8, 48.0 (d, J = 5.0 Hz), 42.5, 40.7, 34.8; ^{31}P NMR (121 MHz, $CDCl_3$) δ 17.1 (s); IR (KBr) 3140, 2900, 1570, 1500, 1450, 1430, 1410, 1325, 1150, 1090, 980, 850, 720 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 381.1 (MH^+, 10.1), 383.1 (MH^+ + 2, 2.6), 385.1 (MH^+ + 4, 1.0); HRMS (FAB) *m*/*z* calcd for $C_{13}H_{20}N_4O_3PCl_2$ (MH^+) 381.0650, found 381.0643; HRMS (FAB) *m*/*z* calcd for $C_{13}H_{20}N_4O_3P^{35}Cl^{37}Cl$ (MH^+ + 2) 383.0621, found 383.0612.

3-Methoxymethoxy-3-(4-nitrophenyl)propan-1-ol (21). A solution of 1-(4-nitrophenyl)prop-2-en-1-ol (1.94 g, 10.8 mmol) in dry dichloromethane (40 mL) at 0 °C was treated sequentially with DIEA (11.33 mL, 64.8 mmol) and chloromethyl methyl ether (4.94 mL, 60.8 mmol). The reaction mixture was stirred at room temperature for 24 h and quenched with 5% $NaHCO_3$. Dichloromethane was evaporated in vacuo, and the aqueous solution was extracted with ethyl ether (40 mL × 3). The extractions were combined, washed with brine, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by flash column chromatography (hexanes/ethyl acetate, 8:1 to 6:1) to give 1-methoxymethoxy-1-(4-nitrophenyl)-2-propene as a yellow oil (2.31 g, 95%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (dd, J = 1.8, 6.9 Hz, 2H), 7.54−7.51 (m, 2H), 5.90−5.78 (m, 1H), 5.39−5.27 (m, 2H), 5.18 (d, J = 6.6. Hz, 1H), 4.78 (d, J = 4.8 Hz, 1H), 4.61 (d, J = 5.7 Hz, 1H), 3.36 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 148.3, 147.6, 137.1, 127.7, 123.8, 118.2, 94.0, 55.7; IR (film) 3020, 2920, 2880, 1580, 1500, 1330, 1130, 1080, 1020, 900, 835 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 224.1 (MH^+, 22.4), 194.1 (MH^+ − 30, 1.5), 208.1 (MH^+ − 15, 3.1), 192.1 (MH^+ − 31, 1.3), 162.1 (MH^+ − OMOM, 77.5); HRMS (FAB) *m*/*z* calcd for $C_{11}H_{14}NO_4$ (MH^+) 224.0923, found 224.0924.

To a solution of 1-methoxymethoxy-1-(4-nitrophenyl)-2-propene (742 mg, 3.3 mmol) in freshly distilled THF (15 mL) at 0 °C was added dropwise a solution of borane in THF (1 M, 3.3 mL). After the reaction solution was stirred at 0 °C overnight, 3 N NaOH (3.5 mL) was added, followed by the dropwise addition of 30% hydrogen peroxide (3.5 mL). The turbid reaction mixture was stirred at 0 °C for 30 min, and the remaining hydrogen peroxide was destroyed by the addition of a solution of sodium bisulfite. Ethyl acetate (100 mL) was added, and the organic phase was washed with saturated aqueous $NaHCO_3$ and saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The crude product was purified by flash column chromatography (petroleum ether/ethyl acetate, 2:1 to 1:1) to afford the desired product **21** as a yellow oil (625 mg, 78%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (dd, J = 1.8, 6.8 Hz, 2H), 7.53−7.50 (m, 2H), 4.95 (dd, J = 4.5, 8.9 Hz, 1H), 4.62 (d, J = 6.6 Hz, 1H), 4.52 (d, J = 6.9 Hz, 1H), 3.83−3.77 (m, 1H), 3.75−3.71 (m, 1H), 3.38 (s, 3H), 2.21 (br s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 149.5, 147.7, 127.4, 123.9, 95.1, 75.9, 59.7, 56.0, 40.4; IR (film) 3400, 2950, 1500, 1330, 1130, 1080, 1010 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 242.1 (MH^+, 19.6), 210.1 (M − 31, 25.8), 224.1 (MH^+ − 18, 5.6); HRMS (FAB) *m*/*z* calcd for $C_{11}H_{16}NO_5$ (MH^+) 242.1028, found 242.1030.

3-Amino-1-(4-nitrophenyl)propan-1-ol (22). To a stirred solution of 3-methoxymethoxy-3-(4-nitrophenyl)propane-1-ol (128 mg, 0.53 mmol) in dry dichloromethane (10 mL) at 0 °C was added TEA (0.22 mL, 1.59 mmol) and methane sulfonyl chloride (80 μL, 1.06 mmol). After being stirred for 15 min, the reaction solution was diluted with ethyl ether (100 mL). The organic solution was washed with saturated aqueous $NaHCO_3$, saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The crude product was dissolved in dry DMF (10 mL) and was treated with NaN_3 (207 mg, 3.18 mmol) and 15-crown-5 (cat.). The reaction solution was stirred at room temperature for 4.5 h and partitioned between ethyl ether and water. The organic phase was washed with saturated aqueous $NaHCO_3$ and saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The crude product was purified by flash column chromatography (petroleum ether/ethyl acetate, 4:1 to 3:1) to afford 3-methoxymethoxy-3-(4-nitrophenyl)-1-propyl azide as a yellow oil (129 mg, 91%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (dd, J = 1.8, 6.8 Hz, 2H), 7.52 (dd, J = 0.3, 6.9 Hz, 2H), 4.83 (dd, J = 4.5, 9.0 Hz, 1H), 4.60 (d, J = 6.9 Hz, 1H), 4.51 (dd, J = 1.2, 6.8 Hz, 1H), 3.51−3.40 (m, 2H), 3.37 (s, 3H), 2.07−2.02 (m, 1H), 1.93−1.89 (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 149.1, 147.8, 127.5, 124.0, 95.0, 74.5, 56.0, 47.8, 37.1; IR (film) 2955, 2070, 1580, 1500, 1330, 1135, 1080, 1020 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 267.2 (MH^+, 4.8), 207.1 (3.6), 198.1 (5.6); HRMS (FAB) *m*/*z* calcd for $C_{11}H_{15}N_4O_4$ (MH^+) 267.1093, found 267.1082.

To a solution of 3-methoxymethoxy-3-(4-nitrophenyl)-1-propyl azide (4.13 g, 15.45 mmol) in THF (80 mL, 0.5% water) was added triphenylphosphine (4.12 g, 15.45 mmol). The reaction solution was stirred at room temperature for 24 h before being concentrated in vacuo. The crude product was purified by flash column chromatography to afford 3-methoxymethoxy-3-(4-nitrophenyl)-1-propylamine as a yellow oil (2.69 g, 72%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (dd, J = 2.1, 6.9 Hz, 2H), 7.50 (d, J = 8.7 Hz, 2H), 4.83 (dd, J = 4.8, 8.4 Hz, 1H), 4.59 (d, J = 6.9 Hz, 1H), 4.50 (dd, J = 0.3, 6.9 Hz, 1H), 3.37 (s, 3H), 2.83 (t, J = 6.9 Hz, 2 H), 2.00−1.93 (m, 1H), 1.82−1.75 (m, 1H), 1.31 (br s, 2H); IR (film) 2900, 1630, 1580, 1500, 1330, 1130, 1080, 1000, 900, 830, 680 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 241.1 (MH^+, 100.0), 225.1 (1.9), 209.1 (1.5); HRMS (FAB) *m*/*z* calcd for $C_{11}H_{17}N_2O_4$ (MH^+) 241.1188, found 241.1182.

To a solution of 3-methoxymethoxy-3-(4-nitrophenyl)-1-propylamine (1.0 g, 4.17 mmol) in dry dichloromethane (50 mL) at −50 °C was added a solution of B-bromocatecholborane in dichloromethane (0.245 N, 17 mL). The reaction mixture was allowed to warm to −20 °C for 2 h and treated with glacial acetic acid (0.24 mL, 4.17 mmol). After the mixture was stirred at room temperature for another 7 h, the reaction was quenched with 3 N NaOH (15 mL). The organic phase was separated, and the aqueous phase was extracted with dichloromethane (30 mL × 3). The organic phases were combined, washed with saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The crude product was purified by flash column chromatography (chloroform/methanol, 9:1 to 8:1) to afford the desired product **22** as a yellow solid (629 mg, 77%): mp (CH_3Cl−MeOH) 126−127.5 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 (dd, J = 2.0, 6.9 Hz, 2H), 7.52−7.47 (m, 2H), 5.03 (dd, J = 2.7, 8.7 Hz, 1H), 3.12−3.06 (m, 1H), 3.07−2.92 (m, 1H), 1.99−1.81 (m, 1H), 1.67−1.41 (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 152.9, 147.1, 126.5, 123.6, 75.2, 40.8, 39.1; IR (film) 3330, 3260, 3100, 2880, 2850, 1575, 1490, 1400, 1330, 1300, 1275, 1075, 1085, 1050, 1000, 935, 810, 730, 680 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 197.1 (MH^+, 30.5), 181.0 (1.8); HRMS (FAB) *m*/*z* calcd for $C_9H_{13}N_2O_3$ (MH^+) 197.0926, found 197.0939.

2-[Bis(2-chloroethyl)amino]-6-(*p*-nitrophenyl)-2*H*-1,3,2-oxazaphosphorinane 2-Oxide (3). To a solution of 3-amino-1-(4-nitrophenyl)-1-propanol (131 mg, 0.67 mmol) in anhydrous ethyl acetate (20 mL) at 0 °C was added a solution of bis(2-chloroethyl)-phosphoramidic dichloride (173 mg, 0.67 mmol) and triethylamine (185 μL, 1.34 mmol) in ethyl acetate (5 mL). The reaction solution

was stirred at room temperature for 48 h and filtered to remove the precipitate. The filtrate was washed with saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by flash column chromatography (chloroform/methanol, 30:1 to 15:1) to give the desired product as two diastereoisomers.

***cis*-(±)-3:** yellow solid (79 mg, 34%); mp ($CHCl_3$−MeOH) 125−127 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (d, J = 6.9 Hz, 2H), 7.62 (d, J = 8.7 Hz, 2H), 5.50−5.40 (m, 1H), 1H), 3.80−3.60 (m, 6H), 3.52−3.35 (m, 5H), 2.20−1.95 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 147.9, 147.3 (d, J = 6.5 Hz), 126.6, 124.0, 80.3 (d, J = 6.8 Hz), 48.6 (d, J = 3.8 Hz), 42.1 (d, J = 2.7 Hz), 40.6, 34.4 (d, J = 9.2 Hz); ^{31}P NMR (121 MHz, $CDCl_3$) δ 11.2 (s); IR (film) 3400, 3140, 2920, 2820, 1580, 1490, 1420, 1325, 1220, 1195, 1095, 1075, 1020, 965, 890, 840, 830, 790, 725 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 382.2 (MH^+, 4.1), 384.2 (MH^+ + 2, 2.9), 386.0 (MH^+ + 4, 3.9); HRMS (FAB) *m*/*z* calcd for $C_{13}H_{19}N_3O_4PCl_2$ (MH^+) 382.0490, found 382.0479; HRMS (FAB) *m*/*z* calcd for $C_{13}H_{21}N_3O_4P^{35}Cl^{37}Cl$ (MH^+ + 2) 384.0461, found 384.0459.

***trans*-(±)-3:** yellow solid (77 mg, 33%); mp ($CHCl_3$−MeOH) 138−140 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (dd, J = 1.8, 6.8 Hz, 2H), 7.62 (d, J = 9.6 Hz, 2H), 5.63 (d, J = 11.1 Hz, 1H), 1H), 3.65−3.30 (m, 10H), 3.10 (br s, 1H), 2.10−1.80 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 147.8, 147.3 (d, J = 9.5 Hz), 126.3, 124.0, 77.9 (d, J = 5.7 Hz), 48.8 (d, J = 4.6 Hz), 42.4, 41.0 (d, J = 2.3 Hz), 33.4 (d, J = 3.4 Hz); ^{31}P NMR (121 MHz, $CDCl_3$) δ 14.6 (s); IR (film) 3400, 3120, 2920, 2760, 1590, 1500, 1435, 1330, 1200, 1080, 940, 900, 730 cm^{-1}; MS (FAB^+, 3NBA) *m*/*z* (relative intensity) 382.1 (MH^+, 3.3), 384.2 (MH^+ + 2, 1.7), 386.0 (MH^+ + 4, 3.9); HRMS (FAB^+) *m*/*z* calcd for $C_{13}H_{19}N_3O_4PCl_2$ (MH^+) 382.0490, found 382.0482; HRMS (FAB^+) *m*/*z* calcd for $C_{13}H_{21}N_3O_4P^{35}Cl^{37}Cl$ (MH^+ + 2) 384.0461, found 384.0462.

3-(*tert*-Butyldiphenylsilyloxy)-1-(4-nitrophenyl)propan-1-ol (23). To a cooled solution of 1-(4-nitrophenyl)propane-1,3-diol (630 mg, 2.55 mmol) and imidazole (866 mg, 12.7 mmol) in DMF (15 mL) at −30 °C was added slowly dropwise *tert*-butyldiphenylsilyl chloride (683 μL, 2.63 mmol). The reaction solution was stirred at −30 to −20 °C for an additional 1.2 h and quenched by the addition of water (5 mL). The solution was diluted with ethyl acetate (100 mL), washed with saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by flash column chromatography (hexanes/acetone, 9:1 to 7:1) to give the desired product **23** as a yellow oil (1.06 g, 95%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (d, J = 8.1 Hz, 2H), 7.70−7.30 (m, 2H), 5.20−5.10 (m, 1H), 4.05 (br s, 1H), 3.90−3.80 (m, 2H), 2.00−1.90 (m, 2H), 1.10 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 152.0, 147.3, 135.6, 132.8, 132.7, 130.1, 128.0, 126.5, 123.7, 73.2, 62.9, 40.5, 27.0, 19.2; IR (film) 3400, 2940, 2920, 1840, 1500, 1410, 1335, 1100, 685 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 436.2 (MH^+, 2.2), 418.1 (MH^+ − 18, 2.0), 378.1 (MH^+ − 56, 1.5); HRMS (FAB) *m*/*z* calcd for $C_{25}H_{30}NO_4Si$ (MH^+) 436.1944, found 436.1932.

3-(*tert*-Butyldiphenylsilyloxy)-1-(4-nitrophenyl)propyl Azide (24). To a solution of 3-(*tert*-butyldiphenylsilyloxy)-1-(4-nitrophenyl)propane-1-ol (5.6 g, 12.8 mmol) and triphenylphosphine (4.36 g, 16.1 mmol) in THF (80 mL) at 0 °C was added DEAD (2.89 g, 16.6 mmol) and a solution of hydrazoic acid in benzene (37.7 mL, 1.2 M). The reaction mixture was stirred at room temperature for 2 h, quenched by adding saturated $NaHCO_3$ (20 mL), and poured into ethyl acetate (200 mL). The organic phase was washed with saturated $NaHCO_3$, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by flash column chromatography (hexanes/acetone, 10:1) to give the desired product **24** as a yellow oil (5.69 g, 97%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 (d, J = 8.7 Hz, 2H), 7.66−7.53 (m, 5H), 7.39−7.28 (m, 7H), 4.84 (dd, J = 6.3, 8.1 Hz, 1H), 3.80−3.70 (m, 1H), 3.56−3.50 (m, 1H), 1.92−1.83 (m, 2H), 1.04 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 147.8, 147.2, 135.6, 133.4, 130.0, 127.9, 127.8, 124.1, 62.2, 59.9, 39.3, 27.0, 19.3; IR (film) 2890, 2820, 2070, 1500, 1405, 1325, 1235, 1080, 775, 675 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 461.3 (MH^+, 2.1), 419.3 (MH^+ − 42, 3.6), 403.2 (MH^+ − 56, 17.1).

3-(*tert*-Butyldiphenylsilyloxy)-1-(4-nitrophenyl)propylamine (25). To a solution of 3-(*tert*-butyldiphenylsilyloxy)-1-(4-nitrophenyl)propyl azide (300 mg, 0.655 mmol) in methanol (6 mL) were added propane-1,3-dithiol (0.33 mL, 3.28 mmol) and triethylamine (0.46 mL, 3.28 mmol). The reaction solution was stirred at room temperature for 12 h and concentrated in vacuo. The residue was purified by flash column chromatography (chloroform/methanol, 30:1) to afford the desired product **25** as a yellow oil (198 mg, 70%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.15 (dd, J = 2.1, 6.6 Hz, 2H), 7.68−7.36 (m, 12H), 4.33 (t, J = 6.8 Hz, 1H), 3.76−3.64 (m, 2H), 1.95−1.80 (m, 2H), 1.72 (br s, 2H), 1.08 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 153.7, 135.6, 133.6, 129.9, 127.8, 127.4, 123.8, 61.2, 53.1, 41.9, 27.0, 19.3; IR (film) 3040, 2920, 2840, 1650, 1585, 1500, 1410, 1325, 1080, 835, 805, 720, 680 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 435.2 (MH^+, 35.3), 377.1 (MH^+ − 56, 13.1), 257.1 (MH^+ − 78, 5.8); HRMS (FAB) *m*/*z* calcd for $C_{25}H_{31}N_2O_3Si$ (MH^+) 435.2104, found 435.2119.

3-(4-Nitrophenyl)-3-amino-1-propanol (26). To a solution of 3-(*tert*-butyldiphenylsilyloxy)-1-(4-nitrophenyl)propylamine (200 mg, 0.46 mmol) in THF (25 mL) at 0 °C was added dropwise a 1 M solution of tetrabutylammonium fluoride in THF (2.3 mL). The solution was stirred at room temperature for 1 h, after which a saturated $KHSO_4$ was added to acidify the solution to pH 6. After extraction with ethyl ether, the aqueous solution was basified with 3 N NaOH and extracted with dichloromethane (40 mL × 3). The organic phase was dried over Na_2SO_4 and evaporated in vacuo, and the residue was purified by flash column chromatography (chloroform/methanol, 50:1 to 40:1; the chloroform was saturated with ammonium hydroxide) to give the desired product **26** as a yellow solid (74 mg, 82%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.23 (d, J = 9.0 Hz, 2H), 7.51 (d, J = 9.0 Hz, 2H), 4.34−4.25 (m, 1H), 3.81 (t, J = 5.25 Hz, 2H), 2.16 (br s, 3H), 1.95−1.89 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 153.4, 126.9, 124.1, 61.6, 55.5, 40.1; IR (film) 3300, 2900, 1580, 1495, 1330, 1040, 835, 730, 680 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 197.1 (MH^+, 100.0), 180.1 (MH^+ − 18, 13.9), 181.1 (MH^+ − 17, 9.8); HRMS (FAB) *m*/*z* calcd for $C_9H_{13}N_2O_3$ (MH^+) 197.0926, found 197.0946.

2-[Bis(2-chloroethyl)amino]-4-(4-nitrophenyl)-2*H*-1,3,2-oxazaphosphorinane 2-Oxide (4). To a solution of 3-(4-nitrophenyl)-3-amino-1-propanol (65 mg, 0.33 mmol) in anhydrous ethyl acetate (40 mL) at 0 °C was added a solution of bis(2-chloroethyl)-phosphoramidic dichloride (103 mg, 0.4 mmol) and triethylamine (111 μL, 0.8 mmol) in ethyl acetate (10 mL). The reaction solution was stirred at room temperature for 48 h and filtered to remove the precipitate. The filtrate was washed with saturated NaCl, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by flash column chromatography (hexanes/ethyl acetate, 3:1 for *cis*-(±)-**4**, followed by chloroform/methanol, 30:1 for *trans*-(±)-**4**) to give the desired product as two diastereoisomers.

***cis*-(±)-4:** yellow solid (17 mg, 14%); mp ($CHCl_3$−MeOH) 118−120 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16 (d, J = 9.0 Hz, 2H), 7.71 (d, J = 9.0 Hz, 2H), 4.74 (t, J = 7.2 Hz, 1H), 1H), 4.31−4.16 (m, 2H), 3.64−3.33 (m, 8H), 3.09 (d, J = 3.6 Hz, 1H), 2.26−2.21 (m, 1H), 2.04−1.96 (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 150.5, 147.7, 127.6, 124.2, 64.9 (d, J = 6.8 Hz), 56.7, 48.7 (d, J = 4.6 Hz), 42.3, 33.2 (d, J = 8.4 Hz); ^{31}P NMR (121 MHz, $CDCl_3$) δ 9.6 (s); IR (film) 3350, 3180, 2900, 1700, 1585, 1500, 1325, 1210, 1110, 1090, 970, 930, 840, 720, 680 cm^{-1}; MS (FAB, 3NBA) *m*/*z* (relative intensity) 382.0 (MH^+, 56.7), 384.0 (MH^+ + 2, 38.8), 386.0 (MH^+ + 4, 3.9); HRMS (FAB) *m*/*z* calcd for $C_{13}H_{19}N_3O_4PCl_2$ (MH^+) 382.0490, found 382.0491; HRMS (FAB) *m*/*z* calcd for $C_{13}H_{19}N_3O_4P^{35}Cl^{37}Cl$ (MH^+ + 2) 384.0461, found 384.0467.

***trans*-(±)-4:** yellow solid (21.3 mg, 17%); mp ($CHCl_3$−MeOH) 139.5−141 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (d, J = 8.1 Hz, 2H), 7.54 (d, J = 8.1 Hz, 2H), 4.81 (dd, J = 4.8, 9.9 Hz, 1H), 4.65−4.56 (m, 1H), 3.70−3.48 (m, 8H), 2.85 (br s, 1H), 2.00−1.92 (m 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 150.0 (d, J = 12.2

Hz), 149.9, 120.1, 124.4, 66.2 (d, $J = 5.7$ Hz), 56.7, 48.6 (d, $J = 5.0$ Hz), 42.4, 34.7 (d, $J = 3.0$ Hz); ^{31}P NMR (121 MHz, $CDCl_3$) δ 14.2 (s); IR (KBr) 3447, 3112, 2995, 2876, 1521, 1449, 1349, 1222, 1193, 1109, 914, 874, 750 cm^{-1}; MS (FAB, 3NBA) *m/z* (relative intensity) 382.0 (MH^+, 65.9), 384.0 ($MH^+ + 2$, 42.9), 386.0 ($MH^+ + 4$, 6.1); HRMS (FAB) *m/z* calcd for $C_{13}H_{19}N_3O_4PCl_2$ (MH^+) 382.0490, found 382.0464; HRMS (FAB) *m/z* calcd for $C_{13}H_{19}N_3O_4PCl_2$ (MH^+) 382.0490, found 382.0464; HRMS (FAB) *m/z* calcd for $C_{13}H_{19}N_3O_4P^{35}Cl^{37}Cl$ ($MH^+ + 2$) 384.0461, found 384.0440.

Stability Test in Aqueous Buffer. A 1 mg sample of the substrate was dissolved in 1 mL of 50 mM sodium phosphate buffer (pH 7.4) containing 10% DMSO and incubated at 37 °C. Aliquots were withdrawn at different time intervals for 72 h and subjected to reversed-phase HPLC analysis.

***E. coli* Nitroreductase Assay.** The substrate (0.2 mM) was incubated with 1 mM NADH at 37 °C in 10 mM sodium phosphate buffer (pH 7.0) in a total volume of 250 μL. The reaction was initiated by the addition of 1.8 μg of *E. coli* nitroreductase. For compound (±)-**7**, 2 mM cysteine was added to protect the nitroreductase enzyme from inactivation by the phosphoramide mustard released upon activation (the addition of 2 mM cysteine had little effect on the half-life of 5-aziridinyl-2,4-dinitrobenzamide under the same conditions). Aliquots were withdrawn and analyzed by reversed-phase HPLC analysis. The half-life of reduction by *E. coli* nitroreductase was calculated on the basis of the disappearance of the substrate assuming pseudo-first-order kinetics.

Cell Culture and Antiproliferative Assays in Vitro. V79 Chinese hamster lung fibroblasts were grown in monolayer culture in DMEM containing 10% fetal calf serum and 4 mM glutamine. Cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2 and subcultured twice weekly by trypsinization. A bicistronic eukaryotic expression vector containing the coding regions for *E coli* nitroreductase together with puromycin acetyl transferase (conferring puromycin resistance) driven from a single CMV promoter was constructed by cloning into the XhoI site of the vector pIRES-P (EMBL:Z75185; 1) using conventional techniques. Insert orientation and identity were confirmed by diagnostic restriction digests and dideoxy sequencing using a Sequenase II kit (Amersham Pharmacia Biotech, St. Albans, Herts, U.K.). The V79 cells were transfected with the bicistronic vector, and the positive clones were selected in growth medium containing 10 μg/mL puromycin and maintained under selective pressure. Cells expressing *E. coli* nitroreductase in exponential phase of growth were trypsinized, seeded in 96-well plates at a density of 1000 cells/well, and permitted to recover for 24 h. V79 cells transfected with vector only were used as the controls. Serial dilutions of the drug solution were performed in situ, and cells were then incubated with drug for 3 days at 37 °C. The plates were fixed and stained with SRB before reading with optical absorption at 570 nm; results were expressed as a percentage of control growth. IC_{50} values are the concentration required to reduce cell number to 50% of control and were obtained by interpolation. For the short drug exposure experiments, cells were exposed to each test drug for 1 h and culture media was then replaced with drugfree, fresh medium for continued incubation. Cell viability assay was performed 3 days after drug addition. For the control experiment using 4-hydroperoxycyclophosphamide, sealed microtiter plates were used to prevent the volatile metabolite from contaminating neighboring wells.[39]

SKOV3 human ovarian carcinoma cells grown in HEPES-buffered DMEM with 10% FCS were infected with an E1, E3-deleted replication-defective adenovirus vector vPS1233 expressing the wild type *E. coli* nitroreductase from the CMV promoter, using infection ratios of 10 or 100 plaque forming units (pfu) per cell. Cells were plated in 96-well plates (at 15 000 or 20 000 cells/well in different experiments) and incubated for 2 days to allow for nitroreductase expression. The used medium was exchanged with fresh medium containing a range of prodrug concentrations up to 1000 μM. After 18 h of incubation, the medium was replaced with fresh medium (without prodrugs). An MTT assay was performed 3 days after adding prodrug to assess cell viability.

SKOV NTR cells were generated by infecting SKOV3 cells with the retrovirus rv.RD18.LNC-nr[38] and selecting clones of transduced cells with 1 mg/mL G418. NTR expression was confirmed by Western blots. For prodrug sensitivity assays, SKOV NTR or parental SKOV3 cells were plated in 96-well plates at 20 000 cells per well and allowed to adhere for 24 h. The medium was exchanged for fresh medium containing a range of prodrug concentrations. After 18 h of exposure to prodrug, the medium was removed and replaced with fresh medium lacking prodrug, and cell survival was determined by MTT assay after an additional 2 days.

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Supporting Information Available: NMR, MS, and IR data of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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