JOC The Journal of Organic Chemistry

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Further Evidence on the Favorable Role of the Anomeric Effect on the Cleavage of HepDirect and Cyclophosphamide Prodrugs

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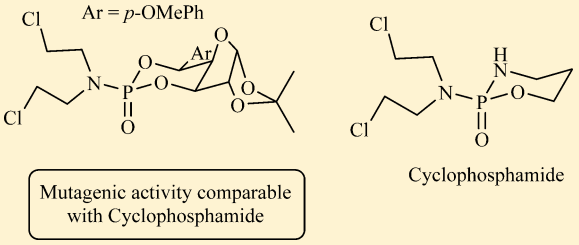
Supporting Information

ABSTRACT: On the basis of previous conformational and configurational studies of 4-aryl-substituted cyclophosph(on)ates derived from D-xylofuranose derivatives, wherein it was proposed that the anomeric effect is involved in the spontaneous isomerization of the P atom and the C4 carbon, and consequently, this unusual behavior was associated with the cleavage of the HepDirect prodrugs. We synthesized an analogous series of 2-amino-2-oxo-1,3,2-dioxaphosphorinanes and performed a conformational and configurational analysis in solution and the solid state followed by an examination of their mutagenic activity. The results showed that the 2-amino-2-oxo-1,3,2-dioxaphosphorinanes with the largest mutagenic activity contain either a 4-methoxyphenyl or 4-fluorophenyl group at C4 carbon and presented a major chair conformation, which is prone to weaken the C4—O3 bond via the anomeric effect and facilitates the cleavage for the release of the biologically active metabolite.



INTRODUCTION

A number of cyclic and acyclic phosphates and P-heterocyclic analogues have proven to be excellent anticancer and antiviral agents when employed via the prodrug strategy.[1] One remarkable example is cyclophosphamide (Cytoxan), which is probably the most successful synthetic prodrug prepared for the treatment of several cancer types.[2] Other powerful prodrugs are the HepDirect agents, which are six-membered ring phosph(on)ates that contain an aryl group at the C4 position and a nucleoside nucleus at the phosphorus atom and were designed for targeting phosph(on)ates drugs in the liver (Figure 1).[3]

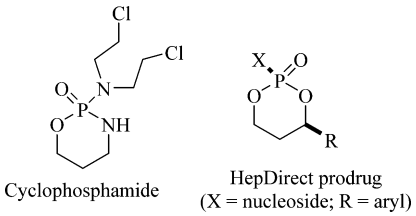


Figure 1. Cyclophosphamide and nucleoside phosph(on)ates (HepDirect) are biologically active prodrugs.

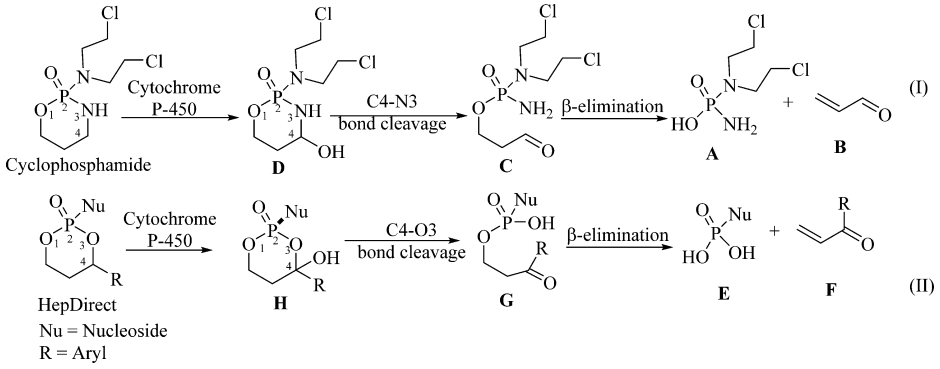
Interestingly, for cyclophosphamide the mechanistic pathway for the liberation of the bioactive agent is quite similar to that of HepDirect prodrugs.[2,3] Starting from cyclophosphamide, the biologically active phosphoramide mustard **A** is produced by elimination of acrolein **B** from the corresponding aldophosphamide **C**, which is formed by C4—N3 bond cleavage of the corresponding carbinolamine **D** after selective oxidation at the C4 carbon with cytochrome P-450. For comparison, in the case of the HepDirect prodrugs the active nucleoside phosphate **E** is formed by a very similar metabolic sequence: oxidation at C4 followed by C4—O3 bond cleavage to form the ketophosphate **G**, which then eliminates the unsaturated aryl ketone **F** and delivers the biologically active nucleoside **E** (Scheme 1).

Our research group has been interested in the conformational and configurational analysis of 1,3,2-dioxaphosphorinane compounds,[4] and recently, we found that certain 4-aryl phosphates and phosphonates derived from D-xylofuranose derivatives can undergo spontaneous isomerization at the phosphorus atom and the C4 carbon centers.[4d−f] Although the epimerization at chiral P-centers is known,[5] this is not the case for the corresponding isomerization of the C4 position. Detailed synthetic and structural studies by NMR spectroscopy and single-crystal X-ray diffraction analysis evidenced the existence of an anomeric effect that weakens the C4—O3 bond of

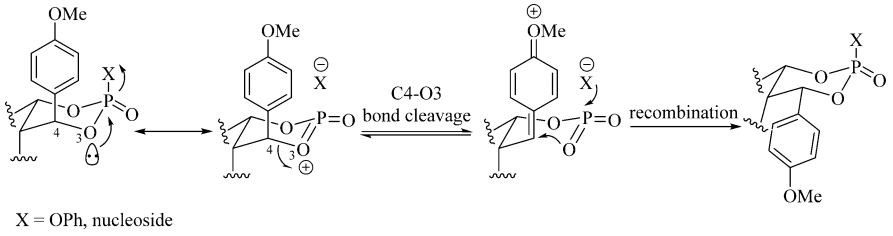
Received: August 1, 2014
Published: September 19, 2014

   dx.doi.org/10.1021/jo501772g | J. Org. Chem. 2014, 79, 9647−9654

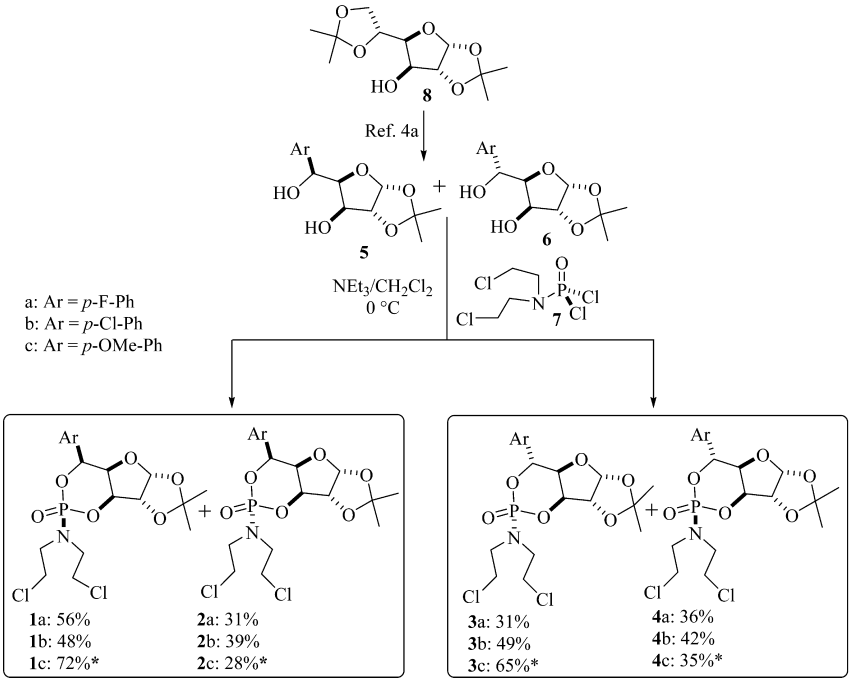
Scheme 1. Prodrug Cleavage Mechanisms for Cyclophosphamide (I) and HepDirect (II)



Scheme 2. Anomeric Effect in the Spontaneous Isomerization of 4-Aryl Phosphates via C4—O3 Bond Cleavage



Scheme 3. Synthesis of the 2-Oxo-2-[*N*,*N*-Bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes Derived from D-Xylofuranoses 1—4



the phosphate ring, favoring the bond cleavage and thus leading to the C4 isomerization (Scheme 2).[4d,f]

On the basis of these results, we postulated the following hypothesis: "*The anomeric effect might be involved in the mechanistic course of the HepDirect prodrugs cleavage*".[4d] This suggests that cytochrome P-450 might not be solely responsible for the activation of both the HepDirect prodrugs and Cyclophosphamide. The present contribution not only attempts to prove these observations further but also provides new findings on the mechanistic course of the cleavage of P-heterocycle prodrugs that might help to design novel anticancer and antiviral prodrugs. To this end, we designed and prepared a series of 2-oxo-2-[*N*,*N*-bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes derived from D-xylofuranose.[6] Since these P-heterocycles can release a nitrogen mustard alkylating agent, then their mutagenic activity can be tested by using the *Salmonella typhimurium* TA 1535 test system.[7] Therefore, their mutagenic activity, for which the release of the phosphoramide mustard is the primary cause of mutagenicity,[8] will arise from either metabolic activation or the anomeric effect. In this context, the biological experiments in the presence and absence of enzymatic activation, and comparison with commercially available Cyclophosphamide provided further relevant information for the study.

dx.doi.org/10.1021/jo501772g | J. Org. Chem. 2014, 79, 9647−9654

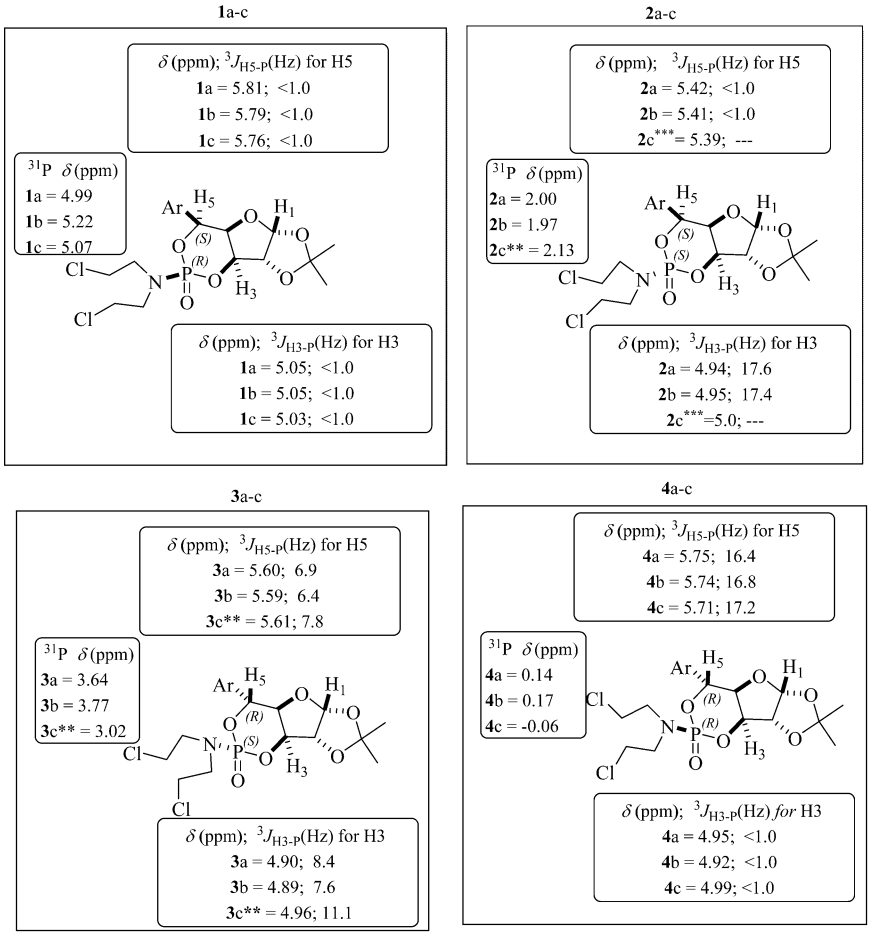


Figure 2. Representative NMR data* for the 2-oxo-2-[N,N-bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes derived from D-xylofuranose **1**−**4** (a, Ar = *p*-F-Ph; b, Ar = *p*-Cl-Ph; c, Ar = *p*-OMe-Ph). *NMR data extracted from spectra recorded at 400 MHz for ^{1}H and 121 MHz for ^{31}P in $CDCl_3$. ** Data obtained from the crude reaction mixture. ***Overlapped signal.

RESULTS AND DISCUSSION

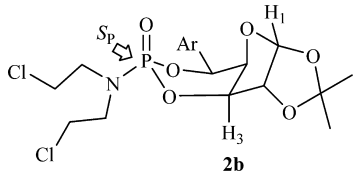
The synthesis of the 2-oxo-2-amino-1,3,2-dioxaphosphorinanes **1**−**4** was accomplished in good yields by a phosphorylation reaction of the respective 5-aryl-D-xylofuranose derivatives **5** and **6** using the dichlorophosphoryl reagent **7** in the presence of triethylamine. While the corresponding 1,3-diols **5** and **6** were obtained from diacetone-D-glucose **8** in a sequential hydrolysis−oxidation−Grignard addition protocol,[4a,d] the phosphoryl reagent **7** was prepared in situ from the commercially available phosphorus oxychloride ($POCl_3$) and bis(2-chloroethyl)amine hydrochloride in the presence of triethylamine and DMAP (Scheme 3).

Since the absolute stereochemistry at C5 of precursor alcohols **5** and **6** is known,[4] the configuration at P atom as well as the major conformation of the respective dioxaphosphorinane ring for **1**−**4** was determined by NMR studies and X-ray crystallographic analysis. The relevant NMR data are shown in Figure 2. Because the 2-oxo-2-amino-1,3,2-dioxaphosphorinanes **2c** and **3c** are spontaneously converted into **1c** and **4c**, respectively, the chemical yield and the NMR data were determined from the reaction crude.[4d−f]

The NMR data presented in Figure 2 are, in general, quite similar to the NMR data reported previously for a series of related 2-oxo-1,3,2-dioxaphosphorinanes.[4,9] For compounds **1a**−**c**, the vicinal coupling constants $^{3}J_{H5P}$ and $^{3}J_{H3P}$ have values below 1.0 Hz, which indicates that both hydrogen atoms H3 and H5 are axially or pseudoaxially oriented, suggesting that the chair conformation is dominant for these compounds. Larger coupling constants for one of these hydrogen atoms (e.g., $^{3}J_{H3P} \approx 17$ Hz for **2a**−**c**) would indicate that the dioxaphosphorinane ring deviates from the chair conformation.[10] Therefore, while the 2-amino-2-oxo-1,3,2-dioxaphosphorinanes **1a**−**c** feel comfortable in the chair conformation, the dioxaphosphorinane analogues **2a**−**c** prefer a boat conformation. Additionally, by examining the ^{31}P NMR spectroscopy data it was found that the signals of compounds **2a**−**c** are upfield shifted when compared to their diastereomeric congeners **1a**−**c**, and according to Gorensteiń criteria,[11] it can be anticipated that the phosphoryl oxygen is trying adopt the axial orientation in the **2a**−**c** series, leading thus to the boat conformation.[4b] Consequently, in compounds **2a**−**c**, the amine group at the P atom is anti-oriented to H1, and therefore, S_P is the correct absolute configuration. For **2a**−**c**, both the S_P absolute configuration and the boat conformation were corroborated by an X-ray crystallographic study of **2b**[12] (Figure 3).

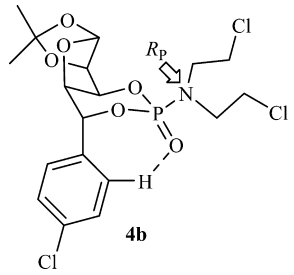
For the corresponding diastereomeric 2-amino-2-oxo-1,3,2-dioxaphosphorinane pairs **3a**−**c** and **4a**−**c** an analogous conformational analysis was performed. For **4a**−**c**, the vicinal coupling constants ($^{3}J_{H5P} \approx 17$ Hz and $^{3}J_{H3P} \approx 1$ Hz) suggest that H5 is oriented preferentially in an equatorial position and H3 in an axial orientation, giving a major chair conformation. This was confirmed by the single-crystal X-ray diffraction analysis of **4b**,[13] which allowed us to establish the absolute configuration at the P atom (R_P) (Figure 4).

dx.doi.org/10.1021/jo501772g | J. Org. Chem. 2014, 79, 9647−9654



2b
(Ar = *p*-Cl-Ph)

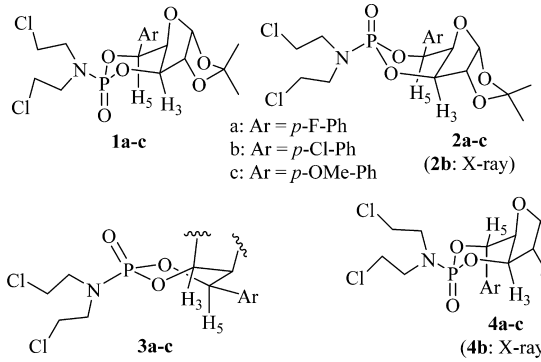
Figure 3. 2-Amino-2-oxo-1,3,2-dioxaphosphorinane **2b** in boat conformation. (X-ray structure in Supporting Information.)



4b

Figure 4. 2-Amino-2-oxo-1,3,2-dioxaphosphorinane **4b** in boat conformation. (X-ray structure in Supporting Information.)

Additionally, the crystallographic study of **4b** showed a weak C−H···O interaction between the *ortho*-hydrogen atom of the aryl group and the oxygen phosphoryl atom C−H···O═P (Σ van der Waals radii for H and O = 2.75 Å; C−H, 0.95 Å; H···O, 2.58 Å; C···O, 3.48 Å; C−H···O, 156°).[14] This intramolecular interaction might be the driving force to compensate the 1,3-diaxial interactions that should perturb the chair conformation.[4e] Finally, the close coincidence among the H5−P and H3−P vicinal coupling ($^3J_{H5P} \approx 7$ Hz and $^3J_{H3P} \approx 8$ Hz) for 2-amino-2-oxo-1,3,2-dioxaphosphorinanes **3a−c** suggest that the 1,3,2-dioxaphosphorinane ring is found very twisted, but in a different form than their diastereomeric congeners **2a−c** (Figure 5). All of the major conformations for the 2-amino-2-oxo-1,3,2-dioxaphosphorinanes **1−4** are depicted in Figure 5.



1a-c **2a-c** (**2b**: X-ray) **3a-c** **4a-c** (**4b**: X-ray)

Figure 5. Major conformers of 2-oxo-2-[*N*,*N*-bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes derived from D-xylofuranose **1-4**.

Mutagenicity Testing. In order to provide further insight into the reaction mechanism of the cleavage of HepDirect prodrugs (and also cyclophosphamide), we evaluated the mutagenic activity of selected 2-oxo-2-[*N*,*N*-bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes using the *Salmonella typhimurium* tester strain TA1535 described by Maron and Ames[17] (for details, see the Experimental Section).

The results of the mutagenic tests are summarized in Table 1. Figures 6 and 7 illustrate the results obtained for

Table 1. Mutagenic Activity of the 2-Oxo-2-[*N*,*N*-bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes Derived from D-Xylofuranose Studied Herein, in the TA 1535 *Salmonella typhimurium* Ames Tester Strain

	compound																	
	1a		**1b**		**1c**		**2a**		**2b**		**3b**		**4a**		**4b**		**4c**	
	no. of revertants/plate[a,b]																	
(μg/plate)	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0	17 ± 3	15 ± 3	18 ± 4	16 ± 3	23 ± 6	**15 ± 2**	14 ± 1	15 ± 0	19 ± 3	13 ± 2	12 ± 1	17 ± 2	28 ± 7	21 ± 7	19 ± 3	16 ± 2	20 ± 1	15 ± 1
100	24 ± 5	33 ± 12	29 ± 4	30 ± 6	300 ± 36	**510 ± 56**	19 ± 5	35 ± 6	16 ± 1	24 ± 3	29 ± 5	90 ± 16	96 ± 17	179 ± 9	13 ± 2	101 ± 7	207 ± 17	253 ± 30
200	30 ± 2	44 ± 16	43 ± 6	48 ± 10	362 ± 44	**629 ± 46**	24 ± 2	37 ± 5	22 ± 4	26 ± 3	31 ± 5	122 ± 18	175 ± 31	250 ± 35	25 ± 8	110 ± 6	268 ± 11	345 ± 53
250	32 ± 10	64 ± 13	35 ± 4	55 ± 8	456 ± 47	**603 ± 3**	28 ± 1	46 ± 2	27 ± 2	34 ± 2	33 ± 6	110 ± 24	233 ± 19	283 ± 7	27 ± 5	115 ± 10	294 ± 37	380 ± 23
500	35 ± 3	78 ± 10	40 ± 3	53 ± 5	526 ± 16	**607 ± 97**	21 ± 3	39 ± 3	31 ± 1	44 ± 5	43 ± 16	107 ± 6	250 ± 8	435 ± 25	50 ± 18	125 ± 22	402 ± 20	473
positive control[c]	79 ± 6	869 ± 77	115 ± 30	917 ± 79	41 ± 15	421 ± 124	52 ± 4	540 ± 50	65 ± 6	486 ± 75	31 ± 6	383 ± 69	48 ± 7	459 ± 82	39 ± 14	433 ± 33	58 ± 5	802 ± 31

[a]Mean of revertant colonies found in two independent experiments (three replicate/experiment). [b]The S9 mixture was prepared from the livers of rats previously treated with phenobarbital/β-naphthoflavone. [c]Cyclophosphamide (500 μg/plate).

dx.doi.org/10.1021/jo501772g | J. Org. Chem. 2014, 79, 9647−9654

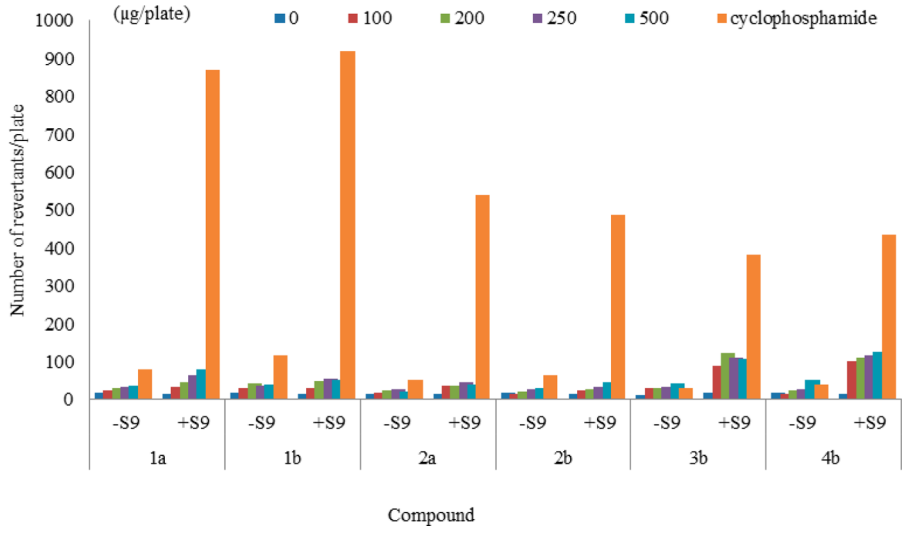


Figure 6. Graphical representation of the results obtained for the 2-oxo-2-[*N*,*N*-bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes derived from D-xylofuranose which were nonmutagenic.

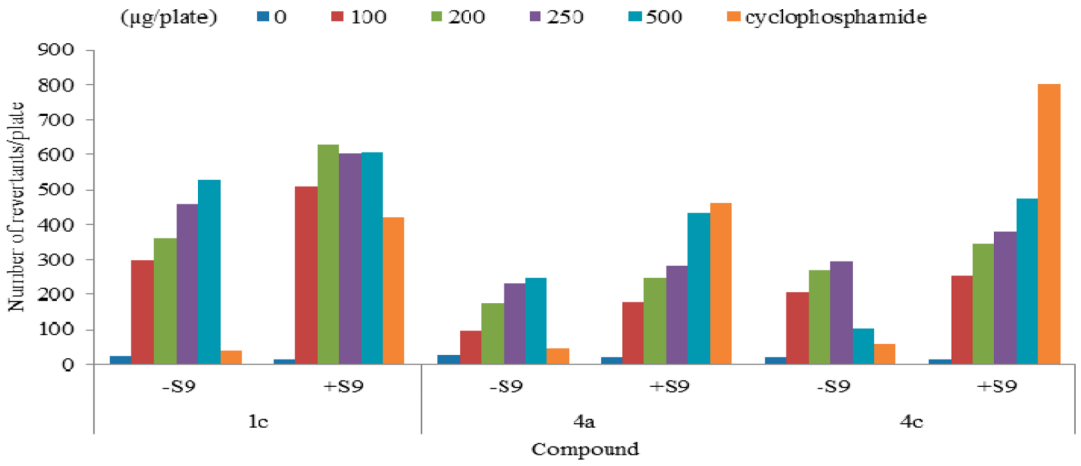


Figure 7. Graphical representation of the results obtained for the 2-oxo-2-[*N*,*N*-bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes derived from D-xylofuranose which were mutagenic.

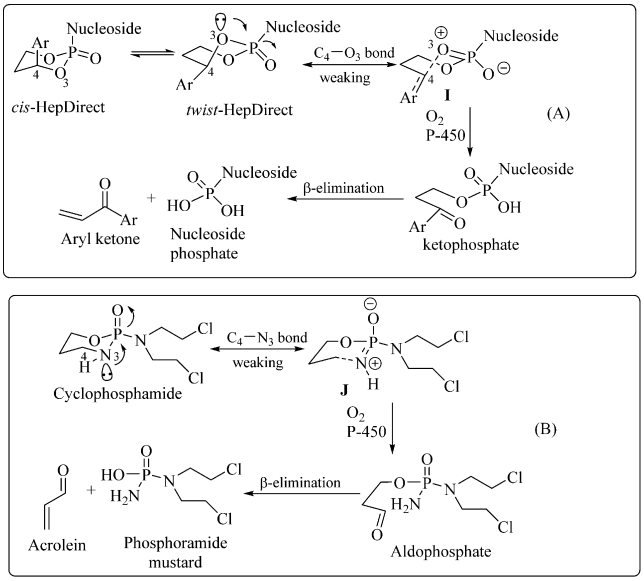
the 2-amino-2-oxo-1,3,2-dioxaphosphorinanes which were nonmutagenic and mutagenic, respectively.

The data given in Table 1 and Figures 6 and 7 show that the mutagenicity of the tested compounds depends on the stereochemistry at the P and C4 atoms and the 1,3,2-dioxaphosphorinane conformation. For example, while componds **2a** and **2b** having an S_P,4*S* absolute configuration and a major boat conformation were not mutagenic (Figure 6), the R_P,4*R* isomers **4a** and **4c**, with a major chair-conformation, were shown to be mutagenic upon metabolic activation (Figure 7). This occurs particularly for **4a**, which was shown to be as mutagenic as cyclophosphamide at 500 μg/plate (Figure 7). Interestingly, whereas compounds **1a** and **1b**, which are both comfortable in the chair conformation, were not mutagenic, compound **1c** was not only more mutagenic than cyclophosphamide at only 200 μg/plate under metabolic activation but also highly mutagenic without metabolic activation (Figure 7).

Erion's research group previously documented that the cleavage of the HepDirect prodrugs depends strongly on the stereochemistry of the P atom and C4 carbon, but no comments regarding the influence of the ring conformation were stated.[3] For example, the metabolic oxidation of their prodrugs require *cis*-configuration between the aryl group on carbon C4 and the nucleoside on the P atom. Furthermore, on the basis of the configurational and conformational analysis of structurally related 1,3,2-dioxaphosphorinanes,[4b,15] we deduced that the corresponding *cis*-phosphate prodrugs presented a twisted conformation in both solution and the solid state. Then, taking into account the remarkable high mutagenicity shown by **1a** in both the presence and absence of metabolic activation, it is reasonable to propose that the cleavage of the HepDirect prodrugs is mediated not only by the cytochrome P-450 but also by the anomeric effect via the weakened C4—O3 bond (**I**) that favors its cleavage into the ketophosphate and the release of the nucleoside phosphate (box A in Scheme 4). A similar interpretation can be carried out for the case of cyclophosphamide: only the chair conformation is prone to delocalize the nonbonding lone pair electrons from the nitrogen (now instead of the oxygen) in order to weaken the C4—N3 bond (**J**) to generate aldophosphamide and then the phosphoramide mustard (box B in Scheme 4). Moreover, the origin of the selective C4—N3 bond cleavage in cyclophosphamide can be attributed to the stronger lone-pair donor character of the nitrogen atom in comparison to oxygen, whereas in the case of

dx.doi.org/10.1021/jo501772g | J. Org. Chem. 2014, 79, 9647−9654

Scheme 4. Anomeric Effect on the Cleavage of HepDirect Prodrugs (A) and Cyclophosphamide (B)



the HepDirect prodrugs the selectivity is due to the presence of the aryl group at the C4 position that mimics the lone pair donor character of the nitrogen atom. This latter premise would justify the high mutagenic activity of the fluorinated 2-amino-2-oxo-1,3,2-dioxaphosphorinane **4c** when compared with its chlorinated analogue **4b**, especially if it is considered that the fluorine substituent is a better lone pair donor toward the aromatic ring than the chlorine substituent.[16]

CONCLUSIONS

In summary, we have presented experimental evidence, based on mutagenic activity of selected 2-oxo-2-[*N*,*N*-bis(2-chloroethyl)]-4-aryl-1,3,2-dioxaphosphorinanes derived from D-xylofuranose, that suggests that both the conformation of the 1,3,2-dioxaphosphorinane ring and the effective electron-donating of the substituent placed in para-position on the aromatic ring at C4 carbon are responsible for the biological activity. Since the conformation of these compounds depends mainly on the anomeric effect, and also taking into account of the high mutagenicity of **1c**, **4a**, and **4c** in the absence of metabolic activation, we can establish that not only the cytochrome P-450 but also the anomeric effect plays a significant role in the prodrug cleavage mechanism of both HepDirect and Cyclophosphamide prodrugs. The findings presented herein offers also new perspectives for the design of other P-heterocyclic anticancer and antiviral prodrug candidates. We are currently working on this approach and developing novel prodrugs which are also biologically tested. The results will be reported in due course.

EXPERIMENTAL SECTION

General Methods. All reagents were obtained from commercial sources and used without purification. Solvents were used as technical grade and freshly distilled prior to use. NMR studies were carried out with 400 and 300 MHz equipment. Internal references (TMS) for ^{1}H and ^{13}C chemical shifts are stated in parts per million. COSY, HSQC, and NOESY experiments have been carried out in order to assign the ^{1}H and ^{13}C NMR spectra completely. High-resolution mass spectra were performed in HRMS FAB-QMS ion mode.

General Procedure for Synthesis of 1,3-Dioxaphosphorinanes. To a solution of bis(2-chloroethyl)amine hydrochloride (1.0 mmol), triethylamine (3.0 mmol), and 4-(dimethylamino)pyridine (1.0 mmol) in dry CH_2Cl_2 (30 mL) at 0 °C was added dropwise phosphoryl chloride (1.2 mmol) dissolved in 2 mL of dry CH_2Cl_2. The reaction mixture was allowed to react for 2 h at room temperature and concentrated under reduced pressure. The residue was dissolved in ethyl acetate, and the formed solids were filtered off. The organic solution was evaporated, and the residue dissolved in dry CH_2Cl_2 (2 mL) and added to a solution of the corresponding 1,3-diol **5** or **6** (0.50 mmol) and triethylamine (1.25 mmol) in dry CH_2Cl_2 (30 mL) at 0 °C. The reaction mixture was stirred for 3 h and concentrated under reduced pressure; the residue was washed with ethyl acetate and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (230−400 mesh) with *n*-hexane−EtOAc to afford the corresponding 2-amino-2-oxo-1,3,2-dioxaphosphorinanes.

(5S,R_P)-1,2-O-Isopropylidene-5-(4-fluorophenyl)-3,5-O-[N-bis(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**1a**): yellow syrup; 131.1 mg (56%); $[\alpha]^{20}_D = -0.3$ (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.29 (s, 3H), 1.44 (s, 3H), 4.48 (m, 4H), 3.63 (m, 4H), 4.29 (apparent q, J = 1.8 Hz, 1H), 4.67 (d, J = 3.9 Hz, 1H), 5.05 (d, J = 2.1 Hz, 1H), 5.81 (s, 1H), 5.99 (d, J = 3.6 Hz, 1H), 7.08 (m, 2H), 7.41 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.8, 26.2, 41.6, 48.8 (δ, J = 4.5 Hz), 60.1, 74.3 (d, J = 4.5 Hz), 75.7 (d, J = 4.5 Hz), 80.7 (d, J = 5.7 Hz), 83.4 (d, J = 12.5 Hz), 104.4, 112.4, 115.2 (d, J = 21.6 Hz), 128.3 (d, J = 7.9 Hz), 131.6 (dd, J = 9.1, 3.4 Hz), 162.55 (d, J = 162.55 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 4.99; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{18}H_{24}Cl_2FNO_6P$ 470.0702, found 470.0727.

(5S,S_P)-1,2-O-Isopropylidene-5-(4-fluorophenyl)-3,5-O-[N-bis(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**2a**): white solid; 72.7 mg (31%); mp = 162−164 °C; $[\alpha]^{20}_D = +31.4$ (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31 (s, 3H), 1.43 (s, 3H), 3.42 (m, 4 H), 3.63 (m,4H), 4.52 (dd, J = 3.6, 1.5 Hz, 1H), 4.85 (d, J = 3.6 Hz, 1H), 4.94 (dd, J = 17.6, 3.7 Hz, 1H), 5.42 (s, 1H), 6.20 (d, J = 3.9 Hz, 1H), 7.08 (m, 2H), 7.48 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.2, 26.8, 41.8, 77.1(d, J = 6.9 Hz), 79.3 (d, J = 6.8 Hz), 83.1 (d, J = 6.8 Hz), 84.3, 105.6, 112.6, 115.6 (d, J = 21.6 Hz), 128.9 (d, J = 8.0 Hz), 131.6, 163.0 (d, J = 247 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 2.0; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{18}H_{24}Cl_2FNO_6P$ 470.0702, found 470.0732.

(5R,S_P)-1,2-O-Isopropylidene-5-(4-fluorophenyl)-3,5-O-[N-bis(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**3a**): yellow syrup; 73 mg (31%); $[\alpha]^{20}_D = +12.25$ (c = 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (s, 3H), 1.46 (s, 3H), 63.34−3.48 (m, 4H), 3.57−3.63 (m, 4H), 4.55 (m, 1H), 4.85 (d, J = 3.9 Hz, 1H), 4.90 (dd, J = 8.4, 3.6 Hz, 1H), 5.60 (dd, J = 6.9, 3.9 Hz, 1H), 6.19 (d, J = 3.6 Hz, 1H), 7.05−7.17 (m, 2H), 7.37−7.46 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.2, 26.8, 41.8, 41.8, 49.6, 49.6 79.6 (d, J = 7.9 Hz), 79.8 (d, J = 5.7 Hz), 82.3 (d, J = 6.8 Hz), 84.5 (d, J = 6.8 Hz), 105.3, 112.7, 116.3 (d, J = 21.7 Hz), 128.1 (d, J = 9.1 Hz), 129.4 (d, J = 9.1 Hz), 163 (d, J = 248.2 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 3.6; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{18}H_{24}Cl_2FNO_6P$ 470.0702, found 470.0731.

(5R,R_P)-1,2-O-Isopropylidene-5-(4-fluorophenyl)-3,5-O-[N-bis(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**4a**): white solid; 84.5 mg (36%); mp = 150−154 °C; $[\alpha]^{20}_D = +0.7$ (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (s, 3H), 1.47 (s, 3H), 3.48 (m, 4H), 3.62 (m, 4H), 4.23 (m, 1H), 4.69 (d, J = 3.6 Hz, 1H), 4.95 (s, 1H), 5.75 (d, J = 16.4 Hz, 1H), 6.04 (d, J = 3.6 Hz, 1H), 7.12 (m, 2H), 7.64 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.0, 26.4, 41.9, 49.2 (d, J = 4.5 Hz), 76.6 (d, J = 5.7 Hz), 78.2 (d, J = 5.7 Hz), 81.5 (d, J = 6.8 Hz), 83.9 (d, J = 11.4 Hz), 104.2, 112.9, 115.9 (d, J = 21.7 Hz), 128.3 (d, J = 7.9 Hz), 132.3 (d, J = 3.4 Hz), 162.8 (d, J = 247.1 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 0.14; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{18}H_{24}Cl_2FNO_6P$ 470.0702, found 470.0726.

(5S,R_P)-1,2-O-Isopropylidene-5-(4-chlorophenyl)-3,5-O-[N-bis(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**1b**): yellow syrup; 116.7 mg (48%); $[\alpha]^{20}_D = -2.9$ (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (s, 3H), 1.45 (s, 3H), 3.48 (m, 4H), 3.63

dx.doi.org/10.1021/jo501772g | J. Org. Chem. 2014, 79, 9647−9654

(m, 4H), 4.27 (apparent q, J = 1.6 Hz, 1H), 4.65 (d, J = 3.6 Hz, 1H), 5.05 (d, J = 2.4 Hz, 1H), 5.79 (s, 1H), 5.97 (d, J = 3.6 Hz, 1H), 7.37 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.0, 26.5, 41.8, 49.1 (δ, J = 4.6 Hz), 74.4 (d, J = 4.5 Hz), 75.8 (d, J = 4.6 Hz), 80.9 (d, J = 4.5 Hz), 83.5 (d, J = 12.1 Hz), 104.6, 112.7, 127.9, 128.7, 134.2 (d, J = 9.1), 134.6; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 5.2; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{18}H_{24}Cl_3NO_6P$ 486.0407, found 486.0379.

(5S,S_P)-1,2-O-Isopropylidene-5-(4-chlorophenyl)-3,5-O-[N-bis(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**2b**): white solid; 95 mg (39%); mp = 186−188 °C; $[\alpha]^{20}_D$ = +29.2 (c = 1 $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31 (s, 3H), 1.42 (s, 3H), 3.42 (m, 4H), 3.62 (m, 4H), 4.51 (m, 1H), 4.85 (d, J = 3.6 Hz, 1H), 4.95 (dd, J = 17.4, 3.9 Hz, 1H), 5.41 (s, 1H), 6.18 (d, J = 3.6 Hz, 1H), 7.39 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.2, 26.9, 41.7, 49.2 (d, J = 4.6 Hz), 76.9 (d, J = 6.8 Hz), 79.2 (d, J = 5.7 Hz), 83.0 (d, J = 6.8 Hz), 84.3, 105.6, 112.6, 128.3, 128.8, 134.3 (d, J = 8.0 Hz), 134.9; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 1.97; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{18}H_{24}Cl_3NO_6P$ 486.0407, found 486.0379.

(5R,S_P)-1,2-O-Isopropylidene-5-(4-chlorophenyl)-3,5-O-[N-bis(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**3b**): yellow syrup; 119.2 mg (49%); $[\alpha]^{20}_D$ = +20.5 (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (s, 3H), 1.45 (s, 3H), 3.43 (m, 4H), 3.60 (m, 4H), 4.52 (td, J = 3.6, 1.6 Hz, 1H), 4.84 (d, J = 3.6 Hz, 1H), 4.89 (dd, J = 7.6, 3.6 HZ, 1H), 5.59 (dd, J = 6.4, 4.0 Hz, 1H), 6.18 (d, J = 3.6 Hz, 1H), 7.35 (m, 2H), 7.43 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.2, 26.8, 41.8, 49.5 (d, J = 4.6 Hz), 79.5 (d, J = 79.5 HZ), 79.6 (d, J = 8.0 Hz), 82.3 (d, J = 6.9 Hz), 84.4 (d, J = 6.8 Hz), 105.3, 112.7, 127.4, 129.3, 135.1, 135.2 (d, J = 6.8 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 3.77; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{18}H_{24}Cl_3NO_6P$ 486.0407, found 486.0377.

(5R,R_P)-1,2-O-Isopropylidene-5-(4-chlorophenyl)-3,5-O-[N-bis(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**4b**): white solid; 102 mg (42%); mp = 152−153 °C; $[\alpha]^{20}_D$ = +7.0 (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (s, 3H), 1.46 (s, 3H), 3.47 (m, 4H), 3.61 (m, 4H), 4.22 (m, 1H), 4.69 (d, J = 3.6 Hz, 1H), 4.92 (s, 1H), 5.74 (d, J = 16.8 Hz, 1H), 6.04 (d, J = 3.6 Hz, 1H), 7.40 (m, 2H), 7.59 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.0, 26.4, 41.9, 49.2 (d, J = 5.4 Hz), 76.5 (d, J = 5.7), 78.2 (d, J = 4.6 Hz), 81.4 (d, J = 6.9 Hz), 83.9 (d, J = 11.5 Hz), 104.3, 112.9, 127.7, 129.2, 134.9, 134.9 (d, J = 8.0 Hz), 134.9; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 0.17; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{18}H_{24}Cl_3NO_6P$ 486.0407, found 486.0385.

(5S,R_P)-1,2-O-Isopropylidene-5-(4-methoxyphenyl)-3,5-O-[N-bis-(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**1c**): yellow syrup; 118.1 mg (49%); $[\alpha]^{20}_D$ = −8.6 (c = 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (s, 3H), 1.44 (s, 3H), 4.46 (m, 4H), 3.60 (m, 4H), 3.81 (s, 3H), 4.28 (apparent d, J = 1.2 Hz, 1H), 4.65 (d, J = 3.9 Hz, 1H), 5.05 (s, 1H), 5.76 (s, 1H), 5.99 (d, J = 3.6 Hz, 1H), 6.92 (d, J = 9.0 Hz, 2H), 7.36 (d, J = 9.0 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.0, 26.5, 41.8, 49.1 (d, J = 4.6 Hz), 55.2, 74,7 (d, J = 3.4 Hz), 76.3 (d, J = 4.5 Hz), 80.9 (d, J = 5.7 Hz), 83.5 (d, J = 11.3 Hz), 104.6, 112.6, 113.8, 127.9 (d, J = 9.1 Hz), 128.1, 128.5, 159.8; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 5.07; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{19}H_{27}Cl_2NO_7P$ 482.0902, found 482.0878.

(5R,R_P)-1,2-O-Isopropylidene-5-(4-methoxyphenyl)-3,5-O-[N-bis-(2-chloroethyl)amino)phosphoryl]-α-D-xylofuranose (**4c**): yellow syrup; 77 mg (32%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (s, 3H), 1.46 (s, 3H), 3.47 (m 4H), 3.60 (m, 4H), 3.82 (s, 3H), 4.25 (apparent q, J = 1.6 Hz, 1H), 4.69 (d, J = 3.6 Hz, 1H), 4.99 (d, J = 1.6 Hz, 1H), 5.71 (d, J = 17.2 Hz, 1H), 6.03 (d, J = 3.6 Hz, 1H), 6.95 (d, J = 9.2 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.0, 26.4, 41.9, 49.3 (d, J = 4.6 Hz), 55.3, 76.6, 78.3 (d, J = 5.7 Hz), 81.9 (d, J = 6.8 Hz), 84.1 (d, J = 10.2 Hz), 104.2, 112.7, 114.3, 127.8, 128.4, 159.9; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ −0.06; HRMS (FAB-QMS) $[M + H]^+$ calcd for $C_{19}H_{27}Cl_2NO_7P$ 482.0902, found 482.0874.

Mutagenicity Test Conditions. All of the experiments were carried out with the *Salmonella typhimurium* tester strain TA1535 using the plate incorporation assay described by Maron and Ames.[17] Phenotypic markers were tested as described by Maron and Ames.[17] Each compound were tested at 100, 200, 250, and 500 μg/Petri dish in the presence and absence of metabolic activation mediated by an S9 mixture prepared from livers of rats previously treated with phenobarbital/β-naphthoflavone. The S9 mixture was prepared according to Ames.[7,17] We followed the OECD guideline (test no. 471) for testing of chemicals in bacterial reverse mutation tests. Spontaneous reversion as well as positive control for mutagenicity was included in each experiment. Cyclophosphamide was the positive mutagenic control in the presence of the S9 mixture. A tested compound was considered mutagenic when the number of induced revertant colonies was increased by doubled in control plates, which is consistent with a dose-dependent mutagenic effect.[18] The data in Table 1 showcase the revertant colonies ± SD found in two independent experiments (three replicated plates/experiment). Data from spontaneous reversion of TA1535 strain as well as those from positive control in the presence and absence of S9 mixture are included.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra; X-ray data for **2b** and **4b** (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge financial support from Consejo Nacional de Ciencia y Tecnología (CONACyT), project no. 151611. We also thank Sandra L. Hernández Ojeda for technical assistance.

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(12) Crystal data for $C_{18}H_{23}Cl_3NO_6P$, M_r = 486.69, 0.32 × 0.39 × 0.42 mm^3, monoclinic, space group $P2_1$, a = 6.2184(15) Å, b = 16.744(4) Å, c = 10.777(3) Å, β = 91.444(4)°, V = 1121.8(5) Å^3, Z = 2, δ = 1.441, $2\theta_{max}$ = 25, 3954 independent reflections (R_{int} = 0.057), R_1 = 0.060 for 3643 reflections with $I > 2\sigma(I)$ and wR_2 = 0.1344 for all data, 264 parameters, GOF = 1.17. CCDC 1015038.
(13) Crystal data for $C_{18}H_{23}Cl_3NO_6P$, M_r = 486.69, 0.24 × 0.27 × 0.28 mm^3, orthorhombic, space group $P2_12_12_1$, a = 11.2788(11) Å, b = 12.8154(13) Å, c = 14.6320(14) Å, V = 2114.9(4) Å^3, Z = 4, δ = 1.529, $2\theta_{max}$ = 25, 3722 independent reflections (R_{int} = 0.042), R_1 = 0.039 for 3635 reflections with $I > 2\sigma(I)$ and wR_2 = 0.088 for all data, 271 parameters, GOF = 1.09. CCDC 1015039.
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