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Yvain Gerard, Aurélien Voissiere, Caroline Peyrode, Marie-Josephe Galmier, Elise Maubert, et al.. Design, synthesis and evaluation of targeted hypoxia-activated prodrugs applied to chondrosarcoma chemotherapy. *Bioorganic Chemistry*, 2020, 98, pp.103747. 10.1016/j.bioorg.2020.103747. hal-02866765

HAL Id: hal-02866765

<https://uca.hal.science/hal-02866765v1>

Submitted on 19 Nov 2020

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Design, synthesis and evaluation of targeted hypoxia-activated prodrugs applied to chondrosarcoma chemotherapy

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Keywords: Hypoxia-activated prodrug, Phosphoramidate, Quaternary ammonium, Chondrosarcoma, Proteoglycan

Abstract

The tumor microenvironment in chondrosarcoma (CHS), a chemo- and radio-resistant cancer provides unique hallmarks for developing a chondrosarcoma targeted drug-delivery system. Tumor targeting could be achieved using a quaternary ammonium function (QA) as a ligand for aggrecan, the main high negative charged proteoglycan of the extracellular matrix of CHS, and a 2-nitroimidazole as trigger that enables hypoxia-responsive drug release. In a previous work, **ICF05016** was identified as efficient proteoglycan-targeting hypoxia-activated prodrug in a human extraskeletal myxoid chondrosarcoma model in mice and a first study of the structure-activity relationship of the QA function and the alkyl linker length was conducted. Here, we report the second part of the study, namely the modification of the nitro-aromatic trigger and the position of the proteoglycan-targeting ligand at the aromatic ring as well as the nature of the alkylating mustard. Synthetic approaches have been established to functionalize the 2-nitroimidazole ring at the *N*-1 and *C*-4 positions with a terminal tertiary alkyl amine, and to perform the phosphorylation step namely through the use of an amine borane complex, leading to phosphoramidate and isophosphoramidate mustards and also to a phosphoramidate mustard bearing four 2-chloroethyl chains. In a preliminary study using a reductive chemical activation, QA-conjugates, except the 4-nitrobenzyl one, were showed to undergo efficient cleavage with release of the corresponding mustard. However *N,N,N*-trimethylpropylamimium tethered to the *N*-1 or *C*-4 positions of the imidazole seemed to hamper the enzymatic reduction of the prodrugs and all tested compounds featured moderate selectivity toward hypoxic cells, likely not sufficient for application as hypoxia-activated prodrugs.

1. Introduction

Surgical resection with wide margins remains the mainstay for the management of chondrosarcoma, the malignant cartilage tumor [1]. For patients with inoperable primary disease, metastases, or relapse, a lack of appropriate therapy persists due to chemo- and radio-resistance related to the extensive cartilaginous extracellular matrix expressed by tumor cells, a physical semi-permeable barrier, and to hypoxia. [1–4] To circumvent this problem, new therapeutic tools have been developed such as: isocitrate dehydrogenase, m-TOR, tyrosine kinase and Hedgehog pathway inhibitors, but so far no drug have been approved for clinical use [5,6]. Our strategy aims at providing a dual targeted therapy of chondrosarcoma leveraging two specific hallmarks of this tumor, namely hypoxia and hyaline extracellular matrix, more precisely, its high negative fixed charge density due to the presence of proteoglycans (PGs). This latter

feature of the chondrosarcoma extracellular matrix is due to the numerous sulfate and carboxylate anionic functions of aggrecan (≈ 250 MD), the most abundant PG in chondrosarcoma with approximately 150 glycosaminoglycan side chains of chondroitin sulfate and keratan sulfate [7].

Hypoxia is a common feature of various solid tumors influencing many aspects of cancer biology, including increased resistance to therapy and disease progression through angiogenesis, glycolytic switch in metabolism, and amplified anti-apoptotic and metastatic potentials [8]. Therefore, the development of cancer-targeted therapies directed against hypoxia is of great importance, especially with hypoxia-activated prodrugs (HAP)[9], like AQ4N[10], apaziquone[11,12], SN30000[13,14], evofosfamide (TH-302)[15–20], PR-104[21,22] or TH-4000[23] for example (Figure 1). HAPs are designed to be bioactivated by one-electron oxygen-sensitive reductases, such as NADPH cytochrome P450 oxidoreductase (POR), to specifically release active cytotoxic species in hypoxic regions of the tumor microenvironment, using a “trigger” or “oxygen concentration sensor” [24–28]. Nitroaromatic-based HAP are well reported in the literature, the 2-nitroimidazole moiety being by far the most widely documented trigger [24–26,29–32].

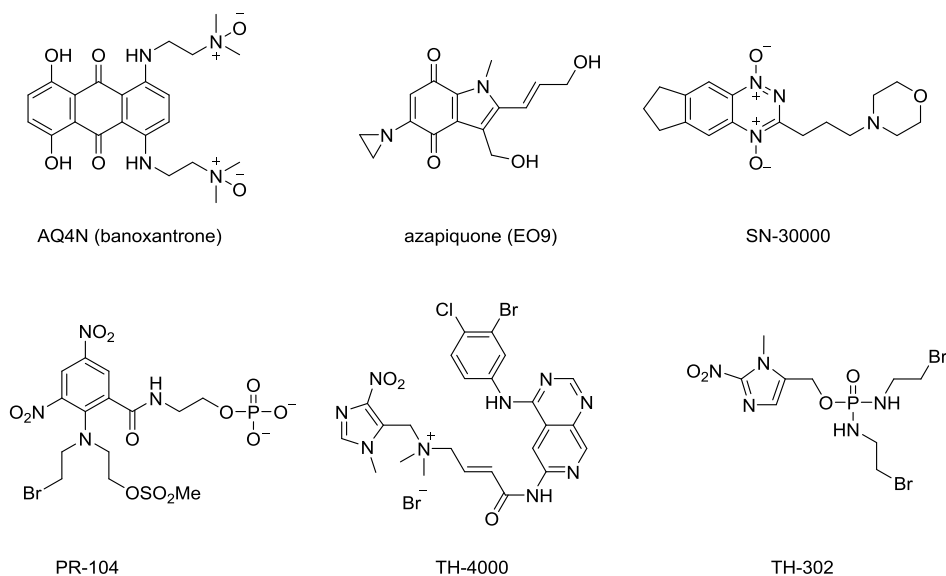


Figure 1: Structures of some hypoxia-activated prodrugs reviewed in literature

This dual targeted therapy for chondrosarcoma was previously investigated, by conjugation of a hypoxia-activated prodrug to a quaternary ammonium function (QA) validated as PG-targeting ligand[33,34] and led to the identification of the first PG-targeted HAP, named **ICF05016**[35], a phosphorodiamidic mustard functionalized with a QA function and a nitroimidazole heterocycle, which may undergo different reductive processes, causing the

release of the cytotoxic mustard (Figure 2). Aggrecan binding of **ICF05016** by surface plasmon resonance (SPR), as well as hypoxic-cytotoxicity ratio (HCR; IC₅₀ under normoxia vs. IC₅₀ under hypoxia) on the HEMC-SS (human extraskelatal myxoid chondrosarcoma) cell line were confirmed. In a HEMC-SS tumor-bearing mice model, **ICF05016** treatment provided a significant tumor growth inhibition, a decrease in the mitotic index and an increase in DNA damages predominantly found in pimonidazole-positive hypoxic areas, with no associated hematological side effects.

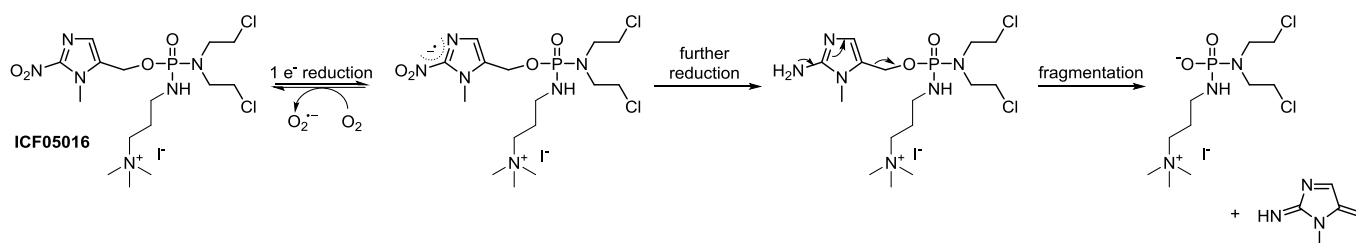


Figure 2: Proposed mechanism of **ICF05016** activated via enzymatic metabolism under hypoxic condition

Based on these results, a first lead optimization study of **ICF05016** was initiated and recently published. This work concerned the modification of the structure of the QA function and the length of the alkyl linker (Figure 3, axis 1) [36]. Thirty QA-phosphoramidate mustard conjugates were synthesized and a preliminary screening by SPR revealed that affinity depends more on the type of QA function, than on the linker length and highlighted the positive impact of a benzyl QA function on the affinity to aggrecan. For all tested prodrugs, hypoxic selectivity was maintained and even increased, compared to the lead **ICF05016**, mainly with compound **ICF05089** that emerged as the most effective QA HAPs. Herein, we extend this study to a series of prodrugs differing both by the hypoxia-sensitive trigger, the position of the *N,N,N*-trimethylpropylammonium targeting moiety (*N*-1 or *C*-4 series), or the nature of the cytotoxic agent (Figure 3, axis 2).

In one hand, we were interested in the reduction of the sensitive nitroaromatic trigger. Although the nitroimidazole appears as the most widely studied, different other cycles have been used in HAP design. Concerning HAP undergoing clinical trials, the 2-nitroimidazole trigger is used in the structure of TH-302 and TH-4000, whereas in the case of PR-104, the cytotoxin is released by bioreduction of a 4-nitrophenyl group. Many other nitroaryl-based HAP were studied in preclinical models, like camptothecin, paclitaxel, doxorubicine conjugates [29,30,37] and tyrosine kinase inhibitors [31,32]. In addition to widespread used 4-nitrophenyl and 2- or 5-nitroimidazole groups, nitrofuran and nitrothiophene groups have also been used as bioreduction-sensitive moieties. The most important considerations in the choice of the bioreductive trigger may be the hypoxic threshold required for activation and the propensity to undergo bioreduction, in part correlated with the trigger's electrochemical reduction potential. The nitro-compounds are encompassed in class II HAP, which depends on severe hypoxia for activation. The 1-methyl-2-nitroimidazole group, with an estimated reduction potential of -390/-

400 mV [38,39], should undergo easier reduction than the 4-nitrobenzyl group (showing a lower reduction potential, $E = -490$ mV), and a harder reduction than 2-nitrofuran ($E = -330$ mV). To define the best trigger in our series of PG-targeted HAP, the selected subset 2-nitroimidazole-, 2-nitrofuran- and 4-nitrobenzyl-based conjugate were synthesized.

In a second hand, we were interested in the position of the *N,N,N*-trimethylpropylammonium targeting chain, so far tethered to the phosphorodiamidic mustard. To this end, we synthesized compounds bearing this chain on the *N*-1 or *C*-4 position of the 2-nitroimidazole ring. This strategy will allow the possibility to release under hypoxic conditions only the alkylating agent free of the PG-targeting ligand.

Our first attempts to validate the concept of PG-targeted HAP were focused on phosphoramidate mustard. Cyclophosphamide belongs with ifosfamide and trofosfamide to the oxazaphosphorines alkylating class, commonly used in clinical practice for their wide spectrum of antineoplastic activity. Cyclophosphamide and ifosfamide both require metabolic activation from the cytochrome P450 to subsequently produce phosphoramidate and isophosphoramidate mustards (PM, IPM), which induce DNA cross-linking. These active nitrogen mustards PM and IPM show an isomeric structure with one or two 2-chloroethyl groups linked to the same nitrogen. We also decided to synthesize isomeric analogs of PM derivatives **15a** and **26a** (schemes 2 and 3), with IPM **15c** and **26c**, and also a phosphoramidate mustard bearing four 2-chloroethyl chains (TPM) (**15b** and **26b**).

A preliminary screening of all the final QA-conjugates produced was performed to evaluate their suitability as substrates for reduction-mediated cleavage (chemical and enzymatic) leading to the release of the phosphorodiamidic mustard, as well as their *in vitro* hypoxia cytotoxicity ratio.

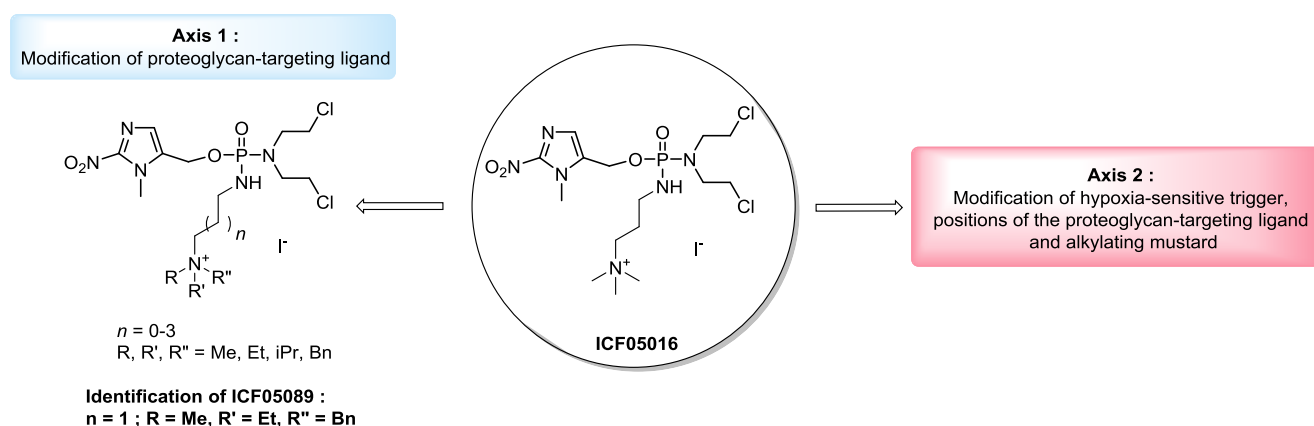


Figure 3: Pharmacomodulation of compound **ICF05016**

2. Results and discussion

2.1. Chemistry

The reagents required to tether the 4-nitrobenzyl- and 2-nitrofuran-based groups to phosphoramidic mustard are readily available from commercial sources, contrary to the 1-methyl-2-nitroimidazole precursor **5** previously described for the synthesis of **ICF05016** and its derivatives [35,36]. Also, 2-hydroxymethyl-5-nitrofuran **4** was obtained by reduction of 5-nitro-2-furaldehyde with NaBH₄ in 79% yield. As for **ICF05016**, compounds **3a-b** were obtained after phosphorylation of the appropriate hydroxymethyl nitroaromatic by the phosphoramidic dichloride **1** followed by the introduction of the *N,N*-dimethylaminopropyl chain by substitution of the remaining chloride by 3-(*N,N*-dimethylamino)propylamine and finally quaternisation of the tertiary amine function using excess of methyl iodide (Scheme 1).

Our first attempts to validate the concept of PG-targeted HAP were focused on phosphoramidic mustard then followed by the synthesis of isomeric analogs of PM derivatives **15a** and **26a**, with TPM and IPM alkylating moieties. To that end, a similar sequence of phosphorylation and quaternisation used for compounds **3a-b** was considered to obtain targeted compounds **15a** and **26a**, starting from the appropriate alcohol **10** and **21**, respectively (Scheme 2 and 3). We previously reported the synthesis of alcohol precursor **5** [36] using O'Connor *et al.* optimized method [40,41]. The same synthetic sequence was applied to obtain the alcohol **10**, common precursor of the *N*-1 functionalized derivatives with a 3-(*N,N*-dimethylamino)propyl chain instead of a methyl group. The first step of the synthesis of derivative **10** was a nucleophilic substitution involving 3-(*N,N*-dimethylamino)propylamine and ethylbromo acetate [42]. Then the secondary amine of **6** was protected by formylation with ethyl formate under basic conditions [43] to give compound **7** in good yield. The next three steps were performed without intermediate purification: (i) *C*-formylation of **7** at the α -carbon position using sodium hydride (added carefully in order to avoid reaction thermal runaway and explosion) and ethyl formate in anhydrous THF, (ii) removal of the unwanted *N*-formyl group under reflux in concentrated water-alcohol hydrochloric acid solution, and finally (iii) Markwald cyclisation with cyanamide under reflux at pH 3. According to this procedure, aminoimidazole **8** was obtained after silica gel column chromatography with 21% yield. The nitro group was then introduced by diazotization of the aromatic amine in the presence of an excess of sodium nitrite with good yield (71%). Due to low stability of product **9**, reduction of the ester function with lithium borohydride (formed *in situ* via the metathesis reaction between sodium borohydride and lithium bromide) needs to be performed immediately after purification. Indeed, prolonged storage of compound **9** at 4 °C led to the formation of a tetrahydroimidazo[1,2-*a*]pyrimidine derivative **11** (supporting data), resulting from a nucleophilic aromatic substitution of the nitro group by the tertiary amine of the ester **9**, probably allowed by the presence of the electron withdrawing ester in the 5-position,

followed by demethylation of the QA intermediate. Lee *et al.* [44] already described a similar S_NAr reaction by tertiary amine with 2,4-dichloropyrimidines, further substituted with an electron-withdrawing substituent, followed by *in situ* *N*-dealkylation of the intermediate. Even if compound **9** was engaged quickly in the next step, the desired alcohol **10** was only obtained in 7% yield, while the major fraction isolated was identified as the corresponding amine-borane complex (45% yield). The structure of the borazane **10-BH₃** was validated using mass spectrometry and X-Rays diffraction (supporting data); the HRMS spectrum showed the protonated complex, m/z 243.1616, and the sodium adduct at m/z 265.1434. After different attempts of complex dissociation under acidic [45,46] or basic [47] conditions, the best results were obtained with boiling ethanol [48,49]. Both reactions, reduction with lithium borohydride, followed after work-up by complex dissociation in boiling ethanol afforded after silica gel purification the alcohol **10** from **9** in 49% yield.

Starting from **10**, initial attempts to synthesize phosphorodiamidates **14a-b** following published synthetic methods from Matteucci *et al.* [43] and previously adapted for **ICF05016** derivatives [36], resulted respectively in ineffective reaction or rapid degradation of the synthesized products, as observed by phosphorus NMR monitoring. Assuming that these results may be due to the nucleophilicity of the tertiary amine on the *N*-1 position, we decided to use the amine-borane complex **10-BH₃**, described as stable in the presence of strong bases such as *n*-butyllithium [49], to perform the phosphorous coupling reaction. The alcohol **10-BH₃** was deprotonated with LiHMDS at -78 °C and the appropriate phosphorous compound **1** or **12** (synthesized as described in the literature [50,51]) was then added. For compound **14a-BH₃**, the reaction was carried out at -78 °C in THF for 1 h before bubbling of ammonia gas and was almost quantitative. To obtain compound **14b-BH₃**, the reaction had to be warmed to rt after the addition of compound **12**. With these conditions, the kinetic follow-up by phosphorus NMR showed a complete reaction after 20 h, leading to compound **14b-BH₃** in 70% yield. For the last isophosphorodiamidate analog **14c-BH₃**, a Mitsunobu reaction was considered as previously described [20] to access to evofosfamide and needed the prior synthesis of the isophosphorodiamidic acid (**IPM**). This kind of compound is usually synthesized by hydrolysis of the corresponding phosphorodiamidyl chloride [20,40]. In all our attempts, this methodology appeared not reliable and we decided to focus our attention to a two-steps sequence involving synthesis of the corresponding benzyl ester followed by hydrogenation as described in the literature [52] and leading to **IPM** in 80% overall yield (supporting data). However, Mitsunobu conditions between **IPM** and **10-BH₃** led to a poor yield of the desired product **14c-BH₃** (5%) while a large amount of starting material **10-BH₃** (76%) has been recovered. To circumvent this problem, we turned our attention on a two-step one-pot reaction, previously described by Misiura *et al.* [53] for a 4-substituted benzyl alcohol, and involving the phosphorylation of **10-BH₃** using phosphorus oxychloride in the presence of TEA, to yield the dichlorophosphate intermediate, which subsequently was treated, without isolation, with two equivalents of 2-

chloroethylamine hydrochloride followed by another base addition. This sequence successfully afforded compound **14c-BH₃** in moderate yield (48%). As expected with the use of phosphorus oxychloride and previously published with phosphorus trichloride[20], the side product 1-*N*-[3-(*N,N*-dimethylamino)propyl]-2-nitro-5-chloromethylimidazole **13** was also isolated (10% yield).

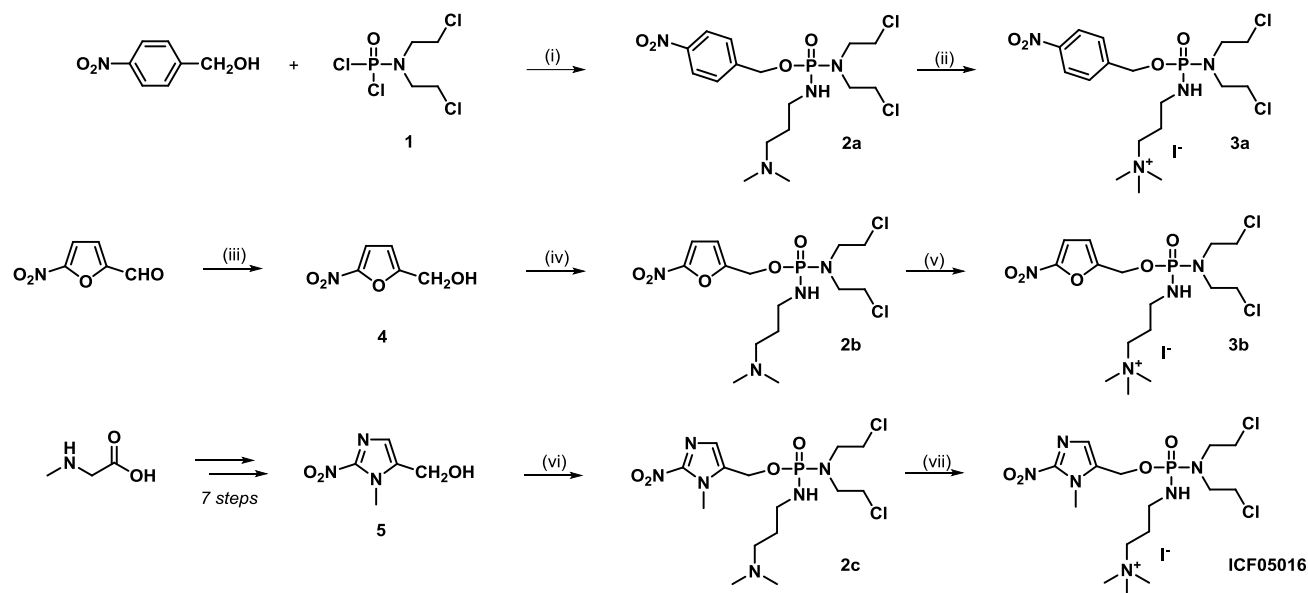
To dissociate the boron complex, we tried similar conditions as described for the alcohol **10-BH₃**, namely heating in ethanol but also acidic conditions. Unfortunately, only several by-products and low or no amount of desired product were observed, highlighting the instability of phosphorodiamidate under heating or acidic conditions. To remove the borane group, a transchelation reaction with 1,4-diazabicyclo[2.2.2]-octane (DABCO; 5 eq.) was then assessed using the method described by Tam *et al.*[54] slightly modified due to the instability of the phosphorodiamidate intermediate. Indeed, the number of equivalents of DABCO was increased (5 eq. vs 1) to decrease reaction time and the free amine was directly alkylated using an excess of methyl iodide (due to the presence of excess of DABCO). Following this procedure, quaternary ammonium **15a-c** were obtained with 75%, 21% and 57% yield, respectively including one-pot borazane dissociation and alkylation steps.

For the 4-*C*-(*N,N*-dimethylamino)propylamino) derivatives **26a-c**, alcohol **5** was functionalized using a Stille coupling reaction. According to the procedure outlined in Scheme 3, the imidazole cycle was first brominated in the 4-position with NBS[55], followed by protection of the hydroxyl function using tri(isopropyl)silylchloride to afford the silylether **17**. Then the Stille coupling reaction was performed with 66% yield following a procedure described by Jiao *et al.*[55] using tetrakis(triphenylphosphine)palladium as catalyst in anhydrous DMF and *N,N*-dimethyl-*N*-[3-(tributylstannyl)-2-propenyl]amine (**18**). The latter was synthesized by hydrostannylation of *N,N*-dimethylpropargylamine according to the methodology developed for the *N,N*-diethyl analog [56]. Under the Stille conditions used, compound **19** was obtained only as the (*E*) isomer, starting either from the pure (*E*) isomer or the isomers mixture of **18**. After silylether deprotection of compound **19** using TBAF, reduction with diimide was favored to selectively reduced the double bond of compound **20** without impacting on the nitro function [57]. First we tried to use potassium azodicarboxylate (PADA) as described in the literature[55] but no reduction reaction was evidenced according to TLC monitoring. A method involving *p*-tosylhydrazine in refluxing pyridine to generate *in situ* diimide [58] successfully converted the alkene **20** to its saturated analog **21** in 67% yield. These two last steps could also be performed in reverse order, *i.e.* reduction before silyl deprotection, but this sequence involving intermediate compound **22** led to a lower overall yield (*i.e.* 11% vs 60% for the two steps).

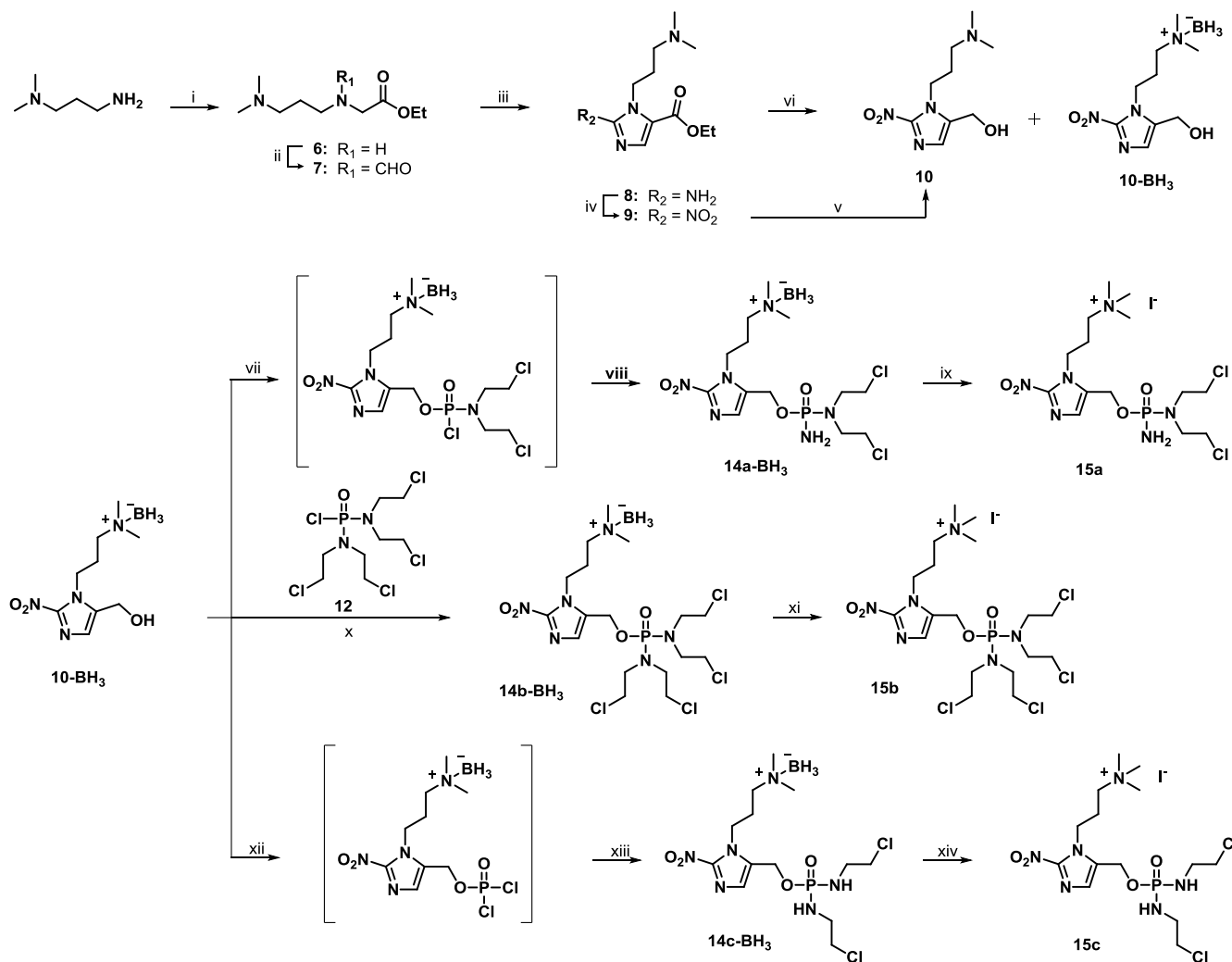
To access respectively to the cyclophosphamide mustards derivatives **23** and **24a** starting from the alcohols **20** and **21**, we tried initially our previous method used for the synthesis of **ICF05016** from dichloride **1**. Under these conditions, phosphorodiamidate intermediates **23** and **24a** were obtained with 31% and 30% yields, respectively. Each compound was further methylated with

methyl iodide under basic conditions to yield the corresponding quaternary ammoniums **25** and **26a**. Nevertheless, the phosphorylation reaction with both alcohols **20** and **21** was not reproducible. Therefore and based on the previous results obtained in the *N*-1 series, we explored the use of amine borane complex to further perform phosphorylation. The complex **21-BH₃** was obtained in 70% yield from compound **21** and its structure was confirmed by X-rays diffraction (supporting data) (note that borane complex of alkene **20** was not studied due to the presence of the double bond). The phosphorylation from borazane complex **21-BH₃** afforded compounds **24a-c-BH₃** in yield ranging from 33% to 63%. The boron complex of **24a-c** was then dissociated using DABCO and the tertiary amine directly converted onto the corresponding QA function using methyl iodide to afford final products **26a-c**.

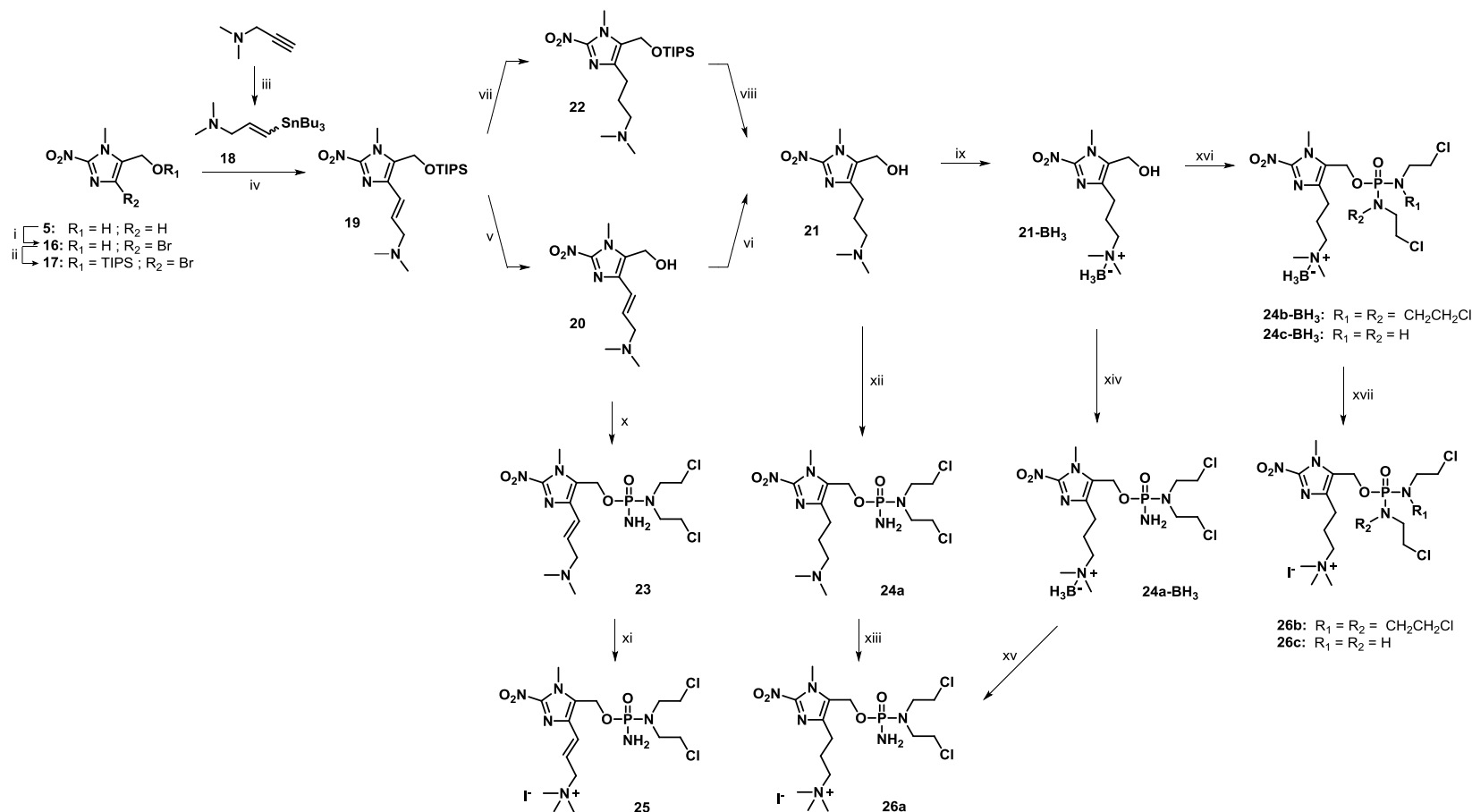
All the seven PG-targeted prodrugs **15a-c**, **25** and **26a-c** were purified by reverse phase (C18) preparative column and freeze-dried in aliquots before further evaluation. Analytical purity was evaluated by the absence of impurities revealed in the ¹H and ¹³C spectra combined with ³¹P impurities estimated at less than 5% in global integrals.



Scheme 1: Reagents and conditions: (i) (a) $\text{LiN}(\text{TMS})_2$, anh. THF, $-78\text{ }^\circ\text{C}$, 30 min, (b) 3-(*N,N*-dimethylamino)propylamine, anh. THF, $-78\text{ }^\circ\text{C}$, 40 min, **57%**; (ii) methyl iodide, anh. THF, rt, 3 h, **99%**; (iii) NaBH_4 , THF/ H_2O , $0\text{ }^\circ\text{C}$, rt, 1h30, **79%**; (iv) (a) $\text{LiN}(\text{TMS})_2$, bis(2-chloroethyl)phosphoramidic acid dichloride (**1**), anh. THF, $-78\text{ }^\circ\text{C}$, 30 min, (b) 3-(*N,N*-dimethylamino)propylamine, anh. THF, $-78\text{ }^\circ\text{C}$, 2 h, **42%**; (v) CH_3I , anh. THF, rt, 12 h, **81%**; (vi) (a) $\text{LiN}(\text{TMS})_2$, bis(2-chloroethyl)phosphoramidic dichloride (**1**), anh. THF, $-78\text{ }^\circ\text{C}$, 40 min, (b) 3-(*N,N*-dimethylamino)propylamine, anh. THF, $-78\text{ }^\circ\text{C}$, 20 min, **43%**; (vii) CH_3I , anh. THF, rt, 3h30, **99%**.



Scheme 2: Reagents conditions: (i) $BrCH_2CO_2Et$, K_2CO_3 , PhMe, 0 °C then rt, 18 h, **54%**; (ii) HCO_2Et , K_2CO_3 , EtOH, rt, 25 h, **90%**; (iii) (a) HCO_2Et , NaH (60%), THF, cyclohexane, rt, 3 h; (b) HCl (37%), EtOH, reflux, 2 h; (c) NH_2CN , EtOH/ H_2O (70/30) (v/v), pH = 3.0, reflux, 90 min, **21%**; (iv) $NaNO_2$, AcOH (66% vol. in H_2O), 0 °C then rt, 2 h, **71%**; (v) (a) $NaBH_4$, LiBr, THF/MeOH (80/20) (v/v), H_2O , 0 °C then rt, 17 h; (b) EtOH, reflux, 4 h, **49%**; (vi) $NaBH_4$, LiBr, THF/MeOH (80/20) (v/v), H_2O , 0 °C then rt, 15 h, **7%** of **10** and **45%** of **10-BH₃**; (vii) (a) LiHMDS, anh. THF, -78 °C, 5 min; (b) *N,N*-bis(2-chloroethyl)phosphoramidic acid dichloride (**1**), anh. THF, -78 °C, 1 h; (viii) NH_3 , -78 °C, 2 min, **93%**; (ix) (a) DABCO, anh. THF, 30-40 °C, 1 h; (b) CH_3I , 0 °C to rt, 2 h 30, **75%**; (x) (a) LiHMDS, anh. THF, -78 °C, 5 min; (b) compound **12**, anh. THF, -78 °C, 1 h then rt, 20 h, **70%**; (xi) (a) DABCO, anh. THF, 40 °C, 1 h; (b) CH_3I , 0 °C to rt, 2 h, **21%**; (xii) $POCl_3$, Et_3N , DCM, -78 °C, 1 h; (xiii) $NH_2CH_2CH_2Cl.HCl$, TEA, -78 °C to 5 °C, 18 h, **48%**; (xiv) (a) DABCO, anh. THF, 40 °C, 1 h; (b) CH_3I , 0 °C to rt, 1 h 30, **57%**.



Scheme 3. Reagents conditions: (i) NBS, DMF, 60 °C, 2 h 30, **82%**; (ii) TIPSCl, imidazole, DMF, rt, 18 h, **97%**; (iii) Bu₃SnH, AIBN, toluene, 85°C, 3 h 30, reflux, 1 h, **85%**; (iv) [Pd(PPh₃)₄], 105 °C, 1 h 30, **66%**; (v) TBAF, anh. THF, 0 °C, 90 min, **89%**; (vi) TsNHNH₂, K₂CO₃, pyridine, reflux, 3 h, **67%**; (vii) TsNHNH₂, K₂CO₃, pyridine, reflux, 19 h, **24%**; (viii) TBAF, THF, 0 °C, 1 h, **47%**; (ix) BH₃.THF, anh. CHCl₃, 0 °C, 1 h, **70%**; (x) (a) LiHMDS, anh. THF, -78 °C, 5 min; (b) *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (**1**), anh. THF, -78 °C, 45 min; (c) NH₃, -78 °C, 2 min, **31%**; (xi) CH₃I, K₂CO₃, anh. ACN, rt, 1 h 45, **65%**; (xii) (a) LiHMDS, anh. THF, -78 °C, 5 min; (b) *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (**1**), anh. THF, -78 °C, 1 h 15; (c) NH₃, -78 °C, 2 min, **30%**; (xiii) CH₃I, THF anh., rt, 7 h, **52%**; (xiv) (a) LiHMDS, anh. THF, -78 °C, 5 min; (b) *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (**1**), anh. THF, -78 °C, 1 h; (c) NH₃, -78 °C, 2 min, **63%**; (xv) (a) DABCO, anh. THF, 40°C, 1 h; (b) CH₃I, 0 °C, 2 h, **46%**; (xvi) (a) LiHMDS, anh. THF, -78 °C, 5 min; (b) *N,N,N',N'*-tetrakis(2-chloroethyl)phosphoramidic acid chloride (**12**), anh. THF, -78 °C to 5 °C, 24 h, **24b-BH₃: 33%** or (a) POCl₃, TEA, anh. DCM, -78 °C, 1 h; (b) NH₂CH₂CH₂Cl.HCl, TEA, -78 °C to 5 °C, 15 h, **24c-BH₃: 41%**; (xvii) (a) DABCO, anh. THF, 40 °C, 1 h; (b) CH₃I, 0 °C, 1-2 h, **26b: 35%**; **26c: 56%**.

2.2. Biological assessments for HAP screening

2.2.1 Reductive Chemical Activation

The stability of all QA-compounds in aqueous media was tested first as this parameter is essential for the biological evaluation of HAP. Under physiological conditions (cacodylate buffer pH 7.4, 37 °C) and in the absence of a reductant system, no cleavage or degradation over a 24 h period was observed by ^{31}P NMR experiments. To evaluate the sensitivity to reduction of the nitroheteroaromatic rings, the three phosphoramidate prodrugs, **ICF05016**, **3a** and **3b**, were evaluated in terms of reductive chemical activation by ^{31}P NMR experiments. In the presence of three equivalents of sodium dithionite (0.1 M cacodylate buffer, pH 7.4), a reductant system used to mimic bioreduction in hypoxic tissue, the two prodrugs **ICF05016** and **3b** were rapidly activated (Table 1 and Figure 4A). The signals related to the prodrugs **ICF05016** and **3b** disappeared with a half-time of about 3-4 min leading to the rapid formation of a signal at 12 ppm corresponding to the active cytotoxic specie, the substituted bis(2-chloroethyl)phosphorodiamidate anion [36] (as exemplified on Figure 4B for **ICF05016**). In these conditions, only 15% of the prodrug **3a** were reduced (Table 1 and Figure 4A). Nine equivalents of reducing agent were required for a total activation. As expected with the estimated reduction potential for the 1-methyl-2-nitroimidazole group, compound **ICF05016** was more easily reduced compared to the 4-nitrobenzyl analog **3a**. In addition, reductions of **ICF05016** and 2-nitrofurane derivatives **3b** were similar.

For compounds **15a-c**, **25** and **26a-c**, the imidazole ring is functionalized on the C-4 or N-1 position with a *N,N,N*-trimethylpropylaminium or *N,N,N*-trimethylpropyl-2-enaminium that may hamper reductive activation of the nitro group due to steric hindrance. To ascertain the reductive activation of the compounds and release of the corresponding PM, ^{31}P kinetic experiments were firstly carried out on compounds **15a**, **25**, **26a**. Cleavage studies were also performed with compounds **15b** and **15c**, bearing respectively IPM and TPM, in order to evaluate the impact of the phosphorodiamidic mustard on the reduction and confirm the release of the three corresponding mustards. The three prodrugs **15a-c** functionalized on the N-1 position showed a similar reduction profile with a short half-life (< 1 min) (Table 1 and Figure 4A). While compound **26a** bearing the *N,N,N*-trimethylpropylaminium chain on the C-4 position showed comparable kinetic (half-life of about 5 minutes) as the lead compound **ICF05016**, the alkene analog **25** displayed a slower reduction profile with a half live exceeding 1 h. For all compounds, ^{31}P prodrugs signals observed around 17-19 ppm disappeared with the concurrent appearance of a resonance near 12 ppm (as exemplified on Figure 4B for **ICF05016**). The rapid kinetics of reduction of the imidazole prodrugs **15a-c** and the high reactivity of the corresponding mustards, gave rapidly rise to the disappearance of the first signals at 11-12 ppm and the formation of a variety of by-products. In order to clearly assign the signal at 11-12 ppm to the corresponding mustard anions, reduction experiments were performed with the lower reduction-sensitivity 4-

nitrobenzyl analogs **27a-c** already described in the literature [53,59,60] (synthesis in supporting data). Indeed for compound **27c**, a maximum of 40% of reduction was observed in the presence of 3 equivalents of sodium dithionite compared with more than 95% for compound **15c**, both giving rise to the same signal at a chemical shift of 11.2 ppm. The latter signal is clearly assigned to **IPM** as demonstrated with pure synthesized **IPM** added in the reduction medium of **27c** at 5 min, leading to an increase of the signal level in the spiked medium (Figure 4C). Similar experiments have been carried out for the other QA-phosphorodiamidates proving each time the release of the corresponding mustard and confirming the relevance of these compounds as reduction-activated prodrugs (supporting data, Figure S1).

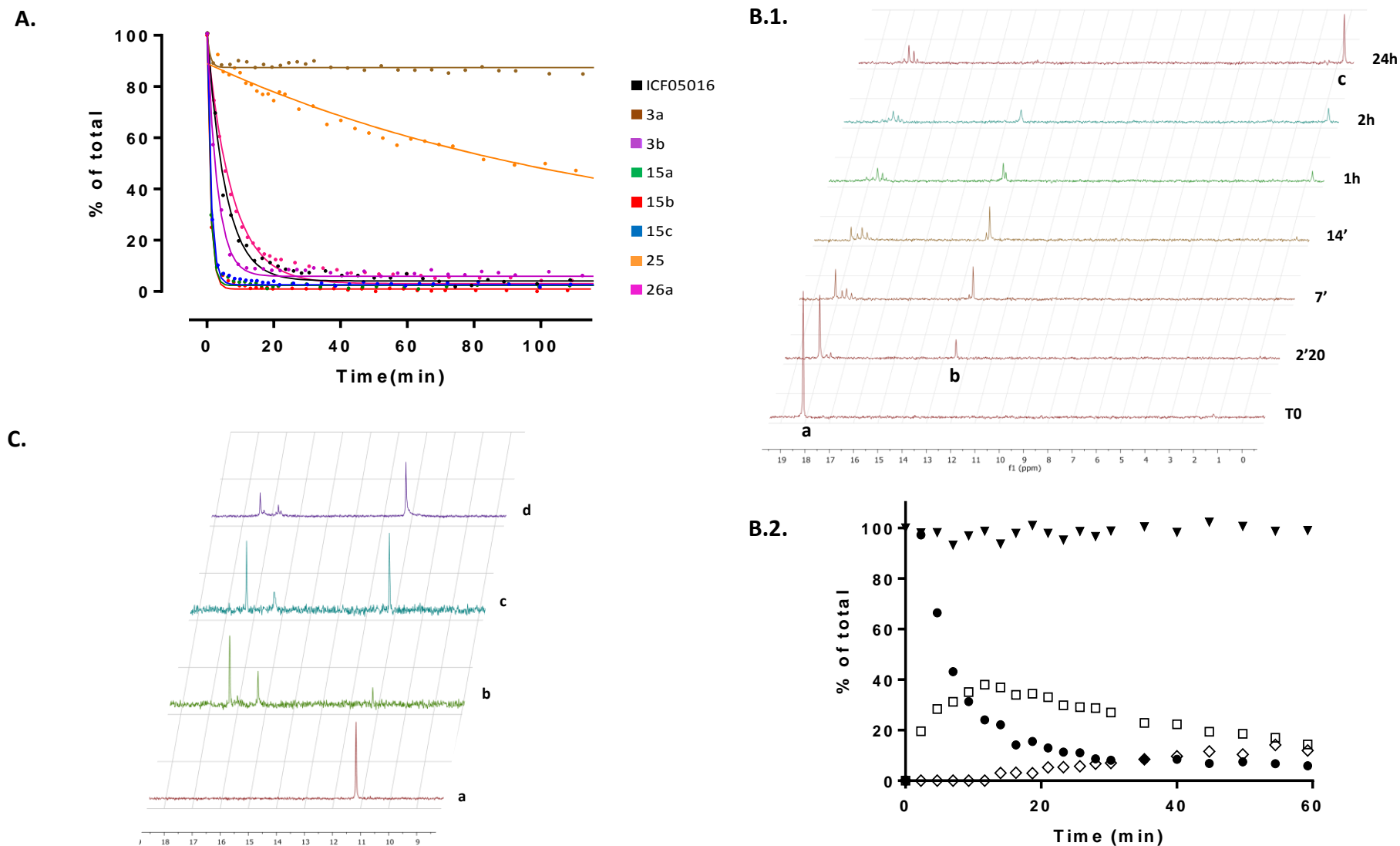


Figure 4: Drug release in reductive conditions (3 eq. sodium dithionite, $\text{CH}_3\text{CN}/0.1$ M cacodylate buffer, pH 7.4, 37°C) assessed by ^{31}P NMR as a function of time: **A. Percent of phosphorodiamidate prodrugs remaining after addition of sodium dithionite in cacodylate buffer; **B.1.** Stacked ^{31}P NMR spectra; a. prodrug **ICF05016**, 18.22 ppm; b. phosphoramidate anion, 12.4 ppm (proven by MS-HPLC, data not shown); c. inorganic phosphate, 0 ppm. Other signals are relative to solvolysis, buffer and reductive adducts or transient unknown by-products. Chemical shifts are reported relative to the Ph_3PO reference; **B.2.** Representative time course of **ICF05016** cleavage (●), substituted bis(2-chloroethyl)phosphorodiamidate anion formation (□) and inorganic phosphate formation (◇) upon incubation of these prodrugs with sodium dithionite in cacodylate buffer; stability of **ICF05016** in cacodylate buffer (▼). Data points were measured from NMR ^{31}P areas (experiments were performed in duplicate); **C.** ^{31}P NMR spectrum of: a. pure synthesized **IPM** (11.2 ppm), b. **27c** reduction medium at 5 min, c. **27c** reduction medium at 5 min spiked with pure synthesized **IPM**, d. **15c** reduction medium at 5 min.**

Compound	Chemical reduction ^[a]		NTR activation ^[b]		Cytotoxicity ^[c]		HCR ^[e]
	Half-live [min]	Amount of substrate after 24 h [%]	Amount of substrate after 15 min [%]	Amount of substrate after 2 h [%]	IC ₅₀ (μM) ^[d]		
					Normoxia	Hypoxia	
ICF05016	4	4 ± 0.5	13 ± 8	6 ± 3	29.3 ± 8.0	4.3 ± 1.2**	6.8[35]
3a	NC	86 ± 1	49 ± 23	30 ± 9	56.9 ± 13.1	29.7 ± 3.8*	1.9
3b	2.5	6 ± 1	2 ± 2	<2	0.9 ± 0.3	0.9 ± 0.3	1.1
15a	< 1	2 ± 0.5	98	96	6.7 ± 0.3	5.2 ± 1.5	1.3
15b	< 1	1 ± 0.5	94 ± 1	75 ± 1	7.2 ± 2.4	7.3 ± 2.5	1.0
15c	< 1	2 ± 0.5	100	97 ± 3	12.9 ± 5.7	3.6 ± 1.5*	3.6
25	90	17 ± 2	60 ± 3	41 ± 2	2.7 ± 0.3	1.6 ± 0.2**	1,7
26a	5	3 ± 1	90 ± 1	69 ± 2	4.4 ± 1.1	3.1 ± 0.2	1.4
26b	-	-	82 ± 3	50 ± 2	10.4 ± 1.8	4.4 ± 2.1**	2.4
26c	-	-	92 ± 5	76 ± 6	6.9 ± 0.9	3.6 ± 1.7*	1.9

Table 1. Chemical reduction, NTR activation and *in vitro* potency on HEMC-SS cells in normoxic (21% O₂) and hypoxic (N₂, O₂ < 0.3%) conditions of HAP derivatives. [a] Chemical reduction and subsequent fragmentation of HAP derivatives (3 eq. sodium dithionite, CH₃CN/0.1 M cacodylate buffer, pH 7.4, 37 °C) assessed by ³¹P NMR as a function of time, Half-life times were determined using GraphpadPrism; [b] Enzymatic reduction and subsequent fragmentation of HAP derivatives (17 μM) by incubation with NADPH (0.9 mM) and NTR (25 μg/mL) in sodium phosphate buffer (10 mM, pH 7.4, 37 °C), calculated from RP-HPLC chromatogram peak areas at 322 nm as a function of time (experiments were performed in triplicate); [c] Cultures were exposed to drugs for 24 h followed by incubation for 48 h in normoxia; [d] IC₅₀ (Growth Inhibiting Concentration 50) values are an average of at least three independent experiments with standard error; [e] HCR: Hypoxic-Cytotoxic Ratio = IC₅₀ in normoxia/IC₅₀ in hypoxia. Notes: significant difference vs. normoxic condition, * p<0.05 and ** p<0.01. NC: not calculable.

2.2.2. Bioreductive activation using nitroreductase enzyme

Nitroaromatic-based HAP are activated through nitroreductases (NTR) that catalyze the reduction of nitro compounds using NAD(P)H. Reduction with NTRs to form nitroso, hydroxylamine and amine metabolites and subsequent release of the mustard can occur *via* two different pathways. Type I NTRs are oxygen-insensitive enzymes and catalyze two electron reduction of the nitro group, while type II NTRs are oxygen-sensitive enzymes and performs single electron reduction. For experimental setting not affected by the environmental oxygen level avoiding the futile-redox cycle, the prodrugs **3a-b**, **15a-c**, **25**, **26a-c** were evaluated, compared to **ICF05016**, as substrate of an oxygen-insensitive I NTR from *E. coli* to verify enzymatic reductive activation. The prodrugs dissolved in PBS (pH 7.4) were incubated with this I NTR and NADPH as cofactor at 37 °C, aliquots were withdrawn at various time points and the biological reduction process was monitored by HPLC (Figure 5 and Table 1). The reduction was estimated on the basis of the disappearance of the prodrugs. In phosphate buffer supplemented with NADPH, no enzyme-free reaction was observed by HPLC analysis. Compared to the lead compound **ICF05016**, **3b** was readily reduced in these conditions with less than 5% of the prodrug being detectable already after 15 min. The bioreductive process *via* NTR was somewhat milder with all other derivatives. As previously noted, 4-nitrophenyl derivative **3a** was the less sensitive to reduction. Moreover, in the *N*-1 series of prodrugs **15a-c** nearly no bioreduction appended, with 75 to 95% of the prodrugs being unchanged after 2 h of reaction. Prodrugs **25** and **26a-c** of the *C*-4 series, revealed to be better substrates of NTR with 40%, 70%, 50% and 80% respectively of the native molecule at 2 h. These results highlighted that *N,N,N*-trimethylpropylamimium tethered to the *N*-1 or *C*-4 position of the imidazole seemed to hamper the enzymatic reduction of the prodrug. Like **ICF05016**, compounds **3b** appeared as the best candidate for hypoxia-activated prodrugs according to this bioreductive assay.

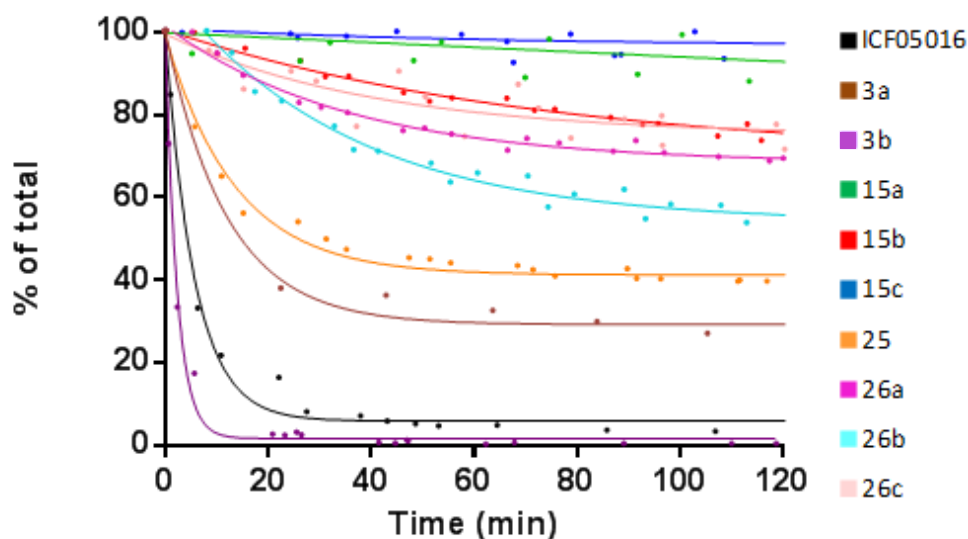


Figure 5: Percent of prodrugs remaining after incubation in phosphate buffer (PBS 1x, 37 °C, pH = 7.4) with nitroreductase in the presence of NADPH; Data points were calculated from RP-HPLC chromatogram peak areas at 322 nm and experiments were performed in triplicate. The lines represent the best-fit values calculated from GraphPadPrism.

2.2.3. Cytotoxicity evaluation in normoxic versus hypoxic conditions

The nine prodrugs were evaluated for their *in vitro* cytotoxicity under normoxic and hypoxic (<0.3% O₂) conditions using human myxoid extraskeletal chondrosarcoma cell line (HEMC-SS). The IC₅₀ values (*i.e.*, Growth Inhibiting Concentration 50: IC₅₀) of the tested compounds were determined and listed in Table 1, in comparison with **ICF05016** as a positive control. For all newly synthesized derivatives except benzyl compound **3a**, the IC₅₀ values ranged between 0.9 and 12.9 μM whatever the oxygenation conditions. The higher selectivity ratio between normoxia and hypoxia (HCR) was observed for compound **15c** (HCR = 3.6), respectively to a value of 6.8 for **ICF05016**. All tested compounds featured moderate selectivity toward hypoxic cells, likely not sufficient for application as hypoxia-activated prodrugs. The limited HCR of these molecules compared to **ICF05016** or evofosfamide (HCR = 23)[36] might be attributed either to partial trigger cleavage under the *in vitro* hypoxic assay conditions, leading to low concentration of the cytotoxic mustard, or conversion to less cytotoxic derivatives, which may cause decreased hypoxia cytotoxicity. Compounds may have be substrates of other enzymes besides oxygen-sensitive reductases even in normoxic conditions, which may cause increased normoxia

cytotoxicity. Although bioreductive assay highlighted compound **3b** as suitable for hypoxia-activated prodrugs, this was not confirmed by cytotoxicity values obtained which were equivalent in normoxic and hypoxic conditions.

In conclusion, a series of seven phosphoramidate-based HAP were prepared and *in vitro* assays demonstrated almost similar cytotoxicities whatever the oxygen conditions used and no efficient enzymatic cleavage in the presence of oxygen-insensitive nitroreductase, except for the furane compound **3b**. These results highlighted that *N,N,N*-trimethylpropylammonium tethered to the *N*-1 or *C*-4 positions of the imidazole seemed to hamper efficient reductive enzymatic activation, essential for the drug release to hypoxic tumor cells. Moreover, in this series no correlation between sensitivity to enzymatic reduction and hypoxic selectivity against H-EMC-SS cells was obtained, as namely observed with compound **3b** and already reported in literature[30,31]. Even if, the enzymatic cleavage experiment allowed obtaining a first *in vitro* result on the sensitivity of molecule to undergo reductive cleavage, this experiment remains far away from the cytotoxicity test, given the variety of different mechanisms underlying the prodrug activity and selectivity in the entire biological environment.

4. Experimental protocols

4.1. Synthesis

All commercially available reagents and solvents were purchased at the following commercial suppliers: Sigma Aldrich (Saint-Quentin Fallavier, France), Acros Organics (Geel, Belgium), Fisher Scientific (Illkirch, France), Carlo Erba Reagents (Val de Reuil, France), VWR (Fontenay-sous-Bois, France) and Alfa Aesar (Karlsruhe, Germany) and were used without further purification. All solvents were dried using common techniques. Air and moisture sensitive reactions were carried out under anhydrous argon atmosphere. Analytical thin-layer chromatography (TLC) was performed on precoated silica gel aluminium plates (60 F₂₅₄, 0.2 mm thick, Macherey or SDS) using the indicated solvent mixture expressed as volume/volume ratios. The plates were visualized with ultraviolet light (254 nm) and (or) by development with ninhydrine ethanolic solution (0.5%) or a 4-(4'-nitrobenzyl)pyridine (NBP)/potassium hydroxide dyeing reagent used for the revelation of alkylating agents (the chromatography plate was immersed in the NBP solution (2.5% in acetone), heated for few min and then immersed in potassium hydroxide solution (10% in methanol)). Column chromatography was performed on silica gel 60A normal phase, 35-70 μm (Merck or SDS or Carlo Erba). Uncorrected melting points (mp) were measured on an electrothermal capillary Digital Melting Point Apparatus (IA9100, Bibby Scientific, Roissy, France). Infrared spectra (IR) were recorded in the range 4000-600 cm^{-1} on a IS10 spectrophotometer with attenuated total reflectance (ATR) accessory Nicolet (Fisher Scientific). Nuclear magnetic resonance spectra (^1H NMR and ^{13}C NMR) were performed on a Bruker AM 200 spectrometer (200 MHz for ^1H , 50 MHz for ^{13}C), a Bruker Avance DPX300 spectrometer (300 MHz for ^1H , 75 MHz for ^{13}C) or a Bruker DRX 500 spectrometer (500 MHz for ^1H , 125 MHz for ^{13}C) (Bruker Biospin SAS, Wissembourg, France). Chemical shift values (δ) are quoted in parts per million (ppm) and calibrated to the deuterated solvent reference peak for ^1H and ^{13}C spectra. ^{31}P NMR spectra (202 MHz) were recorded on a Bruker Avance 500 apparatus with broadband ^1H decoupling and chemical shifts were reported relative to a 1% phosphoric acid solution in deuterium oxide as a coaxial reference (0 ppm). Coupling constants (J) are quoted in Hz. To describe spin multiplicity, standard abbreviations such as s, d, dd, t, q, qt, hept, td, m, br.s referring to singlet, doublet, doublet of doublet, triplet, quartet, quintet, heptuplet, doublet of triplet, multiplet, broad singlet respectively, are used. When necessary, chemical shifts assignments in ^1H and ^{13}C spectra were supported by two dimensional NMR experiments (^1H - ^1H COSY and ^1H - ^{13}C HSQC). Compounds were analyzed by High-Resolution Mass Spectrometry in positive mode (HRMS, Waters[®] Micromass[®] Q-ToF micro[™] Mass Spectrometer, UCA-Partner, Clermont Auvergne University, Clermont-Ferrand, France or MS, Bruker[®] Esquire, Wissembourg, France). Preparative reverse phase high performance liquid chromatography was performed on a CombiflashEZprep (Teledyne ISCO). The purification of QA-derivatives was carried out on a C18 column (Teledyne, Redisep Prep C18, 100 \AA , 5 μm , 200 x 250 mm, flow rate = 15 mL/min, eluent mixture: $\text{H}_2\text{O}/\text{MeCN}$ (v/v), λ = 254 and 323 nm).

Abbreviations: ACN, acetonitrile; DCM, dichloromethane; NBP, 4-(4'-nitrobenzyl)pyridine; NBS, *N*-bromosuccinimide; TBAF, tetrabutylammonium fluoride; TEA, triethylamine; THF, tetrahydrofuran; rt, room temperature.

4-nitrobenzyl *N,N*-bis(2-chloroethyl)-*N'*-[3-(dimethylamino)propyl]phosphorodiamidate (**2a**). To a solution of 4-nitrobenzoic acid (1 g, 6.53 mmol) in anhydrous THF (10 mL) was added dropwise lithium bis(trimethylsilyl)amide (1 M in THF, 7.18 mL, 7.18 mmol) at -78 °C under an inert atmosphere. The reaction mixture was stirred around 5 min at -78 °C, and a solution of bis(2-chloroethyl)phosphoramidic dichloride (**1**) (1.84 g, 7.12 mmol) in anhydrous THF (20 mL) previously cooled at -78 °C, was added all at once at the same temperature (T_0). The reaction mixture was stirred at -78 °C for 30 min, and a solution of 3-(*N,N*-dimethylamino)propylamine (2 eq.) was added. The reaction was completed after an additional 40 min. These reaction times were determined by ^{31}P NMR monitoring for each compound. The reaction was stopped by the addition of water (40 mL) and the reaction mixture was slowly warmed to rt. The aqueous layer was extracted three times with ethyl acetate (3 × 40 mL). The organic layers were combined, dried over magnesium sulfate, filtered and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (EtOAc/EtOH, 50/50, v/v + 2% NH_4OH) to afford compound **2a** (1.64 g, 3.72 mmol) as a yellow-orange oil. **Yield** 57%; **Rf** 0.35 (SiO_2 , EtOAc/EtOH, 50/50, v/v, + 12% NH_4OH); **IR** (ATR) ν cm^{-1} 1522, 1347, 1220, 1046; **^1H NMR** (500 MHz, CD_3OD) δ 8.26 (d, 2H, $^3J = 7.7$ Hz, CH_{Ar}), 7.67 (d, 2H, $^3J = 7.7$ Hz, CH_{Ar}), 5.12 (d, 2H, $^3J_{\text{H-P}} = 7.5$ Hz, OCH_2), 3.63-3.70 (m, 4H, $\text{NCH}_2\text{CH}_2\text{Cl}$), 3.35-3.50 (m, 4H, $\text{NCH}_2\text{CH}_2\text{Cl}$), 2.95 (td, 2H, $^3J_{\text{H,H}} = 6.9$ Hz, $^3J_{\text{H-P}} = 10.8$ Hz, CH_2NHP), 2.37-2.44 (m, 2H, CH_2N), 2.26 (s, 6H, CH_3), 1.71 (qt, 2H, $^3J = 7.3$ Hz, $\text{CH}_2\text{CH}_2\text{CH}_2$); **^{13}C NMR** (50 MHz, $\text{DMSO}-d_6$) δ 145.63 (d, $^3J_{\text{C-P}} = 7.3$ Hz, $\text{C}_{\text{Ar}}\text{CH}_2$), 129.21, 124.70 (4C, CH_{Ar}), 67.06 (d, $^2J_{\text{C-P}} = 4.4$ Hz, OCH_2), 57.39 (CH_2NH^+), 50.23 (d, $^2J_{\text{C-P}} = 4.6$ Hz, $\text{NCH}_2\text{CH}_2\text{Cl}$), 44.45 (2C, $\text{N}(\text{CH}_3)_2$), 43.10 (d, $^3J_{\text{C-P}} = 1.4$ Hz, $\text{NCH}_2\text{CH}_2\text{Cl}$), 39.50 (CH_2NHP), 29.10 (d, $^3J = 5.2$ Hz, $\text{CH}_2\text{CH}_2\text{CH}_2$); **^{31}P NMR** (202 MHz, CD_3OD) δ 18.30.

(5-nitrofuran-2-yl)methyl *N,N*-bis(2-chloroethyl)-*N'*-[3-(dimethylamino)propyl]phosphorodiamidate (**2b**) was prepared from alcohol 5-nitro-2-hydroxymethylfuran (**4**) (526 mg, 3.68 mmol) as described for the preparation of **2a** (after addition of 3-(*N,N*-dimethylamino)propylamine, the reaction was stirred for an additional 2h). The crude product was purified by silica gel column chromatography (EtOAc/EtOH, 50/50, v/v with NH_4OH ranging from 0 to 5%) to afford compound **2b** (720 mg, 1.67 mmol) as a yellow-orange oil. **Yield** 48%; **Rf** 0.35 (SiO_2 , EtOAc/EtOH, 50/50, v/v, + 5% NH_4OH); **IR** (ATR) ν cm^{-1} 1504, 1352, 1241, 1034; **^1H NMR** (500 MHz, $\text{DMSO}-d_6$) δ 7.69 (d, 1H, $^3J = 3.7$ Hz, CH), 6.92 (d, 1H, $^3J = 3.7$ Hz, CH), 4.96 (d, 2H, $^3J_{\text{H-P}} = 8.7$ Hz, OCH_2), 4.88 (td, 1H, $^3J_{\text{H,H}} = 6.7$ Hz, $^2J_{\text{H-P}} = 11.7$ Hz, CH_2NHP), 3.61-3.69 (m, 4H, $\text{NCH}_2\text{CH}_2\text{Cl}$), 3.20-3.36 (m, 4H, $\text{NCH}_2\text{CH}_2\text{Cl}$), 2.75 (qd, 2H, $^3J_{\text{H-P}} = 11.0$ Hz, $^3J_{\text{H,H}} = 6.7$ Hz, CH_2NHP), 2.24 (t, 2H, $^3J = 6.8$ Hz, CH_2N), 2.12 (s, 6H, $\text{N}(\text{CH}_3)_2$), 1.53 (qt, 2H, $^3J = 6.9$ Hz, $\text{CH}_2\text{CH}_2\text{CH}_2$); **^{13}C NMR** (50 MHz, $\text{DMSO}-d_6$) δ 154.26 (d, $^3J_{\text{C-P}} = 8.1$ Hz, $\text{C}_{\text{Ar}}\text{CH}_2$), 151.58 ($\text{C}_{\text{Ar}}\text{NO}_2$), 113.69, 113.59 (CH_{Ar}), 58.14 (d, $^2J_{\text{C-P}} = 3.8$ Hz, OCH_2), 54.30 (CH_2NH^+), 48.34 (d, $^2J_{\text{C-P}} = 4.3$ Hz, $\text{NCH}_2\text{CH}_2\text{Cl}$), 42.35 (d, $^3J_{\text{C-P}} = 1.5$ Hz, $\text{NCH}_2\text{CH}_2\text{Cl}$), 41.99 (2C, $\text{N}(\text{CH}_3)_2$), 37.37

($\underline{\text{C}}\text{H}_2\text{NHP}$), 25,94 (d, $^3J_{\text{C-P}} = 5,2$ Hz, $\text{CH}_2\underline{\text{C}}\text{H}_2\text{CH}_2$); ^{31}P NMR (202 MHz, CD_3OD) δ 17.79; MS (ESI) m/z 431.18 [$\text{M}+\text{H}$] $^+$ (calculated for $[\text{C}_{14}\text{H}_{25}\text{Cl}_2\text{N}_4\text{O}_5\text{P}]$ 431,10).

3-((4-nitrobenzyloxy)[bis(2-chloroethyl)amino]phosphoryl)amino)-N,N,N-trimethylpropane-1-aminium iodide (3a). To a solution of amine **2a** (1.34 g, 3.03 mmol) in anhydrous THF (45 mL) under an inert atmosphere, was added methyl iodide (1 mL, 16.1 mmol). Stirring was maintained at rt in a sealed flask for 3 h. After evaporation to dryness under reduced pressure, compound **3a** was obtained analytically pure, as a yellow hygroscopic solid (1.76 g, 3.02 mmol). Yield 99%; IR (ATR) ν cm^{-1} 1520, 1348, 1221, 1046; ^1H NMR (500 MHz, CD_3OD) δ 8.27 (d, 2H, $^3J = 8.7$ Hz, $\underline{\text{C}}\text{H}_{\text{Ar}}$), 7.68 (d, 2H, $^3J = 8.6$ Hz, $\underline{\text{C}}\text{H}_{\text{Ar}}$), 5.17 (d, 2H, $^3J_{\text{H-P}} = 7.4$ Hz, OCH_2), 3.68-3.72 (m, 4H, $\text{NCH}_2\underline{\text{C}}\text{H}_2\text{Cl}$), 3.41-3.50 (m, 6H, $\text{NCH}_2\underline{\text{C}}\text{H}_2\text{Cl}$, CH_2N^+), 3.17 (s, 9H, $\text{CH}_2\text{N}^+(\underline{\text{C}}\text{H}_3)_3$), 3.05 (td, 2H, $^3J_{\text{H,H}} = 6.4$ Hz, $^3J_{\text{H-P}} = 12.0$ Hz, CH_2NHP), 1.97-2.07 (m, 2H, $\text{CH}_2\underline{\text{C}}\text{H}_2\text{CH}_2$); ^{13}C NMR (126 MHz, CD_3OD) δ 149.14 ($\underline{\text{C}}_{\text{Ar}}\text{NO}_2$), 145.55 (d, $^3J_{\text{C-P}} = 7.7$ Hz, $\underline{\text{C}}_{\text{Ar}}\text{CH}_2$), 129.28, 124.71 (4C, CH_{Ar}), 67.16 (d, $^2J_{\text{C-P}} = 4.7$ Hz, OCH_2), 65.83, 65.80, 65.78 ($\text{CH}_2\text{N}^+(\underline{\text{C}}\text{H}_3)_3$), 53.81, 53.78, 53.75 ($\text{CH}_2\text{N}^+(\underline{\text{C}}\text{H}_3)_3$), 50.16 (d, 2C, $^2J_{\text{C-P}} = 4.7$ Hz, $\text{N}(\underline{\text{C}}\text{H}_2\underline{\text{C}}\text{H}_2\text{Cl})_2$), 43.16 (d, 2C, $^3J_{\text{C-P}} = 1.7$ Hz, $\text{N}(\text{CH}_2\underline{\text{C}}\text{H}_2\text{Cl})_2$), 38.79 ($\underline{\text{C}}\text{H}_2\text{NHP}$), 26.35 (d, $^3J_{\text{C-P}} = 4.7$ Hz, $\text{CH}_2\underline{\text{C}}\text{H}_2\text{CH}_2$); ^{31}P NMR (202 MHz, CD_3OD) δ 18.10; HRMS (ESI) m/z 455.24 [M] $^+$ (calculated for $[\text{C}_{17}\text{H}_{30}\text{Cl}_2\text{N}_4\text{O}_4\text{P}]^+$ 455.14).

3-((bis(2-chloroethyl)amino)[(5-nitrofuran-2-yl)methoxy]phosphoryl)amino)-N,N,N-trimethylpropane-1-aminium iodide (3b) was prepared from amine **2b** (115 mg, 0.267 mmol) as described for the preparation of **3a** (reaction time: 12 h) to afford compound analytically pure, as a yellow hygroscopic solid (124 mg, 0.216 mmol). Yield 81%; IR (ATR) ν cm^{-1} 3444, 1500, 1353, 1242, 1218, 1020; ^1H NMR (200 MHz, CD_3OD) δ 7.46 (d, 2H, $^3J = 3.7$ Hz, CH_{Ar}), 6.85 (d, 2H, $^3J = 3.7$ Hz, CH_{Ar}), 5.09 (d, 2H, $^3J_{\text{H-P}} = 8.9$ Hz, OCH_2), 3.65-3.72 (m, 4H, $\text{NCH}_2\underline{\text{C}}\text{H}_2\text{Cl}$), 3.35-3.53 (m, 6H, $\text{NCH}_2\underline{\text{C}}\text{H}_2\text{Cl}$, CH_2N^+), 3.18 (s, 9H, $\text{CH}_2\text{N}^+(\underline{\text{C}}\text{H}_3)_3$), 3.04 (td, 2H, $^3J_{\text{H,H}} = 6.3$ Hz, $^3J_{\text{H-P}} = 12.5$ Hz, CH_2NHP), 2.03-2.10 (m, 2H, $\text{CH}_2\underline{\text{C}}\text{H}_2\text{CH}_2$); ^{13}C NMR (50 MHz, CD_3OD) δ 154,70 (d, $^3J = 7.2$ Hz, $\text{C}_{\text{Ar}}\text{CH}_2$), 153.75 ($\underline{\text{C}}_{\text{Ar}}\text{NO}_2$), 114.38, 113.31 (CH_{Ar}), 65.85, 65.82, 65.80 ($\text{CH}_2\text{N}^+(\underline{\text{C}}\text{H}_3)_3$), 60.14 (d, $^2J_{\text{C-P}} = 4.6$ Hz, OCH_2), 53.85, 53.82, 53.78 ($\text{N}^+(\underline{\text{C}}\text{H}_3)_3$), 50.11 (d, $^2J_{\text{C-P}} = 4.6$ Hz, $\text{NCH}_2\underline{\text{C}}\text{H}_2\text{Cl}$), 43.07 (d, $^3J_{\text{C-P}} = 1.4$ Hz, $\text{NCH}_2\underline{\text{C}}\text{H}_2\text{Cl}$), 38.82 ($\underline{\text{C}}\text{H}_2\text{NHP}$), 26.31 (d, $^3J_{\text{C-P}} = 4.5$ Hz, $\text{CH}_2\underline{\text{C}}\text{H}_2\text{CH}_2$); ^{31}P NMR (202 MHz, CD_3OD) δ 17,97; MS (ESI) m/z 445.21 [M] $^+$ (calculated for $[\text{C}_{15}\text{H}_{28}\text{Cl}_2\text{N}_4\text{O}_5\text{P}]^+$ 445.12).

Ethyl N-[3-(N',N'-dimethylamino)propyl]glycinate (6). To a solution at 0 °C of 3-(N,N-dimethylamino)propylamine (60 mL, 477 mmol) and potassium carbonate (54.5 g, 394 mmol) in toluene (260 mL) was added dropwise a solution of ethyl bromoacetate (26 mL, 234 mmol) in toluene (260 mL) for 3 h 30. The resulting solution was allowed to stirred at rt for 18 h. The reaction mixture was filtrated and the solid was washed with toluene (3 \times 200 mL). The filtrate was evaporated under reduced pressure to afford a slightly yellow liquid. The residue was purified by silica gel column chromatography (eluent: EtOAc/EtOH, 80/20, v/v + 5% TEA) to yield the secondary amine **6** (23.9 g, 127 mmol). Yield 54%; Rf 0.71 (SiO_2 , EtOAc/EtOH, 80/20,

v/v + 5% TEA); **IR (ATR)** ν cm^{-1} 3336, 1737, 1191; **$^1\text{H NMR}$ (200 MHz, CDCl_3)** δ 4.15 (q, 2H, $^3J = 7.1$ Hz, OCH_2), 3.36 (s, 2H, CH_2CO), 2.62 (t, 2H, $^3J = 7.1$ Hz, NHCH_2CH_2), 2.30 (t, 2H, $^3J = 7.2$ Hz, $(\text{CH}_3)_2\text{NCH}_2$), 2.19 (s, 6H, $\text{N}(\text{CH}_3)_2$), 1.98 (s, 1H, NH), 1.64 (qt, $^3J = 7.1$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 1.24 (t, 3H, $^3J = 7.1$ Hz, CH_2CH_3); **$^{13}\text{C NMR}$ (50 MHz, CDCl_3)** δ 172.55 (CO_2), 60.74 (OCH_2), 57.90 ($\text{CH}_2\text{N}(\text{CH}_3)_2$), 51.07 (NHCH_2CO), 48.11 (NHCH_2CH_2), 45.55 (2C, $\text{N}(\text{CH}_3)_2$), 28.06 ($\text{CH}_2\text{CH}_2\text{CH}_2$), 14.29 (CH_2CH_3).

N-formyl-*N*-[3-(*N,N'*-dimethylamino)propyl]glycinate (**7**). To a stirred solution of the diamino ester **6** (24.2 g, 129 mmol) in ethanol (86 mL) were added potassium carbonate (17.7 g, 128 mmol) and ethyl formate (77 mL, 957 mmol). The reaction mixture was stirred for 25 h at rt. The resulting mixture was filtrated and the solid was washed with ethanol (3 \times 50 mL). The filtrate was evaporated under reduced pressure, before addition of water (100 mL) and extraction with ethyl acetate (3 \times 200 mL). The aqueous layer was saturated with sodium chloride and extracted with ethyl acetate (3 \times 200 mL). The organic layers were combined, dried over magnesium sulfate, filtrated and evaporated under reduced pressure to yield the formylated product **7** (25.7 g, 119 mmol) analytically pure as a yellowish liquid. **Yield** 90%; **Rf** 0.85 (Al_2O_3 , EtOAc/EtOH, 80/20, v/v + 0.1% NH_4OH); **IR (ATR)** ν cm^{-1} 1745, 1668, 1196; **$^1\text{H NMR}$ (200 MHz, CDCl_3)** δ (two rotamers) 8.14 and 8.03 (s, 1H, CHO), 4.27-4.10 (m, 2H, OCH_2), 4.04 and 3.98 (s, 2H, NCH_2CO), 3.27-3.08 (t, 2H, $^3J = 6.9$ Hz, $\text{NCHOCH}_2\text{CH}_2$), 2.29-2.16 (m, 8H, $(\text{CH}_2\text{N}(\text{CH}_3)_2)$), 1.77-1.56 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 1.27 and 1.26 (t, 3H, $^3J = 7.1$ Hz, CH_2CH_3); **$^{13}\text{C NMR}$ (50 MHz, CDCl_3)** δ (two rotamers) 168.99, 168.20 (CO_2), 163.00 (CHO), 61.20, 60.87 (OCH_2), 56.27, 55.33 ($\text{CH}_2\text{N}(\text{CH}_3)_2$), 48.79, 43.73 (NCH_2CO), 45.55, 41.70 ($\text{NCHOCH}_2\text{CH}_2$), 44.90 (2C, $\text{N}(\text{CH}_3)_2$), 25.65, 24.81 ($\text{CH}_2\text{CH}_2\text{CH}_2$), 13.73 (CH_2CH_3); **MS** (ESI) m/z 217.14 [$\text{M}+\text{H}$]⁺ (calculated for $[\text{C}_{10}\text{H}_{21}\text{N}_2\text{O}_3]^+$ 217.15)

Ethyl 2-amino-1-N-(3-(*N,N'*-dimethylamino)propyl)-1*H*-imidazole-5-carboxylate (**8**). To a solution of *N*-formyl compound **7** (19.2 g, 88.8 mmol) in an equal mixture of ethyl formate and THF (90 mL) containing cyclohexane (5 mL), was added slowly NaH (60% wt in mineral oil, 5.33 g, 133 mmol) at rt (the reaction was carefully activated by heat until hydrogen gas evolution and color change were observed. At this point, the reaction mixture was cooled down with a water/ice bath). After the addition was completed and hydrogen gas release ceased, the reaction mixture was allowed to stirred at rt for 3 h. The reaction mixture was concentrated under reduced pressure. The obtained solid was suspended in a solution of EtOH (145 mL) containing concentrated aq. HCl 37% (29 mL, 353 mmol) and refluxed for 2 h. The hot reaction mixture was filtered and the resulting white solid was washed with boiling EtOH (3 \times 50 mL). The filtrate was concentrated under vacuum and diluted with a mixture of EtOH/water (170 mL, 70/30, v/v). The pH of the solution was adjusted to 3, using an aqueous 5M solution of NaOH and cyanamide (7.46 g, 177 mmol) was added. The resulting mixture was refluxed for 1 h 30, then cooled to rt and concentrated under reduced pressure to approximately 1/8 of the initial volume. After cooling at 0 $^\circ\text{C}$, the pH of the remaining solution was adjusted to 9-10 with a saturated aqueous solution of potassium carbonate. The mixture was extracted with

ethyl acetate (3 x 100 mL). The organic layers was combined, dried over magnesium sulfate, filtrated and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (eluent: EtOAc/EtOH, 80/20, v/v with 2% TEA) to give the imidazole **8** (4.52 g, 18.8 mmol) as a brown solid. **Yield** 21%; **Rf** 0.36 (SiO₂, EtOAc/EtOH, 80/20, v/v + 2% TEA); **mp** 82-83 °C; **IR (ATR)** ν cm⁻¹ 3317, 3194, 1667, 1539, 1182; **¹H NMR (500 MHz, CD₃OD)** δ 7.35 (s, 1H, H_{Ar}), 4.24 (q, 2H, ³J = 7.1 Hz, OCH₂), 4.11 (t, 2H, ³J = 6.9 Hz, N_{Ar}CH₂), 2.31 (t, 2H, ³J = 6.9 Hz, CH₂N(CH₃)₂), 2.26 (s, 6H, N(CH₃)₂), 1.93 (qt, 2H, ³J = 6.9 Hz, CH₂CH₂CH₂), 1.31 (t, 3H, ³J = 7.1 Hz, CH₂CH₃); **¹³C NMR (126 MHz, CD₃OD)** δ 161.71 (CO₂), 155.38 (C_{Ar}NH₂), 136.40 (CH_{Ar}), 118.84 (C_{Ar}CO), 60.85 (OCH₂), 56.37 (CH₂N(CH₃)₂), 45.08 (2C, N(CH₃)₂), 42.11 (N_{Ar}CH₂), 28.22 (CH₂CH₂CH₂), 14.69 (CH₂CH₃); **HRMS (ESI)** m/z 241.1658 [M+H]⁺ (calculated for [C₁₁H₂₁N₄O₂]⁺ 241.1659)

Ethyl 1-N-[3-(N',N'-dimethylamino)prop-1-enyl]-2-nitro-1H-imidazole-5-carboxylate (9). To a solution of sodium nitrite (13.0 g, 188 mmol) in water (17 mL) cooled around 0 °C, was added dropwise a solution of the amino ester **8** (4.52 g, 18.8 mmol) in glacial acetic acid (33 mL). The reaction mixture was allowed to stir at rt for 2 h. The solution was cooled at 0 °C and a saturated aqueous solution of sodium carbonate (90 mL) was added dropwise until pH 9-10. The reaction mixture was extracted with ethyl acetate (3 x 200 mL), saturated with sodium chloride and extracted with ethyl acetate (2 x 200 mL). The combined organic layers were dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: EtOAc/EtOH, 90/10, v/v + 1% TEA). The product **9** was obtained as a yellow oil (3.63 g, 13.4 mmol). **Yield** 71%; **Rf** 0.55 (SiO₂, EtOAc/EtOH, 90/10, v/v + 1% TEA); **IR (ATR)** ν cm⁻¹ 1722, 1473, 1334, 1283, 1129; **¹H NMR (500 MHz, DMSO-*d*₆)** δ 7.78 (s, 1H, H_{Ar}), 4.72-4.66 (m, 2H, N_{Ar}CH₂), 4.35 (q, ³J = 7.1 Hz, 2H, OCH₂), 2.24 (t, ³J = 6.5 Hz, 2H, CH₂N(CH₃)₂), 2.06 (s, 6H, N(CH₃)₂), 1.96-1.89 (m, 2H, CH₂CH₂CH₂), 1.32 (t, ³J = 7.1 Hz, 3H, CH₂CH₃); **¹³C NMR (126 MHz, DMSO-*d*₆)** δ 158.48 (CO₂), 147.37 (C_{Ar}NO₂), 133.91 (CH_{Ar}), 125.91 (C_{Ar}CO), 61.37 (OCH₂), 56.18 (CH₂N(CH₃)₂), 46.42 (N_{Ar}CH₂), 44.69 (2C, N(CH₃)₂), 27.16 (CH₂CH₂CH₂), 13.91 (CH₂CH₃); **HRMS (ESI)** m/z 271.1400 [M+H]⁺ (calculated for [C₁₁H₁₉N₄O₄]⁺ 271.1401).

1-[1-N-[3-(N',N'-dimethylamino)propyl]-2-nitro-1H-imidazo-5-yl]methanol (10). The ester **9** (3.63 g, 13.4 mmol) was dissolved in a mixture of anhydrous THF/ MeOH (32 mL, 80/20, v/v) cooled at 0 °C. A suspension of NaBH₄ (1.52 g, 40.2 mmol) in THF (75 mL) and a solution of LiBr (3.51 g, 40.6 mmol) in water (20 mL) were cooled at 0 °C, introduced in the same dropping funnel and added dropwise to the nitroimidazole solution, at such a rate that the internal temperature did not exceed 10 °C. The temperature was allowed to rise gradually to rt and the reaction mixture was stirred for 17 h. The reaction mixture was cooled at 0 °C and a saturated aqueous solution of ammonium chloride (40 mL) was added. After the evaporation of the THF under reduced pressure, the pH of the resulting solution was adjusted to 9-10 by the addition of a saturated aqueous solution of sodium carbonate (40 mL) and saturated with brine. The solution was then extracted with ethyl acetate (3 x 200 mL) maintaining the pH of the solution at 10. The combined organic layers were dried over magnesium sulfate, filtered

and evaporated under reduced pressure. The residue was diluted in ethanol (180 mL) and the resulting solution was refluxed for 4 h. After cooling down to rt, ethanol was evaporated under reduced pressure and the residue was purified by silica gel column chromatography (eluent: DCM/methanol, 95/5, v/v + 5% TEA) to yield the alcohol **10** (1.49, 6.53 mmol) as an orange oil. **Yield** 49%; **Rf** 0.18 (SiO₂, DCM/MeOH, 95/5, v/v + 5% TEA); **IR (ATR)** ν cm⁻¹ 3239, 1481, 1335, 1031; **¹H NMR (200 MHz, CDCl₃)** δ 7.03 (s, 1H, H_{Ar}), 5.29 (br.s, 1H, CH₂OH), 4.58 (s, 2H, CH₂OH), 4.46 (t, 2H, ³J = 6.8 Hz, N_{Ar}CH₂), 2.32 (t, 2H, ³J = 6.3 Hz, CH₂N(CH₃)₂), 2.19 (s, 6H, N(CH₃)₂), 2.09 (qt, 2H, ³J = 6.5 Hz, CH₂CH₂CH₂); **¹³C NMR (126 MHz, CD₃OD)** δ 146.72 (C_{Ar}NO₂), 139.33 (C_{Ar}CH₂), 127.92 (CH_{Ar}), 57.19 (CH₂N(CH₃)₂), 54.58 (CH₂OH), 46.55 (N_{Ar}CH₂), 45.18 (2C, N(CH₃)₂), 28.57 (CH₂CH₂CH₂); **HRMS (ESI)** m/z 229.1293 [M+H]⁺ (calculated for [C₉H₁₇N₄O₃]⁺ 229.1295).

3-[(5-hydroxymethyl)-2-nitro-1H-imidazo-1-N'-yl]-N,N-dimethyl-propyl-1-amine borane complex (10-BH₃). The ester **9** (3.35 g, 12.3 mmol) was dissolved in a mixture of THF/ MeOH (30 mL, 80/20, v/v) cooled at 0 °C. A suspension of NaBH₄ (1.36 g, 36.9 mmol) and LiBr (3.18 g, 36.8 mmol) in anhydrous THF (70 mL) was cooled at 0 °C, water (20 mL) was added before dropwise addition to the nitroimidazole solution, at such a rate that the internal temperature did not exceed 10 °C. The reaction mixture was stirred at rt for 15 h. After cooling down to 0 °C, a saturated aqueous solution of ammonium chloride (15 mL) was added to the reaction mixture. The pH of the resulting solution was adjusted to 9-10 by the addition of a saturated aqueous solution of sodium carbonate and saturated with sodium chloride. The solution was then extracted with ethyl acetate (6 x 150 mL) maintaining the pH of the solution at 10. The combined organic layers were dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: DCM/methanol, 97/3, v/v) to yield the alcohol **10** (209 mg, 0.916 mmol, 7%) and its borane complex **10-BH₃** (1.35 g, 5.58 mmol, 45%) as main product. **Rf** 0.75 (SiO₂, DCM/MeOH, 90/10, v/v + 1% TEA); mp 104-105 °C; **IR (ATR)** ν cm⁻¹ 3216, 2386, 2363, 2314, 2271, 1489, 1333, 1166, 1036; **¹H NMR (500 MHz, CDCl₃)** δ 7.11 (s, 1H, H_{Ar}), 4.76 (m, 2H, CH₂OH), 4.57-4.39 (m, 2H, N_{Ar}CH₂), 2.94-2.80 (m, 2H, CH₂N⁺(CH₃)₂), 2.61 (s, 6H, N⁺(CH₃)₂), 2.44-2.24 (m, 2H, CH₂CH₂CH₂); **¹³C NMR (126 MHz, CDCl₃)** δ 145.70 (C_{Ar}NO₂), 136.53 (C_{Ar}CH₂), 128.17 (CH_{Ar}), 61.58 (CH₂N⁺(CH₃)₂), 54.65 (CH₂OH), 52.11 (2C, N⁺(CH₃)₂), 45.69 (N_{Ar}CH₂), 25.78 (CH₂CH₂CH₂); **HRMS (ESI)** m/z 243.1616 [M+H]⁺ (calculated for [C₉H₂₀BN₄O₃]⁺ 243.1623), 265.1434 [M+Na]⁺ (calculated for [C₉H₁₉BN₄NaO₃]⁺ 265.1442).

4-{5-[(amino[bis(2-chloroethyl)amino]phosphoryl)oxy)methyl]-2-nitro-1H-imidazol-1-N'-yl}-N,N-dimethylpropan-1-amine borane complex (14a-BH₃) was prepared from alcohol **10-BH₃** (165 mg, 0.682 mmol) as described for the preparation of **2a** with some differences: the coupling reaction with bis(2-chloroethyl)phosphoramidic acid dichloride (**1**) was performed for 1 h before ammonia gas was bubbled through the reaction for 2 min. The reaction was directly stopped by addition of water (10 mL). The crude product was purified by silica gel column chromatography (eluent: DCM/ethanol, 97/3, v/v) to yield compound **14a-BH₃** (282

mg, 0.634 mmol) as a beige thick oil. **Yield** 93%; **Rf** 0.20 (SiO₂, DCM/EtOH, 97/3, v/v); **IR (ATR)** ν cm⁻¹ 3241, 2380, 2318, 2273, 1484, 1338, 1224, 1168, 1012, 971; **¹H NMR (500 MHz, CDCl₃)** δ 7.25 (s, 1H, H_{Ar}), 5.15 (dd, 1H, ²J_{H-H} = 13.2 Hz, ³J_{H-P} = 7.0 Hz, OCH'), 5.02 (dd, 1H, ²J_{H-H} = 13.2 Hz, ³J_{H-P} = 5.9 Hz, OCH''), 4.51 (ddd, 1H, ²J = 13.6 Hz, ³J = 10.8 Hz, ³J = 5.4 Hz, N_{Ar}CH'), 4.41 (ddd, 1H, ²J = 13.6 Hz, ³J = 10.7 Hz, ³J = 5.5 Hz, N_{Ar}CH''), 3.70-3.65 (m, 4H, N(CH₂CH₂Cl)₂), 3.58-3.44 (m, 4H, N(CH₂CH₂Cl)₂), 3.11 (br.s, 2H, NH₂), 2.91 (ddd, 1H, ²J = ³J = 11.8 Hz, ³J = 5.0 Hz, CH'₂N⁺(CH₃)₂), 2.81 (ddd, 1H, ²J = ³J = 11.8 Hz, ³J = 5.1 Hz, CH''₂N⁺(CH₃)₂), 2.65 and 2.61 (s, 3H, N⁺(CH₃)₂), 2.48-2.38 (m, 1H, CH₂CH'₂CH₂), 2.38-2.27 (m, 1H, CH₂CH''₂CH₂), 1.97-1.30 (m, 3H, BH₃); **¹³C NMR (126 MHz, CDCl₃)** δ 145.86 (C_{Ar}NO₂), 132.74 (d, ³J_{C-P} = 9.1 Hz, C_{Ar}CH₂), 129.78 (CH_{Ar}), 61.50 (CH₂N⁺(CH₃)₂), 55.97 (d, ²J_{C-P} = 3.6 Hz, OCH₂), 53.18, 51.86 (2C, N⁺(CH₃)₂), 48.94 (d, 2C, ²J_{C-P} = 4.9 Hz, N(CH₂CH₂Cl)₂), 45.46 (N_{Ar}CH₂), 42.70 (2C, N(CH₂CH₂Cl)₂), 26.12 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CDCl₃)** δ 16.47; **HRMS (ESI)** m/z 443.1296 [M-H]⁺ (calculated for [C₁₃H₂₇BCl₂N₆O₄P]⁺ 443.1296), 467.1272 [M+Na]⁺ (calculated for [C₁₃H₂₈BCl₂N₆NaO₄P]⁺ 467.1272).

3-{5-[(bis[bis(2-chloroethyl)amino]phosphoryl)oxy)methyl]-2-nitro-1H-imidazol-1-N'-yl}-N,N-dimethylpropan-1-amine borane complex (14b-BH₃). To a stirred solution of the alcohol **10-BH₃** (150 mg, 0.620 mmol) in anhydrous THF (4.5 mL) cooled at -78°C was added dropwise lithium bis(trimethylsilyl)amide (1M in THF, 680 μ L, 0.680 mmol) under an inert atmosphere. After 5 min, a solution of the compound **12** (306 mg, 0.840 mmol) in anhydrous THF (4 mL) previously cooled at -78°C was added dropwise to the reaction mixture. The kinetic of the reaction was monitored by ³¹P NMR. After stirring 1 h at -78 °C, the reaction mixture was warmed to rt and allowed to stir for 20 h. The reaction was stopped by addition of water (10 mL) and the reaction mixture was extracted with ethyl acetate (3 \times 10 mL). The organic layers were combined, dried over magnesium sulfate, filtrated and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (eluent: DCM/methanol, 98/2, v/v) to yield phosphorodiamidate **14b-BH₃** (264 mg; 0.463 mmol) as a colorless thick oil. **Yield** 70%; **Rf** 0.28 (SiO₂, DCM/MeOH, 98/2, v/v); **IR (ATR)** ν cm⁻¹ 2380, 2319, 2273, 1483, 1339, 1219, 1169, 1004, 964; **¹H NMR (500 MHz, CDCl₃)** δ 7.28 (s, 1H, H_{Ar}), 5.17 (d, 2H, ³J_{H-P} = 8.1 Hz, OCH₂), 4.54-4.47 (m, 2H, N_{Ar}CH₂), 3.71-3.56 (m, 8H, N(CH₂CH₂Cl)₂), 3.41 (td, 8H, ³J_{H-P} = 12.9 Hz, ³J_{H-H} = 6.5 Hz, N(CH₂CH₂Cl)₂), 2.87-2.81 (m, 2H, CH₂N⁺(CH₃)₂), 2.61 (s, 6H, N⁺(CH₃)₂), 2.34-2.25 (m, 2H, CH₂CH₂CH₂), 2.00-1.30 (m, 3H, BH₃); **¹³C NMR (126 MHz, CDCl₃)** δ 145.87 (C_{Ar}NO₂), 132.32 (d, ³J_{C-P} = 7.0 Hz, C_{Ar}CH₂), 130.09 (CH_{Ar}), 61.47 (CH₂N⁺(CH₃)₂), 56.38 (d, ²J_{C-P} = 3.8 Hz, OCH₂), 52.47 (2C, N⁺(CH₃)₂), 49.31 (d, 4C, ²J_{C-P} = 4.6 Hz, N(CH₂CH₂Cl)₂), 45.49 (N_{Ar}CH₂), 42.34 (4C, N(CH₂CH₂Cl)₂), 26.18 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CDCl₃)** δ 17,14; **HRMS (ESI)** m/z 593,1088 [M+Na]⁺ (calculated for [C₁₇H₃₄BCl₄N₆NaO₄P]⁺ 593,1089).

3-{5-[(bis[(2-chloroethyl)amino]phosphoryl)oxy)methyl]-2-nitro-1H-imidazol-1-N'-yl}-N,N-dimethylpropan-1-amine borane complex (14c-BH₃). To a solution cooled at -78 °C of phosphorous oxychloride (200 μ L, 2.15 mmol) in anhydrous DCM (5 mL), was added dropwise a solution of the compound **10-BH₃** (500 mg, 2.07 mmol) and TEA (320 μ L, 2.30 mmol) in anhydrous DCM (4 mL). After 1 h of stirring at -78 °C, 2-chloroethylamine hydrochloride (501

mg, 4.35 mmol) was added followed by the dropwise addition of TEA (1.3 mL, 9.33 mmol). The temperature of the reaction mixture was risen gradually from -78 °C to 5 °C for 18 h. The reaction was stopped by the addition of water (30 mL). After layers separation, the organic layer was washed with a saturated aqueous solution of sodium hydrogenocarbonate (30 mL), dried over magnesium sulfate, filtrated and evaporated under reduced pressure to give a pale yellow oil. The residue was purified by silica gel column chromatography (eluent: EtOAc/EtOH, 95/5 then 90/10, v/v) to yield the isophosphorodiamidate **14c-BH₃** (445 mg, 1.00 mmol) as a beige thick oil. **Yield** 48%; **Rf** 0.15 (SiO₂, AcOEt/EtOH, 95/5, v/v); **IR (ATR)** ν cm⁻¹ 3237, 2381, 2318, 2273, 1484, 1338, 1209, 1168, 1008, 972; **¹H NMR (200 MHz, CDCl₃)** δ 7.24 (s, 1H, H_{Ar}), 5.07 (d, 2H, ³J_{H-P} = 7.0 Hz, OCH₂), 4.48-4.42 (m, 2H, N_{Ar}CH₂), 3.61-3.55 (m, 2H, NHCH₂CH₂Cl), 3.45-3.34 (m, 2H, NH), 3.34-3.22 (m, 4H, NHCH₂CH₂Cl), 2.88-2.81 (m, 2H, CH₂N⁺(CH₃)₂), 2.62 (s, 6H, N⁺(CH₃)₂), 2.42-2.32 (m, 2H, CH₂CH₂CH₂), 1.93-1.32 (m, 3H, BH₃); **¹³C NMR (126 MHz, CDCl₃)** δ 145.90 (C_{Ar}NO₂), 132.79 (d, ³J_{C-P} = 8.6 Hz, C_{Ar}CH₂), 129.76 (CH_{Ar}), 61.59 (CH₂N⁺(CH₃)₂), 56.05 (d, ²J_{C-P} = 3.8 Hz, OCH₂), 52.56 (2C, N⁺(CH₃)₂), 45.78 (d, 2C, ³J_{C-P} = 5.4 Hz, NHCH₂CH₂Cl), 45.55 (N_{Ar}CH₂), 43.17 (2C, NHCH₂CH₂Cl), 26.14 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CDCl₃)** δ 15.04; **HRMS** (ESI) *m/z* 443.1295 [M-H]⁺ (calculated for [C₁₃H₂₇BCl₂N₆O₄P]⁺ 443.1296), 467.1270 [M+Na]⁺ (calculated for [C₁₃H₂₈BCl₂N₆NaO₄P]⁺ 467.1272).

3-[5-[(amino[bis(2-chloroethyl)amino]phosphoryl)oxy)methyl]-2-nitro-1H-imidazol-1-N'-yl]-N,N,N-trimethylpropan-1-aminium iodide (15a). To a stirred solution of amine borane complex **14a-BH₃** (39.9 mg, 89.6 μ mol) in anhydrous THF (3 mL) was added DABCO (50.1 mg, 447 μ mol). The reaction mixture was heated at 30-40 °C for 1 h. After cooling at 0 °C, methyl iodide (90 μ L, 1.45 mmol, 15 eq.) was added and the reaction mixture was stirred at rt for 2.5 h. Methyl iodide (30 μ L, 480 μ mol) was added and the reaction was stirred for an additional 1 h. After evaporation under reduced pressure, the residue was purified by semi-preparative flash column chromatography and lyophilized to give the QA compound **15a** (38.4 mg, 67.0 μ mol) as a highly hygroscopic yellow powder. **Purification conditions:** detection at 254 nm and 323 nm, eluent: H₂O/ACN (v/v), 95/5 for 2 min, 95/5 \rightarrow 70/30 for 6 min, 70/30 for 2 min, 70/30 \rightarrow 60/40 for 6 min, 60/40 for 3 min, 60/40 \rightarrow 10/90 for 7 min, 10/90 for 4 min, retention time of 14.2 min; **Yield** 75%; **IR (ATR)** ν cm⁻¹ 3216, 1483, 1338, 1220, 1015, 973, 923; **¹H NMR (200 MHz, CD₃OD)** δ 7.32 (s, 1H, H_{Ar}), 5.19 (dd, 1H, ²J_{H-H} = 13.4 Hz, ³J_{H-P} = 7.7 Hz, OCH'), 5.11 (dd, 1H, ²J_{H-H} = 13.4 Hz, ³J_{H-P} = 7.0 Hz, OCH''), 4.61-4.46 (m, 2H, N_{Ar}CH₂), 3.75-3.67 (m, 4H, N(CH₂CH₂Cl)₂), 3.65-3.39 (m, 6H, N(CH₂CH₂Cl)₂, CH₂N⁺(CH₃)₃), 3.20 (s, 9H, CH₂N⁺(CH₃)₃), 2.55-2.34 (m, 2H, CH₂CH₂CH₂); **¹³C NMR (50 MHz, CD₃OD)** δ 147.08 (C_{Ar}NO₂), 134.88 (d, ³J_{C-P} = 8.1 Hz, C_{Ar}CH₂), 129.97 (CH_{Ar}), 64.41, 64.34 (CH₂N⁺(CH₃)₃), 57.32 (d, ²J_{C-P} = 4.2 Hz, OCH₂), 54.02, 53.94, 53.87 (CH₂N⁺(CH₃)₃), 50.39 (2C, N(CH₂CH₂Cl)₂), 45.26 (N_{Ar}CH₂), 43.18 (d, 2C, ³J_{C-P} = 1.2 Hz, N(CH₂CH₂Cl)₂), 25.24 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CD₃OD)** δ 19.45; **HRMS** (ESI) *m/z* 445.1282 [M]⁺ (calculated for [C₁₄H₂₈Cl₂N₆O₄P]⁺ 445.1281).

3-{5-[[bis[bis(2-chloroéthyl)amino]phosphoryl]oxy)méthyl]-2-nitro-1H-imidazol-1-N'-yl}-N,N,N-triméthylpropan-1-aminium iodide (**15b**) was prepared from amine borane complex **14b-BH₃** (128 mg, 0.225 mmol) as described for the preparation of **15a** with some differences: 15 eq. of methyl iodide initially followed by two times 3.5 eq after 1 h and 1.5 h and stirring for additional 30 min. QA compound **15b** (33.4 mg, 47.8 μ mol) was obtained as a highly hygroscopic yellow powder. **Purification conditions:** detection at 254 nm and 323 nm, eluent: H₂O/ACN (v/v), 95/5 for 2 min, 95/5 \rightarrow 70/30 for 6 min, 70/30 for 2 min, 70/30 \rightarrow 60/40 for 6 min, 60/40 for 3 min, 60/40 \rightarrow 36/64 for 1.5 min, 36/64 for 1.5 min, 36/64 \rightarrow 10/90 for 2 min, 10/90 for 6 min, retention time of 21.1 min; **Yield** 21%; **IR (ATR)** ν cm⁻¹ 1483, 1339, 1230, 1065, 1057, 1027, 971; **¹H NMR (500 MHz, CD₃OD)** δ 7.38 (s, 1H, H_{Ar}), 5.31 (d, 2H, ³J_{H-P} = 8.7 Hz, OCH₂), 4.59 (t, 2H, ³J = 7.8 Hz, N_{Ar}CH₂), 3.76-3.67 (m, 8H, N(CH₂CH₂Cl)₂), 3.65-3.60 (m, 2H, CH₂N⁺(CH₃)₃), 3.52-3.43 (m, 8H, N(CH₂CH₂Cl)₂), 3.21 (s, 9H, CH₂N⁺(CH₃)₃), 2.49-2.39 (m, 2H, CH₂CH₂CH₂); **¹³C NMR (126 MHz, CD₃OD)** δ 147.09 (C_{Ar}NO₂), 134.31 (d, ³J_{C-P} = 6.8 Hz, C_{Ar}CH₂), 130.48 (CH_{Ar}), 64.30, 64.28, 64.24 (CH₂N⁺(CH₃)₃), 57.86 (d, ²J_{C-P} = 4.0 Hz, OCH₂), 53.92, 53.89, 53.86 (CH₂N⁺(CH₃)₃), 50.11 (d, 4C, ²J_{C-P} = 4.6 Hz, N(CH₂CH₂Cl)₂), 45.18 (N_{Ar}CH₂), 43.11 (4C, N(CH₂CH₂Cl)₂), 25.28 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CD₃OD)** δ 18.04; **HRMS (ESI) m/z** 569.1137 [M]⁺ (calculated for [C₁₈H₃₄Cl₄N₆O₄P]⁺ 569.1128).

3-{5-[[bis[(2-chloroéthyl)amino]phosphoryl]oxy)méthyl]-2-nitro-1H-imidazol-1-N'-yl}-N,N,N-triméthylpropan-1-aminium iodide (**15c**) was prepared from amine borane complex **14c-BH₃** (90.0 mg, 0.202 mmol) as described for the preparation of **15a** with some differences: 15 eq. of methyl iodide was added and the reaction completed after 1.5 h at rt. QA compound **15c** (66.6 mg, 0.116 mmol) was obtained as a highly hygroscopic yellow powder. **Purification conditions:** detection at 254 nm and 323 nm, eluent: H₂O/ACN (v/v), 95/5 for 2 min, 95/5 \rightarrow 70/30 for 6 min, 70/30 for 2 min, 70/30 \rightarrow 64/36 for 4 min, 64/36 for 2 min, 64/36 \rightarrow 60/40 for 2 min, 60/40 for 2 min, 60/40 \rightarrow 10/90 for 7 min, 10/90 for 3 min, retention time of 14.5 min; **Yield** 57%; **IR (ATR)** ν cm⁻¹ 3215, 1534, 1483, 1338, 1202, 1186, 1009, 976; **¹H NMR (200 MHz, CD₃OD)** δ 7.31 (s, 1H, H_{Ar}), 5.16 (d, 2H, ³J_{H-P} = 7.9 Hz, CH₂O), 4.77-4.43 (m, 2H, N_{Ar}CH₂), 3.70-3.53 (m, 6H, NHCH₂CH₂Cl, CH₂N⁺(CH₃)₃), 3.28-3.14 (m, 13H, NHCH₂CH₂Cl, CH₂N⁺(CH₃)₃), 2.57-2.34 (m, 2H, CH₂CH₂CH₂); **¹³C NMR (126 MHz, CD₃OD)** δ 147.11 (C_{Ar}NO₂), 134.92 (d, ³J_{C-P} = 7.8 Hz, C_{Ar}CH₂O), 129.89 (CH_{Ar}), 64.43, 64.40, 64.38 (CH₂N⁺(CH₃)₃), 57.73 (d, ²J_{C-P} = 4.6 Hz, C_{Ar}CH₂O), 54.00, 53.97, 53.94 (CH₂N⁺(CH₃)₃), 46.01 (d, 2C, ³J_{C-P} = 5.1 Hz, NHCH₂CH₂Cl), 45.37 (N_{Ar}CH₂), 44.18 (2C, NHCH₂CH₂Cl), 25.28 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CD₃OD)** δ 17.12; **HRMS (ESI) m/z** 455.1281 [M]⁺ (calculated for [C₁₄H₂₈Cl₂N₆O₄P]⁺ 455.1281).

(4-bromo-1-N-méthyl-2-nitro-1H-imidazo-5-yl)méthanol (**16**). To a stirred solution of the imidazole **5** (6.01 g, 38.2 mmol) in anhydrous DMF (45 mL) was added N-bromosuccinimide (7.56 g, 42.5 mmol). The reaction mixture was stirred at 60 °C for 2 h 30. The resulting mixture was cooled to rt and the reaction was stopped by addition of water (250 mL). The yellow/green solid was filtrated and washed with diethyl ether (3 x 30 mL). The solid was dried at 30 °C under vacuum to yield the brominated compound **16** analytically pure (5.84 g, 24.7 mmol). The organic layer of the filtrate was separated and the aqueous layer was saturated using

sodium chloride and extracted with ethyl acetate (2 × 200 mL). The organic layers were combined, washed with brine (500 mL) and water (500 mL) dried over magnesium sulfate, filtrated and evaporated under reduced pressure. The resulting yellow/green solid was dried at 30 °C under vacuum to yield a second fraction of product **16** (1.54 g, 6.52 mmol) analytically pure by ¹H and ¹³C NMR analysis. **Yield** 82%; **mp** 163-164 °C; **Rf** 0.82 (SiO₂, AcOEt/cyclohexane, 80/20, v/v); **IR (ATR)** ν cm⁻¹ 3321, 1488, 1364, 1015; **¹H NMR (500 MHz, DMSO-*d*₆)** δ 4.51 (s, 2H, OCH₂); 3.98 (s, 3H, N_{Ar}CH₃); **¹³C NMR (126 MHz, DMSO-*d*₆)** δ 144.31 (C_{Ar}NO₂); 135.77 (C_{Ar}CH₂); 113.92 (C_{Ar}Br); 52.02 (OCH₂); 35.06 (N_{Ar}CH₃).

4-bromo-1-N-methyl-2-nitro-5-[[triiisopropylsilyl]oxy]methyl]-1H-imidazole (17). To a stirred solution of the alcohol **16** (9.14 g, 38.7 mmol) in anhydrous DMF (50 mL) were added imidazole (7.92 g, 116 mmol) and triisopropylsilyl chloride (9.1 mL, 42.5 mmol) dropwise. The reaction mixture was stirred for 18 h at rt. Then it was cooled at 0 °C and water (20 mL) was added dropwise until precipitation. Water (380 mL) was added at once and the heterogeneous mixture was stirred for 15 min at 0 °C. After filtration, the solid was washed with water (3 × 100 mL) and dried at 35 °C under vacuum to yield the protected alcohol **17** (14.7 g, 37.5 mmol) as a yellow solid. **Yield** 97%; **mp** 66-67 °C; **Rf** 0.69 (SiO₂, cyclohexane/AcOEt, 80/20, v/v); **IR (ATR)** ν cm⁻¹ 1483, 1357, 1081, 1061; **¹H NMR (500 MHz, DMSO-*d*₆)** δ 4.81 (s, 2H, OCH₂), 4.00 (s, 3H, N_{Ar}CH₃), 1.22-1.11 (m, 3H, Si(CH(CH₃)₂)₃), 1.09-1.01 (m, 18H, Si(CH(CH₃)₂)₃); **¹³C NMR (126 MHz, DMSO-*d*₆)** δ 144.55 (C_{Ar}NO₂), 134.46 (C_{Ar}CH₂), 116.61 (C_{Ar}Br), 54.41 (OCH₂), 35.12 (N_{Ar}CH₃), 17.61 (6C, Si(CH(CH₃)₂)₃), 11.29 (3C, Si(CH(CH₃)₂)₃); **HRMS (ESI)** *m/z* 392.1014 [M+H]⁺ (calculated for [C₁₄H₂₇BrN₃O₃Si]⁺ 392.1005).

(Z) and (E) N,N-dimethyl-3-(tributylstannyl)-prop-2-en-1-amine (18). To a stirred solution of *N,N*-dimethylpropargylamine (3.80 mL, 35.3 mmol) in anhydrous toluene (150 mL), was added tributyltin hydride (16.4 mL, 61.0 mmol) and azobisisobutyronitrile (751 mg, 4.57 mmol). The reaction mixture was warmed to 85 °C for 3 h 30 and then under reflux for 1 h. The solvent was removed by evaporation under reduced pressure and the crude was purified by silica gel column chromatography (eluent: cyclohexane/EtOAc/MeOH, 95/4/1, 90/8/2 then 90/6/4, v/v/v), to yield the organotin compound **18** as a yellowish liquid. Pure (*Z*) isomer (1.36 g, 3.63 mmol) and (*E*) isomer (5.20 g, 13.9 mmol) were obtained as well as a mixture fraction of both isomers (4.62 g, 12.3 mmol, (*Z*)/(*E*) = 17/83). **Yield** 85%; **Rf** (*Z*) isomer 0.50, (*E*) isomer 0.33 (SiO₂, cyclohexane/AcOEt/MeOH, 90/6/4, v/v/v); **IR (ATR)** ν cm⁻¹ 2954, 2922, 2871, 2581, 1455, 856; **¹H NMR (200 MHz, CDCl₃)** δ (*Z*) isomer: 6.55 (td, 1H, ³*J*_{cis} = 12.6 Hz, ³*J* = 6.2 Hz, CH=CHCH₂), 6.11-5.90 (td, 1H, ³*J*_{cis} = 12.7 Hz, ⁴*J* = 1.2 Hz, CH=CHCH₂), 2.90 (dd, 2H, ³*J* = 6.2 Hz, ⁴*J* = 1.1 Hz, CH=CHCH₂), 2.22 (s, 6H, N(CH₃)₂), 1.60-1.40 (m, 6H, Sn(CH₂CH₂CH₂CH₃)₃), 1.30 (h, 6H, ³*J*_{H-H} = 7.3 Hz, Sn(CH₂CH₂CH₂CH₃)₃), 1.01-0.81 (m, 15H, Sn(CH₂CH₂CH₂CH₃)₃); **¹H NMR (200 MHz, CDCl₃)** δ (*E*) isomer: 6.11 (d, 1H, ³*J*_{trans} = 19.0 Hz, CH=CHCH₂), 5.97 (td, 1H, ³*J*_{trans} = 18.9 Hz, ³*J* = 4.9 Hz, CH=CHCH₂), 2.97 (d, 2H, ³*J* = 4.8 Hz, CH=CHCH₂), 2.23 (s, 6H, N(CH₃)₂), 1.55-1.15

(m, 12H, Sn(CH₂CH₂CH₂CH₃)₃), 0.98-0.80 (m, 15H, Sn(CH₂CH₂CH₂CH₃)₃); **¹³C NMR (126 MHz, CDCl₃)** δ (Z) isomer: 146.31 (CH=C_HCH₂), 131.39 (C_H=CHCH₂), 65.13 (CH=CH_HCH₂), 45.47 (2C, N(CH₃)₂), 29.34 (3C, ³J_{C-Sn} = 20.4 Hz, Sn(CH₂CH₂CH₂CH₃)₃), 27.47 (3C, Sn(CH₂CH₂CH₂CH₃)₃), 13.82 (3C, Sn(CH₂CH₂CH₂CH₃)₃), 10.61 (3C, ¹J_{C-119Sn} = 341.8 Hz, ¹J_{C-117Sn} = 326.5 Hz, Sn(CH₂CH₂CH₂CH₃)₃); **¹³C NMR (126 MHz, CDCl₃)** δ (E) isomer: 146.03 (CH=C_HCH₂), 131.76 (¹J_{C-119Sn} = 378.8 Hz, ¹J_{C-117Sn} = 361.9 Hz, C_H=CHCH₂), 66.58 (³J_{C-119Sn} = 66.2 Hz, ³J_{C-117Sn} = 64.2 Hz, CH=CH_HCH₂), 45.23 (2C, N(CH₃)₂), 29.24 (3C, ³J_{C-Sn} = 20.6 Hz, Sn(CH₂CH₂CH₂CH₃)₃), 27.39 (3C, ²J_{C-119Sn} = 54.3 Hz, ²J_{C-117Sn} = 52.7 Hz, Sn(CH₂CH₂CH₂CH₃)₃), 13.83 (3C, Sn(CH₂CH₂CH₂CH₃)₃), 9.57 (3C, ¹J_{C-119Sn} = 343.3 Hz, ¹J_{C-117Sn} = 328.1 Hz, Sn(CH₂CH₂CH₂CH₃)₃); **MS** (ESI) *m/z* 376.21 [M+H]⁺ (calculated for [C₁₇H₃₈NSn]⁺ 376.20).

(E)-3-(1-N'-methyl-2-nitro-5-((triisopropylsilyloxy)methyl)-1H-imidazol-4-yl)-N,N-dimethylprop-2-en-1-amine (**19**). To a stirred solution of bromo protected alcohol **17** (2.27 g, 5.79 mmol) in anhydrous DMF (22 mL) were added tetrakis(triphenylphosphine) palladium (0) (1.05 g, 0.909 mmol) and N,N-dimethylamino-3-(tributylstannyl)-prop-2-en-1-amine (**18**) (4.34 g, 11.6 mmol) as mixture of isomers (Z)/(E) (40/60 estimated by ¹H NMR). The reaction mixture was stirred at 105 °C for 90 min. After cooling to rt, the solution was diluted with ethyl acetate (20 mL), filtrated through a pad of Celite 545® and washed with ethyl acetate (4 x 25 mL). Water (400 mL) was added to the filtrate and the aqueous layer obtained after decantation was extracted with ethyl acetate (3 x 300 mL). The organic layers were combined, washed with water (400 mL) and brine (400 mL), dried over magnesium sulfate, filtrated and evaporated under reduced pressure to yield a dark orange oil. The crude product was purified by silica gel column chromatography (eluent: DCM/methanol, 95/5, 93/7 then 90/9, v/v) to yield product **19** (1.51 g, 3.81 mmol) as an orange oil. **Yield** 66%; **Rf** 0.51 (SiO₂, DCM/MeOH, 90/10, v/v); **IR (ATR)** ν cm⁻¹ 1495, 1329, 1081, 1060; **¹H NMR (200 MHz, CDCl₃)** δ 6.59 (td, 1H, ³J_{trans} = 15.5 Hz, ³J = 6.3 Hz, CH=CH_HCH₂), 6.39 (d, 1H, ³J_{trans} = 15.6 Hz, C_H=CHCH₂), 4.80 (s, 2H, OCH₂), 4.04 (s, 3H, N_{Ar}CH₃), 3.08 (d, 2H, ³J = 6.2 Hz, CH=CH_HCH₂), 2.25 (s, 6H, N(CH₃)₂), 1.14-0.90 (m, 21H, Si(CH(CH₃)₂)₃); **¹³C NMR (50 MHz, CDCl₃)** δ 145.10 (C_{Ar}NO₂), 135.30, 133.09 (2C, C_{Ar}C), 127.74 (C_H=CHCH₂), 123.98 (CH=C_HCH₂); 60.74 (CH=CH_HCH₂), 54.01 (OCH₂), 44.31 (2C, N(CH₃)₂); 34.67 (N_{Ar}CH₃); 17.84 (6C, Si(CH(CH₃)₂)₃), 11.74 (3C, Si(CH(CH₃)₂)₃); **HRMS** (ESI) *m/z* 397.2625 [M+H]⁺ (calculated for [C₁₉H₃₇N₄O₃Si]⁺ 397.2629).

(E)-(1-N-methyl-2-nitro-4-[3-(N,N-dimethylamino)prop-1-enyl]-1H-imidazo-5-yl)methanol (**20**).

To a solution of silylether **19** (303 mg, 0.764 mmol) in anhydrous THF (6 mL) cooled at 0 °C was added tetrabutylammonium fluoride (1 M in THF, 1.15 mL, 1.15 mmol). The reaction mixture was stirred for 90 min at 0 °C. After concentration under reduced pressure, the orange oil was purified by silica gel column chromatography (eluent: DCM/methanol, 95/5, v/v then 95/5, v/v + 2% TEA) to yield the alcohol **20** as a yellow oil (164 mg, 0.683 mmol). **Yield** 89%; **Rf** 0.43 (SiO₂, DCM/MeOH, 90/10, v/v + 5% TEA); **IR (ATR)** ν cm⁻¹ 3133, 1499, 1332, 1035; **¹H NMR (200 MHz, CD₃OD)** δ 6.66 (d, 1H, ³J_{trans} = 15.7 Hz, C_H=CHCH₂), 6.53 (td, 1H, ³J_{trans} = 15.6 Hz, ³J_{H-H} = 6.2 Hz, CH=CH_HCH₂), 4.71 (s, 2H, CH₂OH), 4.03 (s, 3H, N_{Ar}CH₃), 3.24 (d, 2H, ³J = 6.1 Hz,

CH=CHCH₂), 2.36 (s, 6H, N(CH₃)₂); ¹³C NMR (50 MHz, CD₃OD) δ 146.43 (C_{Ar}NO₂), 136.67, 135.45 (C_{Ar}C), 128.64 (CH=CHCH₂), 123.98 (CH=CHCH₂), 61.98 (CH=CHCH₂), 52.69 (CH₂OH), 44.84 (2C, N(CH₃)₂), 34.94 (N_{Ar}CH₃); HRMS (ESI) *m/z* 241.1295 [M+H]⁺ (calculated for [C₁₀H₁₇N₄O₃]⁺ 241.1295).

(*E*)-3-(1-*N'*-methyl-2-nitro-5-[[triiisopropylsilyl]oxy]methyl}-1*H*-imidazol-4-yl)-*N,N*-dimethylpropan-1-amine (**22**). To a solution of compound **19** (108 mg, 0.271 mmol) in pyridine (2 mL) were added tosylhydrazine (255 mg, 1.37 mmol) and potassium carbonate (188 mg, 1.36 mmol). The reaction mixture was heated under reflux for 19 h. After cooling to rt, the reaction mixture was filtrated and the solid was washed with DCM (3 x 10 mL). The filtrate was successively washed with brine (2 x 50 mL) and saturated aqueous solution of sodium carbonate (2 x 50 mL). The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The crude product was purified by silica gel chromatography (eluent: gradient of MeOH in DCM 5 to 8%) to yield compound **22** (25.4 mg, 63.7 μmol) as an orange oil. Yield 24%; ¹H NMR (400 MHz, CDCl₃) δ 4.76 (s, 2H, OCH₂), 4.04 (s, 3H, N_{Ar}CH₃), 2.59 (t, 2H, ³*J* = 7.5 Hz, C_{Ar}CH₂CH₂), 2.38 (t, 2H, ³*J* = 7.4 Hz, CH₂N(CH₃)₂), 2.28 (s, 6H, N(CH₃)₂), 1.87 (qt, 2H, ³*J* = 7.5 Hz, CH₂CH₂CH₂), 1.20-0.93 (m, 21H, Si(CH₂(CH₃)₂)₃).

{4-[3-(*N',N'*-dimethylamino)propyl]-1-*N*-methyl-2-nitro-1*H*-imidazo-5-yl}methanol (**21**).

Conditions A: To a solution of compound **20** (688 mg, 2.86 mmol) in pyridine (25 mL) were added tosylhydrazine (2.67 g, 14.3 mmol) and potassium carbonate (1.99 g, 14.4 mmol). The reaction mixture was heated under reflux for 3 h. After cooling to rt, the reaction mixture was diluted with ethyl acetate (15 mL). The solid was filtrated and washed with ethyl acetate (3 x 15 mL). After evaporation of the filtrate under reduced pressure, the crude product was purified by column chromatography on alumina (eluent: gradient from 2 to 20% of EtOH in EtOAc, v/v) to yield compound **21** (464 mg, 1.92 mmol) as a yellow oil. Yield 67%

Conditions B: To a solution of silylether **22** (30.0 mg, 75.2 μmol) in anhydrous THF (1 mL) cooled at 0 °C was added tetrabutylammonium fluoride (1 M in THF, 140 μL, 140 μmol). The reaction mixture was stirred for 1 h at 0 °C. After concentration under reduced pressure, the orange oil was purified by column chromatography on alumina (eluent: EtOAc/EtOH/NH₄OH, 95/4/1, v/v/v) to yield the alcohol **21** as a yellow oil (8.5 mg, 35.4 μmol). Yield 47%; R_f 0.41 (Al₂O₃, AcOEt/EtOH, 90/10, v/v); IR (ATR) ν cm⁻¹ 3290, 1487, 1327, 1020; ¹H NMR (200 MHz, CD₃OD) δ 4.65 (s, 2H, CH₂OH), 4.03 (s, 3H, N_{Ar}CH₃), 2.63 (t, 2H, ³*J* = 7.5 Hz, C_{Ar}CH₂CH₂), 2.43-2.30 (m, 2H, CH₂N(CH₃)₂), 2.24 (s, 6H, N(CH₃)₂), 1.93-1.73 (m, 2H, CH₂CH₂CH₂); ¹³C NMR (50 MHz, CD₃OD) δ 145.76 (C_{Ar}NO₂), 140.60 (C_{Ar}CH₂CH₂), 135.00 (C_{Ar}CH₂OH), 59.60 (CH₂N(CH₃)₂), 52.99 (CH₂OH), 45.32 (2C, N(CH₃)₂), 34.88 (N_{Ar}CH₃), 28.12 (C_{Ar}CH₂CH₂), 25.30 (CH₂CH₂CH₂); MS (ESI) *m/z* 243.14 [M+H]⁺ (calculated for [C₁₀H₁₉N₄O₃]⁺ 243.15).

N,N-dimethyl-3-(5-hydroxymethyl-1-*N'*-methyl-2-nitro-1*H*-imidazo-4-yl)propyl-1-amine borane complex (**21-BH₃**). To a stirred solution of amine **21** (224 mg, 0.925 mmol) in anhydrous chloroform (4.5 mL) cooled at 0 °C was added dropwise a 1 M solution of borane-THF complex

(4.6 mL, 4.60 mmol). The reaction mixture was stirred at 0 °C for 1 h. Anhydrous methanol (4.5 mL) was added and the reaction mixture was stirred 15 min at 0 °C. The reaction mixture was evaporated under reduced pressure and purified by silica gel column chromatography (eluent: DCM/MeOH, 98/2, v/v). The amine-borane complex **21-BH₃** (209 mg; 0.816 mmol) was obtained as beige crystals. **Yield** 70%; **mp** 99-100 °C; **Rf** 0.37 (SiO₂, DCM/MeOH, 98/2, v/v); **IR (ATR)** ν cm⁻¹ 3268, 2379, 2321, 2275, 1490, 1331, 1170, 1027; **¹H NMR (200 MHz, CDCl₃)** δ 4.69 (s, 2H, CH₂OH), 4.06 (s, 3H, N_{Ar}CH₃), 2.88 (br.s, 1H, OH), 2.79-2.68 (m, 2H, CH₂N⁺(CH₃)₂), 2.62 (t, 2H, ³J = 7.5 Hz, C_{Ar}CH₂CH₂), 2.54 (s, 6H, N⁺(CH₃)₂), 2.20-1.98 (m, 2H, CH₂CH₂CH₂), 1.96-0.74 (m, 3H, BH₃); **¹³C NMR (50 MHz, CDCl₃)** δ 144.81 (C_{Ar}NO₂), 139.18, 132.94 (2C, C_{Ar}CH₂), 63.84 (CH₂N⁺(CH₃)₂), 53.13 (CH₂OH), 51.78 (2C, N⁺(CH₃)₂), 34.67 (N_{Ar}CH₃), 24.73 (C_{Ar}CH₂CH₂), 24.09 (CH₂CH₂CH₂); **MS (ESI)** *m/z* 255.15 [M-H]⁺ (calculated for [C₁₀H₂₀BN₄O₃]⁺ 255.16), 279.13 [M+Na]⁺ (calculated for [C₁₀H₂₁BN₄NaO₃]⁺ 279.16). Crystallographic data are available in supporting information.

N,N-bis(2-chloroethyl)-{1-*N'*-methyl-4-[3-(*N''*,*N''*-dimethylamino)prop-1-enyl]-2-nitro-1*H*-imidazol-5-yl}methyl phosphorodiamidate (**23**) was prepared from alcohol **20** (93.0 mg, 0.387 mmol) as described for the preparation of **2a** with some differences: the coupling reaction with bis(2-chloroethyl)phosphoramidic dichloride (**1**) was performed for 45 min before ammonia gas was bubbled through the reaction for 2 min. The reaction was directly stopped by addition of water (10 mL). The crude product was purified by silica gel chromatography (eluent: EtOAc/EtOH, 80/20, v/v, + 5% NH₄OH) to yield compound **23** (52.4 mg; 0.134 mmol) as an orange oil. **Yield** 31%; **Rf** 0.68 (SiO₂, AcOEt/EtOH, 80/20, v/v, + 5% NH₄OH); **IR (ATR)** ν cm⁻¹ 3417, 3222, 1495, 1331, 1220, 1187, 1002, 977; **¹H NMR (200 MHz, CD₃OD)** δ 6.70 (d, 1H, ³J_{trans} = 15.6 Hz, CH=CHCH₂), 6.57 (td, 1H, ³J_{trans} = 15.6 Hz, ³J = 6.0 Hz, CH=CHCH₂), 5.15 (d, 2H, ³J_{H-P} = 8.0 Hz, CH₂O), 4.05 (s, 3H, N_{Ar}CH₃), 3.72-3.58 (m, 4H, N(CH₂CH₂Cl)₂), 3.53-3.33 (m, 4H, N(CH₂CH₂Cl)₂), 3.20 (d, 2H, ³J = 5.9 Hz, CH=CHCH₂), 2.32 (s, 6H, N(CH₃)₂); **¹³C NMR (126 MHz, CD₃OD)** δ 146.92 (C_{Ar}NO₂), 138.20 (C_{Ar}CH=CH), 131.04 (d, ³J_{C-P} = 7.4 Hz, C_{Ar}CH₂O), 130.34 (CH=CHCH₂), 123.29 (CH=CHCH₂), 62.17 (CH=CHCH₂), 55.91 (d, ²J_{C-P} = 4.5 Hz, CH₂O), 50.49 (d, 2C, ²J_{C-P} = 4.8 Hz, N(CH₂CH₂Cl)₂), 45.07 (2C, N(CH₃)₂), 43.12 (2C, N(CH₂CH₂Cl)₂), 35.09 (N_{Ar}CH₃); **³¹P NMR (202 MHz, CD₃OD)** δ 19.37; **MS (ESI)** *m/z* 443.06 [M+H]⁺ (calculated for [C₁₄H₂₆Cl₂N₆O₄P]⁺ 443.11).

N,N-bis(2-chloroethyl)-{1-*N'*-methyl-4-[3-(*N''*,*N''*-dimethylamino)propyl]-2-nitro-1*H*-imidazol-5-yl}methyl-phosphorodiamidate (**24a**) was prepared from alcohol **21** (102 mg, 0.421 mmol) as described for the preparation of **2a** with some differences: the coupling reaction with bis(2-chloroethyl)phosphoramidic dichloride (**1**) was performed for 1 h 15 before ammonia gas was bubbled through the reaction for 2 min. The reaction was directly stopped by addition of water (10 mL). The crude product was purified by silica gel chromatography (eluent: EtOAc/EtOH, 80/20, v/v + 5% TEA) to yield compound **24a** (56.0 mg, 0.126 mmol) as a yellow oil. **Yield** 30%; **Rf** 0.10 (SiO₂, AcOEt/EtOH, 80/20, v/v, + 5% TEA); **IR (ATR)** ν cm⁻¹ 3345, 3258, 1493, 1331,

1208, 1044, 981; $^1\text{H NMR}$ (500 MHz, CD_3OD) δ 5.11 (dd, 1H, $^2J_{\text{H-H}} = 13.5$ Hz, $^3J_{\text{H-P}} = 7.7$ Hz, OCH'), 5.08 (dd, 1H, $^2J_{\text{H-H}} = 13.5$ Hz, $^3J_{\text{H-P}} = 7.7$ Hz, OCH''), 4.04 (s, 3H, $\text{N}_{\text{Ar}}\text{CH}_3$), 3.67 (t, 4H, $^3J = 7.1$ Hz, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 3.52-3.35 (m, 4H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 2.68 (t, 2H, $^3J = 7.6$ Hz, $\text{C}_{\text{Ar}}\text{CH}_2\text{CH}_2$), 2.44-2.37 (m, 2H, $\text{CH}_2\text{N}(\text{CH}_3)_2$), 2.27 (s, 6H, $\text{N}(\text{CH}_3)_2$), 1.93-1.81 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$); $^{13}\text{C NMR}$ (126 MHz, CD_3OD) δ 146.28 ($\text{C}_{\text{Ar}}\text{NO}_2$), 142.14 ($\text{C}_{\text{Ar}}\text{CH}_2\text{CH}_2$), 130.92 (d, $^3J_{\text{C-P}} = 7.7$ Hz, $\text{C}_{\text{Ar}}\text{CH}_2\text{O}$), 59.72 ($\text{CH}_2\text{N}(\text{CH}_3)_2$), 56.18 (d, $^2J_{\text{C-P}} = 4.5$ Hz, CH_2O), 50.45 (d, 2C, $^2J_{\text{C-P}} = 4.8$ Hz, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 45.34 (2C, $\text{N}(\text{CH}_3)_2$), 43.13 (2C, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 35.11 ($\text{N}_{\text{Ar}}\text{CH}_3$), 27.96 ($\text{C}_{\text{Ar}}\text{CH}_2\text{CH}_2$), 25.41 ($\text{CH}_2\text{CH}_2\text{CH}_2$); $^{31}\text{P NMR}$ (202 MHz, CD_3OD) δ 19.22; **MS** (ESI) m/z 445.13 [$\text{M}+\text{H}$] $^+$ (calculated for $[\text{C}_{14}\text{H}_{28}\text{Cl}_2\text{N}_6\text{O}_4\text{P}]^+$ 445.13).

3-{5-[(amino[bis(2-chloroethyl)amino]phosphoryl)oxy)methyl]-1-N'-methyl-2-nitro-1H-imidazol-4-yl}-N,N,N-trimethylprop-2-en-1-aminium iodide (25). To a stirred solution of the phosphoramidate **23** (69.2 mg, 0.156 mmol) in anhydrous ACN (5 mL) were added potassium carbonate (129 mg, 0.933 mmol) and methyl iodide (59 μL , 0.948 mmol). The reaction mixture was stirred at rt for 1 h 45 and filtrated through a PTFE (0.45 μm) filter. The solid was washed with ACN (2 mL). After evaporation of the filtrate and purification by semi-preparative reverse phase column chromatography the quaternary ammonium **25** (59.6 mg, 0.102 mmol) was obtained as a highly hygroscopic lyophilisate. **Purification conditions**: detection: 254 nm and 338 nm; separation time: 30 min; eluent: $\text{H}_2\text{O}/\text{ACN}$ (v/v): 95/5 for 2 min; 95/5 \rightarrow 70/30 for 6 min; 70/30 for 2 min; 70/30 \rightarrow 60/40 for 6 min; 60/40 for 2 min; 60/40 \rightarrow 10/90 for 8 min; 10/90 for 4 min; retention time: 15.4 min; **Yield** 65%; **IR (ATR)** ν cm^{-1} 3403, 3229, 1542, 1497, 1331, 1214, 1187, 1004, 978; $^1\text{H NMR}$ (500 MHz, CD_3OD) δ 7.12 (d, 1H, $^3J_{\text{trans}} = 15.4$ Hz, $\text{CH}=\text{CHCH}_2$), 6.67 (td, 1H, $^3J_{\text{trans}} = 15.4$ Hz, $^3J = 7.7$ Hz, $\text{CH}=\text{CHCH}_2$), 5.20 (d, 2H, $^3J_{\text{H-P}} = 9.6$ Hz, CH_2O), 4.17 (d, 2H, $^3J = 7.7$ Hz, $\text{CH}=\text{CHCH}_2$), 4.07 (s, 3H, $\text{N}_{\text{Ar}}\text{CH}_3$), 3.69-3.58 (m, 4H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 3.49-3.33 (m, 4H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 3.17 (s, 9H, $\text{CH}_2\text{N}^+(\text{CH}_3)_3$); $^{13}\text{C NMR}$ (126 MHz, CD_3OD) δ 147.18 ($\text{C}_{\text{Ar}}\text{NO}_2$), 136.36 ($\text{C}_{\text{Ar}}\text{CH}=\text{CH}$), 133.17 (d, $^3J_{\text{C-P}} = 6.5$ Hz, $\text{C}_{\text{Ar}}\text{CH}_2\text{O}$), 132.31 ($\text{CH}=\text{CHCH}_2$), 119.01 ($\text{CH}=\text{CHCH}_2$), 68.74, 68.72, 68.70 ($\text{CH}=\text{CHCH}_2$), 56.04 (d, $^2J_{\text{C-P}} = 4.7$ Hz, CH_2O), 53.49, 53.46, 53.42 ($\text{CH}_2\text{N}^+(\text{CH}_3)_3$), 50.46 (d, 2C, $^2J_{\text{C-P}} = 4.8$ Hz, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 43.25 (2C, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 35.45 ($\text{N}_{\text{Ar}}\text{CH}_3$); $^{31}\text{P NMR}$ (202 MHz, CD_3OD) δ 19.38; **HRMS** (ESI) m/z 457.1288 [M] $^+$ (calculated for $[\text{C}_{15}\text{H}_{28}\text{Cl}_2\text{N}_6\text{O}_4\text{P}]^+$ 457.1281).

3-{5-[(amino[bis(2-chloroethyl)amino]phosphoryl)oxy)methyl]-1-N'-methyl-2-nitro-1H-imidazol-4-yl}-N,N-dimethylpropan-1-amine borane complex (24a-BH₃) was prepared from alcohol **21-BH₃** (133 mg, 0.519 mmol) as described for the preparation of **14a-BH₃**. The crude product was purified by silica gel chromatography (eluent: DCM/EtOH , 96/4, v/v) to yield compound **24a-BH₃** (150 mg, 0.629 mmol) as an oil. **Yield** 63%; **Rf** 0.20 (SiO_2 , DCM/MeOH , 97/3, v/v); **IR (ATR)** ν cm^{-1} 3231, 2380, 2318, 2272, 1492, 1330, 1227, 1193, 1168, 1003, 980; $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 5.14 (dd, 1H, $^2J_{\text{H-H}} = 13.4$ Hz, $^3J_{\text{H-P}} = 7.7$ Hz, OCH'), 4.99 (dd, 1H, $^2J_{\text{H-H}} = 13.4$ Hz, $^3J_{\text{H-P}} = 6.4$ Hz, OCH''), 4.06 (s, 3H, $\text{N}_{\text{Ar}}\text{CH}_3$), 3.69-3.61 (m, 4H, $\text{N}(\text{CH}_2\text{CH}_2\text{Cl})_2$), 3.54-

3.43 (m, 4H, N(CH₂CH₂Cl)₂), 3.08 (br.s, 2H, NH₂), 2.83-2.72 (m, 2H, CH₂N⁺(CH₃)₂), 2.68 (t, 2H, ³J = 7.4 Hz, C_{Ar}CH₂CH₂), 2.56 and 2.54 (s, 3H, CH₂N⁺(CH₃)₂), 2.13-2.06 (qt, 2H, ³J = 7.7 Hz, CH₂CH₂CH₂); ¹³C NMR (126 MHz, CDCl₃) δ 145.24 (C_{Ar}NO₂), 141.16 (C_{Ar}CH₂CH₂), 129.17 (d, ³J_{C-P} = 8.2 Hz, C_{Ar}CH₂), 63.89 (CH₂N⁺(CH₃)₂), 55.11 (d, ²J_{C-P} = 4.0 Hz, CH₂O), 51.55, 51.48 (CH₂N⁺(CH₃)₂), 48.93 (d, 2C, ²J_{C-P} = 5.0 Hz, N(CH₂CH₂Cl)₂), 42.70 (2C, N(CH₂CH₂Cl)₂), 34.63 (N_{Ar}CH₃), 24.79, 24.00 (C_{Ar}CH₂CH₂); ³¹P NMR (202 MHz, CDCl₃) δ 16.17; MS (ESI) m/z 457.08 [M-H]⁺ (calculated for [C₁₄H₂₉BCl₂N₆O₄P]⁺ 457.15).

3-{5-[(bis[bis(2-chloroethyl)amino]phosphoryl)oxy)methyl]-1-N'-methyl-2-nitro-1H-imidazol-4-yl}-N,N-dimethylpropan-1-amine borane complex (**24b-BH₃**). To a stirred solution of the alcohol **21-BH₃** (120 mg, 0.469 mmol) in anhydrous THF (4 mL) cooled at -78 °C, was added dropwise lithium bis(trimethylsilyl)amide (1 M in THF, 520 μL, 520 μmol) under an inert atmosphere. After 5 min, a solution of compound **12** (189 mg, 519 μmol) in anhydrous THF (2 mL) previously cooled at -78 °C was added dropwise to the reaction mixture. The kinetic of the reaction was monitored by ³¹P NMR. The temperature was allowed to rise gradually to 5 °C and the stirring was maintained for 24 h. The reaction was stopped by addition of water (10 mL) and the reaction mixture was extracted with ethyl acetate (3 × 10 mL). The organic layers were combined, dried over magnesium sulfate, filtrated and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (eluent: DCM/EtOH, 98/2, v/v) to yield phosphorodiamidate **24b-BH₃** (90.7 mg, 0.155 mmol) as a thick oil. Yield 33%; Rf 0.31 (SiO₂, DCM/EtOH, 98/2, v/v); IR (ATR) ν cm⁻¹ 2381, 2318, 2272, 1493, 1331, 1219, 1167, 1088, 973; ¹H NMR (500 MHz, CDCl₃) δ 5.13 (d, 2H, ³J_{H-P} = 8.2 Hz, CH₂O), 4.06 (s, 3H, N_{Ar}CH₃), 3.68-3.54 (m, 8H, N(CH₂CH₂Cl)₂), 3.40 (td, 8H, ³J_{H-P} = 12.6 Hz, ³J_{H-H} = 6.4 Hz, N(CH₂CH₂Cl)₂), 2.85-2.74 (m, 2H, CH₂N⁺(CH₃)₂), 2.67 (t, 2H, ³J = 7.6 Hz, C_{Ar}CH₂CH₂), 2.55 (s, 6H, N⁺(CH₃)₂), 2.18-1.97 (m, 2H, CH₂CH₂CH₂), 1.87-1.25 (m, 3H, BH₃); ¹³C NMR (126 MHz, CDCl₃) δ 145.31 (C_{Ar}NO₂), 141.47 (C_{Ar}CH₂CH₂), 128.53 (d, ³J_{C-P} = 6.5 Hz, C_{Ar}CH₂O), 63.89 (CH₂N⁺(CH₃)₂), 55.36 (d, ²J_{C-P} = 3.9 Hz, CH₂O), 51.55 (2C, N⁺(CH₃)₂), 49.22 (d, 4C, ³J_{C-P} = 4.6 Hz, N(CH₂CH₂Cl)₂), 42.36 (4C, N(CH₂CH₂Cl)₂), 34.66 (N_{Ar}CH₃); 24.65 (C_{Ar}CH₂CH₂), 23.91 (CH₂CH₂CH₂); ³¹P NMR (202 MHz, CDCl₃) δ 17.04; MS (ESI) m/z 583.14 [M-H]⁺ (calculated for [C₁₈H₃₅BCl₄N₆O₄P]⁺ 583.13).

3-{5-[(bis[(2-chloroethyl)amino]phosphoryl)oxy)methyl]-1-N'-methyl-2-nitro-1H-imidazol-4-yl}-N,N-dimethylpropan-1-amine borane complex (**24c-BH₃**). To a solution cooled at -78 °C of phosphorous oxychloride (38 μL, 0.408 mmol) in anhydrous DCM (5 mL), was added dropwise a solution of the alcohol **21-BH₃** (105 mg, 0.410 mmol) and TEA (63 μL, 0.452 mmol) in anhydrous DCM (2 mL). After 1 h of stirring at -78 °C, 2-chloroethylamine hydrochloride (102 mg, 0.885 mmol) was added followed by the dropwise addition of TEA (260 μL, 1.87 mmol). The temperature of the reaction mixture was risen gradually from -78 °C to 5 °C for 15 h. The reaction was stopped by the addition of water (10 mL). After layers separation, the organic

layer was washed with a saturated aqueous solution of sodium hydrogenocarbonate (10 mL), dried over magnesium sulfate, filtrated and evaporated under reduced pressure to give a yellow oil. The residue was purified by silica gel column chromatography (eluent: gradient of MeOH in DCM 0-2%) to yield the isophosphorodiamidate **24c-BH₃** (78.0 mg, 0.170 mmol) as an oil. **Yield** 41%; **Rf** 0.26 (SiO₂, DCM/MeOH, 98/2, v/v); **IR (ATR)** ν cm⁻¹ 3338, 3235, 2381, 2318, 2271, 1493, 1331, 1193, 1167, 1110, 999; **¹H NMR (500 MHz, CDCl₃)** δ 5.03 (d, 2H, ³J_{H-P} = 7.4 Hz, CH₂O), 4.05 (s, 3H, N_{Ar}CH₃), 3.58 (t, ³J = 5.5 Hz, 4H, NHCH₂CH₂Cl), 3.35-3.19 (m, 6H, NHCH₂CH₂Cl), 2.81-2.72 (m, 2H, CH₂N⁺(CH₃)₂), 2.67 (t, 2H, ³J = 7.4 Hz, C_{Ar}CH₂CH₂), 2.54 (s, 6H, N⁺(CH₃)₂), 2.12-2.01 (m, 2H, CH₂CH₂CH₂), 1.77-1.32 (m, 3H, BH₃); **¹³C NMR (126 MHz, CDCl₃)** δ 145.11 (C_{Ar}NO₂), 141.04 (C_{Ar}CH₂CH₂); 129.24 (d, ³J_{C-P} = 7.8 Hz, C_{Ar}CH₂O), 63.82 (CH₂N⁺(CH₃)₂), 55.03 (d, ²J_{C-P} = 4.1 Hz, CH₂O), 51.47 (2C, N⁺(CH₃)₂), 45.87 (d, 2C, ³J_{C-P} = 5.2 Hz, NHCH₂CH₂Cl), 43.10 (2C, NHCH₂CH₂Cl), 34.65 (N_{Ar}CH₃), 24.73 (C_{Ar}CH₂CH₂), 23.97 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CDCl₃)** δ 14.82; **MS** (ESI) *m/z* 457.13 [M-H]⁺ (calculated for [C₁₄H₂₉BCl₂N₆O₄P]⁺ 457.15).

3-{5-[[{amino[bis(2-chloroethyl)amino]phosphoryl]oxy)methyl]-1-*N'*-methyl-2-nitro-1*H*-imidazol-4-yl]-*N,N,N*-trimethylpropan-1-aminium (**26a**). Conditions A: Alkylation of amine **24a** (42.8 mg, 96.1 μ mol) following conditions described for the preparation of **3a** (reaction time: 7 h) followed by purification by semi-preparative reverse phase column chromatography afforded QA-compound **26a** (29.2 mg, 49.7 μ mol) as a highly hygroscopic yellow powder. **Yield** 52%;

Conditions B: Dissociation of the amine borane complex **24a-BH₃** (50.3 mg, 0.110 mmol) and alkylation according to the conditions described for the preparation of **15a** (methyl iodide 15 eq. at rt for 2 h) followed by purification by semi-preparative reverse phase column chromatography afforded QA-compound **26a** (29.5 mg, 50.2 μ mol) as a highly hygroscopic yellow powder. **Yield** 46%; **Purification conditions**: detection: 254 nm and 338 nm; separation time: 30 min; eluent: H₂O/ACN (v/v): 95/5 for 2 min; 95/5 \rightarrow 70/30 for 6 min; 70/30 for 2 min; 70/30 \rightarrow 60/40 for 6 min; 60/40 for 3 min; 60/40 \rightarrow 10/90 for 7 min; 10/90 for 4 min; retention time : 15.4 min; **IR (ATR)** ν cm⁻¹ 3232, 1493, 1332, 1228, 979; **¹H NMR (200 MHz, CD₃OD)** δ 5.16 (dd, 1H, ²J_{H-H} = 13.7 Hz, ³J_{H-P} = 9.3 Hz, OCH'), 5.06 (dd, 1H, ²J_{H-H} = 13.7 Hz, ³J_{H-P} = 9.1 Hz, OCH''), 4.05 (s, 3H, N_{Ar}CH₃), 3.74-3.62 (m, 4H, N(CH₂CH₂Cl)₂), 3.54-3.35 (m, 6H, N(CH₂CH₂Cl)₂, CH₂N⁺(CH₃)₃), 3.15 (s, 9H, CH₂N⁺(CH₃)₃), 2.78 (t, 2H, ³J = 7.3 Hz, C_{Ar}CH₂CH₂), 2.32-2.05 (m, 2H, CH₂CH₂CH₂); **¹³C NMR (126 MHz, CD₃OD)** δ 146.53 (C_{Ar}NO₂), 140.01 (C_{Ar}CH₂CH₂), 131.52 (d, ³J_{C-P} = 6.4 Hz, C_{Ar}CH₂O), 66.97, 66.89, 66.85 (CH₂N⁺(CH₃)₃), 56.42 (d, ²J_{C-P} = 4.8 Hz, CH₂O), 53.82, 53.74, 53.66 (CH₂N⁺(CH₃)₃), 50.41 (d, 2C, ²J_{C-P} = 4.8 Hz, N(CH₂CH₂Cl)₂), 43.18 (2C, N(CH₂CH₂Cl)₂), 35.36 (N_{Ar}CH₃), 24.07 (C_{Ar}CH₂CH₂), 23.33 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CD₃OD)** δ 19.45; **HRMS** (ESI) *m/z* 459.1444 [M]⁺ (calculated for [C₁₅H₃₀Cl₂N₆O₄P]⁺ 459.1438).

3-{5-[[{bis[bis(2-chloroethyl)amino]phosphoryl]oxy)methyl]-1-*N'*-methyl-2-nitro-1*H*-imidazol-4-yl]-*N,N,N*-trimethylpropan-1-aminium iodide (**26b**) was prepared from amine borane complex **24b-BH₃** (90.7 mg, 0.155 mmol) as described for the preparation of **15a** (methyl iodide 15 eq. at rt for 1 h). QA compound **26b** (38.8 mg, 54.5 μ mol) was obtained as a highly hygroscopic yellow powder. **Purification conditions**: detection: 254 nm and 338 nm;

separation time: 30 min; eluent: H₂O/ACN (v/v): 95/5 for 2 min; 95/5 → 70/30 for 6 min; 70/30 for 2 min; 70/30 → 60/40 for 6 min; 60/40 for 3 min; 60/40 → 10/90 for 7 min; 10/90 for 4 min; retention time : 21.7 min; **Yield** 35%; **IR (ATR)** ν cm⁻¹ 1491, 1331, 1210, 973, 959; **¹H NMR (500 MHz, CD₃OD)** δ 5.26 (d, 1H, ³J_{H-P} = 10.0 Hz, CH₂O), 4.08 (s, 3H, N_{Ar}CH₃), 3.76-3.64 (m, 8H, N(CH₂CH₂Cl)₂), 3.51-3.38 (m, 10H, CH₂N⁺(CH₃)₃, N(CH₂CH₂Cl)₂), 3.17 (s, 9H, CH₂N⁺(CH₃)₃), 2.81 (t, 2H, ³J = 7.3 Hz, C_{Ar}CH₂CH₂), 2.27-2.18 (m, 2H, CH₂CH₂CH₂); **¹³C NMR (126 MHz, CD₃OD)** δ 146.72 (C_{Ar}NO₂), 140.48 (C_{Ar}CH₂CH₂), 130.86 (d, ³J_{C-P} = 5.0 Hz, C_{Ar}CH₂O), 67.05, 67.03, 67.91 (CH₂N⁺(CH₃)₃), 56.98 (d, ²J_{C-P} = 4.5 Hz, CH₂O), 53.84, 53.81, 53.78 (CH₂N⁺(CH₃)₃), 50.11 (d, 4C, ²J_{C-P} = 4.7 Hz, N(CH₂CH₂Cl)₂), 43.06 (4C, N(CH₂CH₂Cl)₂), 35.43 (N_{Ar}CH₃), 24.16 (C_{Ar}CH₂CH₂), 23.19 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CD₃OD)** δ 18.24; **HRMS (ESI)** *m/z* 583.1292 [M]⁺ (calculated for [C₁₉H₃₆Cl₄N₆O₄P]⁺ 583.1284).

3-{5-[(bis[(2-chloroethyl)amino]phosphoryl)oxy)methyl]-1-*N'*-methyl-2-nitro-1*H*-imidazol-4-yl}-*N,N,N*-trimethylpropan-1-aminium iodide (**26c**) was prepared from amine borane complex **24c-BH₃** (67.0 mg, 0.146 mmol) as described for the preparation of **15a** (methyl iodide 15 eq. at rt for 2 h). QA compound **26c** (47.9 mg, 81.6 μ mol) was obtained as a highly hygroscopic yellow powder. **Purification conditions:** detection: 254 nm and 338 nm; separation time: 30 min; eluent: H₂O/ACN (v/v): 95/5 for 2 min; 95/5 → 70/30 for 6 min; 70/30 for 2 min; 70/30 → 60/40 for 6 min; 60/40 for 3 min; 60/40 → 10/90 for 7 min; 10/90 for 4 min; retention time : 14.1 min; **Yield** 56%; **IR (ATR)** ν cm⁻¹ 3246, 1496, 1329, 1211, 1002, 990, 979; **¹H NMR (500 MHz, CD₃OD)** δ 5.11 (d, 1H, ³J_{H-P} = 9.4 Hz, CH₂O), 4.06 (s, 3H, N_{Ar}CH₃), 3.57 (t, 4H, ³J = 6.3 Hz, NHCH₂CH₂Cl), 3.47-3.38 (m, 2H, CH₂N⁺(CH₃)₃), 3.20 (td, 4H, ³J_{H-P} = 12.5 Hz, ³J_{H-H} = 6.3 Hz, NHCH₂CH₂Cl), 3.15 (s, 9H, CH₂N⁺(CH₃)₃), 2.79 (t, 2H, ³J = 7.3 Hz, C_{Ar}CH₂CH₂), 2.24-2.16 (m, 2H, CH₂CH₂CH₂); **¹³C NMR (126 MHz, CD₃OD)** δ 146.48 (C_{Ar}NO₂), 140.13 (C_{Ar}CH₂CH₂), 131.52 (d, ³J_{C-P} = 6.5 Hz, C_{Ar}CH₂O), 66.92, 66.94, 66.90 (CH₂N⁺(CH₃)₃), 56.57 (d, ²J_{C-P} = 4.8 Hz, CH₂O), 53.91, 53.88, 53.84 (CH₂N⁺(CH₃)₃), 45.88 (d, 2C, ²J_{C-P} = 5.0 Hz, NHCH₂CH₂Cl), 44.18 (2C, NHCH₂CH₂Cl), 35.44 (N_{Ar}CH₃), 24.12 (C_{Ar}CH₂CH₂), 23.34 (CH₂CH₂CH₂); **³¹P NMR (202 MHz, CD₃OD)** δ 17.20; **HRMS (ESI)** *m/z* 459.1446 [M]⁺ (calculated for [C₁₅H₃₀Cl₂N₆O₄P]⁺ 459.1438).

4.2. Stability measurements in aqueous buffer. Prodrugs were dissolved in Phosphate Buffer Saline (PBS) (10 mM, pH 7.4, 37 °C) containing 0.13% DMSO at a concentration of 17 μ M and incubated at 37 °C. Aliquots were withdrawn at various time points over a 24 h period and directly subjected to RP-HPLC analysis. The HPLC chromatogram peak area at 322 nm was used to calculate the concentration of the remaining prodrug. Analytical RP-HPLC measurements were performed on a HP1100 (Agilent, Palo Alto, CA, USA). The separation was carried out on a C₁₈ column (Phenomenex, Luna C18, 3.0 × 150 mm, 3 μ m) using the following conditions: C₁₈ guard column from Phenomenex (Le Pecq, France) total experiment time: 20 min, flow rate = 0.4 mL/min, eluent mixture: H₂O/ACN (v:v) containing trifluoroacetic acid (15 mM), gradient: 80/20 at 0 min → 75/25 at 3 min, then 75/25 → 60/40 for 2 min, then 60/40 → 50/50 for 5 min, then 50/50 → 80/20 for 1 min, then 80/20 for 9 min.

4.3 ³¹P NMR Kinetics of chemical activation. Prodrug (\approx 10 mg) were dissolved in ACN (90 μ L) and cacodylate buffer (300 μ L, 0.1 M, pH 7.4) while sodium dithionite was dissolved in

cacodylate buffer. 30 μL of the solution containing 3 eq. of sodium dithionite were added to the prodrug solution. The reaction mixture was transferred to a 5 mm NMR tube and the data acquisition was started. Spectra were taken at different time intervals over a 24 h period, and time points for each spectrum were assigned from the initiation of the reaction. Chemical shifts were reported relative to the signal of a Ph_3PO solution (5% in $\text{DMSO-}d_6$) as a coaxial reference (26 ppm). The temperature of the probe was maintained at 37 $^\circ\text{C}$ using the Bruker variable temperature unit. The disappearance of the starting material as well as the appearance of the phosphoramidate anion and their relative concentrations were determined by measuring peak areas.

4.4. Nitroreductase assay. Recombinant *E. coli* nitroreductase and dihydronicotinamide adenine dinucleotide (NADPH, reduced form, tetrasodium salt) were purchased from Sigma–Aldrich. 10 μL of a prodrug stock solution (1.7 mM) in ultra-pure water with 13% DMSO were added to 1000 μL of PBS (10 mM, pH 7.4, 37 $^\circ\text{C}$) containing NADPH. The reaction was initiated by addition of *E. coli* nitroreductase (25 μL of an initial solution, 1.0 mg/mL in PBS; final concentrations: prodrug: 17 μM ; NADPH: 0.9 mM; nitroreductase: 25 $\mu\text{g}/\text{mL}$). Aliquots were withdrawn at various time points over a 2 h period and the reactions were followed by RP-HPLC as a function of time. The separation was carried out in the same conditions as previously described for phosphate buffer stability. The HPLC chromatogram peak area at 322 nm was used to calculate the concentration of the remaining prodrug. Between the time points, all solutions were incubated at 37 $^\circ\text{C}$. Reference solutions containing 10 μL of compound stock solution and 100 μL NADPH solution (9 mM) completed with PBS (1000 μL final volume), were prepared and analyzed under the same conditions in order to distinguish between nitroreductase-based activation and enzyme-free reaction.

4.5. In vitro anti-proliferation assay. Human HEMC-SS chondrosarcoma cell line was obtained from the European Collection of Authenticated Cell Cultures, maintained in DMEM/F12 medium (Life Technologies) supplemented with 10% of fetal calf serum (Dutscher) and 4 $\mu\text{g}/\text{mL}$ gentamicin and maintained in a 5% CO_2 humidified environment at 37 $^\circ\text{C}$. After trypsinization, HEMC-SS cells were seeded in 96-well plates at a density of $2 \cdot 10^4$ cells in 150 μL of culture medium and allowed to adhere overnight. Increasing concentrations of drugs diluted in DMSO (maintaining final DMSO concentration at 0.5% (v/v)) were added. After plate incubation for 24 h in normoxic (21% O_2 , 5% CO_2 , 37 $^\circ\text{C}$) or hypoxic (N_2 , $\text{O}_2 < 0.3\%$, 37 $^\circ\text{C}$) conditions, the culture media was removed and cell layers washed with PBS. Cells were then left to grow for 48 h in normoxic conditions. Viability of cells cultured was quantified by AlamarBlue assay. Cytotoxic activity was expressed as the drug concentration that inhibited cell growth by 50% (IC_{50}). Experiments were performed at least in triplicates. Data are presented as means \pm SD. Statistical significance was determined using Student's a t-Test. Results were considered significant at $p < 0.05$.

Acknowledgements:

The authors would like to thank Fernand Leal for his technical support on IR measurement.

Funding : This work was supported by the Ligue contre le cancer (2017-2018, projet reference R16146CC / RAB16025CCA) and PRT-K, Translational Cancer Research: INCa-DGOS, D-TECT (project reference R15066CC / RPT15003CCA)

Available Supporting Information: Synthesis and characterization data of compounds **1**, **4**, **12**, **PM**, **TPM**, **IPM** and **27a-c**; Identification of compound **11**; Crystallographic data; Reduction experiments with compounds **27a-b**; ¹H NMR, ¹³C NMR and ³¹P NMR Spectroscopic Data for all synthesized compounds.

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