# SYNTHESIS OF $^{15}N$ and $^{17}O$ LABELLED PHOSPHORAMIDE MUSTARDS

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## SUMMARY

The cyclophosphamide metabolite phosphoramide mustard (PM) was synthesized with isotopic enrichment at each nitrogen and oxygen site. Sequential reaction of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride [$Cl_2P(O)N(CH_2CH_2Cl)_2$] with benzyl alcohol and ammonia gave *N,N*-bis(2-chloroethyl)phosphorodiamidic acid phenylmethyl ester [$BzO(H_2N)P(O)N(CH_2CH_2Cl)_2$]. Catalytic hydrogenation of this benzyl ester followed by the addition of cyclohexylamine (CHA) provided PM as the CHA salt. Incorporation of $^{15}NH_3$ into this general scheme gave PM with a $^{15}NH_2$ moiety. Glycine-$^{15}N$ was converted to bis(2-chloro-ethyl)amine-$^{15}N$ hydrochloride which, in turn, provided for *N,N*-bis(2-chloroethyl)phosphoramidic-$^{15}N$ dichloride. Use of this compound in the general synthetic pathway yielded PM•CHA with $^{15}N$ in the mustard moiety. $^{17}O$-Enriched PM was generated through the use of benzyl alcohol-$^{17}O$. To obtain the alcohol, labelled benzaldehyde was made by exchange with $^{17}OH_2$ and was then reduced with sodium borohydride.

***Key words.*** $^{15}N$-Phosphoramide mustard; $^{17}O$-phosphoramide mustard; bis(2-chloro-ethylamine-$^{15}N$; benzyl alcohol-$^{17}O$; cyclophosphamide.

## INTRODUCTION

Cyclophosphamide is clinically effective against a broad spectrum of human cancers. It is generally accepted that this drug undergoes a cascade of metabolic transformations, ultimately generating the DNA bisalkylating agent phosphoramide mustard (PM, 1).[1-3] Through deuterium labelling studies, it has been demonstrated that PM alkylates nucleophiles *via* the intermediacy of electrophilic aziridinium ions.[4] The kinetics of this bimolecular alkylation reaction as well as those of

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competing *intra*molecular alkylation reactions can vary significantly among PM and its analogs.[5,6]

The alkylation chemistry of PM and its attendant intermediates in the alkylation process must be directed by charge densities, particularly those at the nitrogen and oxygen sites. NMR spectroscopy is one technique that can probe electron density; NMR-derived electronic parameters can be expressed in a quantitative ($pK_a$ values) or qualitative (chemical shift comparisons) manner. NMR studies of the electronic modulations at the specific nuclei of interest could be best accomplished using isotopically labelled materials. For this purpose then, we have synthesized the following phosphoramide mustards: 1) PM with $^{15}N$ incorporated in the $NH_2$ functionality [*N',N'*-bis(2-chloroethyl)phosphorodiamidic-$^{15}N$ acid (**1a**)]; 2) PM with $^{15}N$ in the mustard moiety [*N,N*-bis(2-chloroethyl)-phosphorodiamidic-$^{15}N$ acid (**1b**)]; and 3) PM with $^{17}O$ enrichment at the equivalent oxygen atoms [*N,N*-bis(2-chloroethyl)phosphorodiamidic acid-$^{17}O$ (**1c**)].

## DISCUSSION

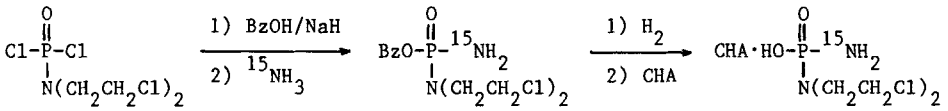
***Synthesis of N',N'-Bis(2-chloroethyl)phosphorodiamidic-$^{15}N$ Acid (1a).*** Using modifications to published syntheses,[7-9] incorporation of $^{15}N$ into the $NH_2$ moiety of PM was accomplished as shown in Scheme I. In short, *N,N*-bis(2-chloroethyl)-phosphoramidic dichloride was reacted sequentially with sodium benzylate and $^{15}NH_3$ (98 atom % $^{15}N$) to provide *N',N'*-bis(2-chloroethyl)phosphorodiamidic-$^{15}N$ acid phenylmethyl ester (**2a**, 14%). Catalytic hydrogenation of **2a** followed by the addition of cyclohexylamine (CHA) and recrystallization gave *N',N'*-bis(2-chloroethyl)phosphorodiamidic-$^{15}N$ acid as the more stable cyclohexylammonium salt (**1a**•CHA, 58%).

The low yield (average: 13 ± 1%) of benzyl phosphorodiamidate **2a** could be improved by a modification[10] which was subsequently investigated using unlabelled materials. This pathway utilized *n*-butyllithium in place of sodium hydride and also employed excess ammonia. In brief, a THF solution of *n*-butyllithium (1 equiv) and benzyl alcohol (1 equiv) was stirred for 2 hrs at -23 °C. The reaction mixture was then added to a THF solution of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (1 equiv) at -23 °C. Over two hours, the reaction mixture warmed to

0 °C and then excess ammonia was added as described in the Experimental Section for the synthesis of **2b**. Flash chromatography (ethyl acetate - hexane, *ca.* 1:1, $R_f$ 0.2) afforded unlabelled **2** in an average yield of 49 ± 2%. A major factor in the increased yield was presumably the presence of excess ammonia. This was apparent in the synthesis of **2b** (*vide infra*) when the yield of labelled phosphorodiamidate increased 2.5-fold as a result of using excess ammonia. However, since the synthesis of **2a** utilized $^{15}N$-ammonia, increased yield would have been offset by increased cost of labelled starting material. On the other hand, a comparison of the yields given by the synthesis of **2b** (36%) and unlabelled **2** (49%) indicated that use of *n*-butyllithium also increased the yield with other factors being equal.

**Scheme I**

[In all schemes: Bz = benzyl; CHA = cyclohexylamine.]




**2a** **1a·CHA**

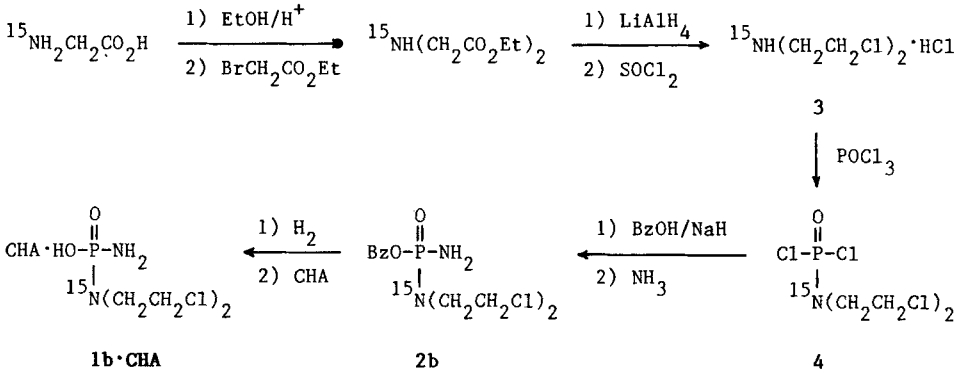
The hydrogenation of **2a** to provide **1a** also gave an impurity which was 15 - 30% of the product mixture (as judged by $^{31}P$ NMR). The impurity was not identified; however, some structural features were apparent from $^{31}P$ NMR spectral data ($\delta$ 9.7, d, $^1J_{31P\text{-}15N}$ = 25 Hz, 0.05 M phosphate, pH 7.4, 5 °C). The sensitivity of the $^{31}P$ chemical shift to pH indicated the presence of a P-OH functionality. In addition, $^{31}P$-$^{15}N$ coupling was evidence for a P-$^{15}NH_2$ moiety. The *absence* of $^{31}P$-$^{15}N$ coupling in the same impurity generated in the synthesis of **2b** (with $^{15}N$ in the mustard group) indicated the absence of a bis(2-chloroethyl)amino group.

The impurity formed during the hydrogenation reaction could be minimized through the use of fresh Pd/C catalyst. On the other hand, varying the reaction time from 1 - 4 hrs did not change the product distribution. Since the impurity was less soluble in ether than the desired product, careful addition of ether to

ethanolic solutions of crude product resulted in the formation of a crystalline mixture enriched in impurity. Pure **1a•CHA** was then isolated from the mother liquor in 58% yield.

***Synthesis of N,N-Bis(2-chloroethyl)phosphorodiamidic-$^{15}N$ Acid (1b).*** For incorporation of $^{15}N$ into the nitrogen mustard functionality of **1b**, the synthesis of bis-(2-chloroethyl)amine-$^{15}N$ hydrochloride (*nor*-nitrogen mustard, **3**) was a key target. As outlined in Scheme II, this intermediate was made from glycine-$^{15}N$ (98 atom % $^{15}N$) using modifications of a previously published pathway for a $^{13}C$ labelled *nor*-nitrogen mustard.[11] Reaction of **3** with phosphorus oxychloride provided *N,N*-bis(2-chloroethyl)phos-phoramidic-$^{15}N$ dichloride (**4**). This was then converted to **1b•CHA** (30%) through the intermediacy of **2b** (36%) as described above for **1a•CHA**.

**Scheme II**

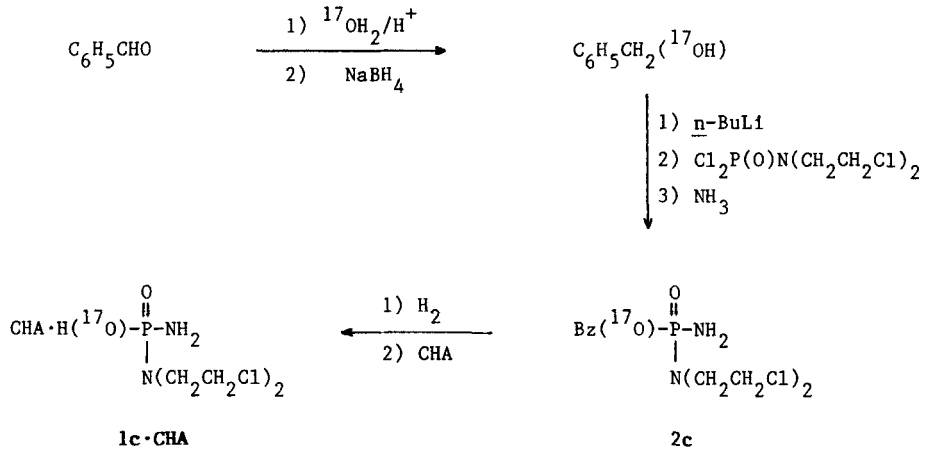



The synthesis of glycine-$^{15}N$ was briefly investigated using $^{15}NH_3$ and chloroacetic acid.[12] Based on our yield of labelled glycine, a modest monetary savings of perhaps 5 - 10% could be realized by starting with the less expensive $^{15}NH_3$.

***Synthesis of N,N-Bis(2-chloroethyl)phosphorodiamidic Acid-$^{17}O$ (1c).*** Literature procedures for the synthesis of $^{18}O$-labelled benzyl alcohol were adapted to $^{17}O$

incorporation.[13-15] Thus, labelled benzaldehyde was made by exchange with $^{17}O$-enriched water (10 atom % $^{17}O$) and this aldehyde was then reduced with sodium borohydride to give benzyl alcohol-$^{17}O$. As shown in Scheme III, benzyl alcohol-$^{17}O$ thus provided for the synthesis of 2c and, ultimately, **1c·CHA**. In the final product, the two oxygen atoms were equivalent and this resulted in label incorporation at both centers.

**Scheme III**




**1c·CHA** **2c**

Isotopic enrichment was measured in 2c by comparing the peak heights and integrated intensities of the $^{31}P$ NMR signals for the compound with a $^{31}P$-$^{17}O$ moiety [($CDCl_3$) $\delta$ 15.5] versus that with $^{31}P$-$^{16}O$ [($CDCl_3$) $\delta$ 15.9]. The ratio was 9:91, respectively, which correlated well with the theoretical, maximum $^{17}O$-enrichment (8.5%) calculated for our experimental conditions. The same result was obtained by comparing the $^{13}C$ NMR peak heights for the benzylic carbons in 2c [($CDCl_3$) $\delta$ 65.3 (9%, $^{13}C$-$^{17}O$) and 67.2 (91%, $^{13}C$-$^{16}O$)]. The consistency of these numbers among different nuclei provided support for the signal assignments. In the $^{31}P$ NMR spectra of final product **1c·CHA** (0.1 M NaCl, 4°C), signal overlap for $^{31}P$-$^{17}O$ ($\delta$ 13.07) and $^{31}P$-$^{16}O$ ($\delta$13.10) prevented a reliable calculation of enrichment. For all the isotopic enrichment measurements by NMR, accurate peak intensities were obtained using gated decoupling during acquisition with long interpulse delays to avoid both saturation and nuclear Overhauser effects.

## CONCLUSION

The work described herein details the syntheses of $^{15}N$- and $^{17}O$-labelled phosphoramide mustards. The syntheses were accomplished by a sequential substitution of each chloride in $P(O)Cl_3$ with ammonia, *nor*-nitrogen mustard, and benzyl alcohol. Incorporation of $^{15}N$ or $^{17}O$ into each of these nucleophiles provided for the title compounds.

## EXPERIMENTAL SECTION

Benzene, cyclohexylamine, phosphorus oxychloride, tetrahydrofuran (THF), thionyl chloride, and triethylamine refer to dried and/or distilled solvents and reagents. Reaction mixtures that did not include water were carried out under nitrogen. Reactions done at 5 °C refer to ice bath conditions while those at -23 °C used a dry ice/$CCl_4$ bath. Unlabelled diethyl iminodiacetate was purchased from Eastman Kodak; unless specified otherwise, all other solvents and reagents were generally purchased from Aldrich Chemical Company or Fisher Scientific Company. Authentic, unlabelled phosphoramide mustard (cyclohexylammonium salt) was a gift from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Analytical TLC employed 2.5 cm x 10 cm plates coated with a 250 $\mu$m layer of silica gel GF (Analtech); $I_2$ and a 254-nm UV lamp were used for component visualization. Silica gel from EM Reagents (< 230 mesh) was used for flash chromatography; all other chromatography columns used silica gel from J.T. Baker Chemicals (60 - 200 mesh). Melting points were generally obtained on a Fisher-Johns Melting Point Apparatus and are uncorrected. Hydrogenations were carried out using a Parr medium-presure shaker-hydrogenator and 50- or 500-mL pressure bottles.

NMR spectra were obtained on 8.5 T Bruker AM360 and 11.8 T Bruker MSL500 spectrometers; post-acquisition processing was accomplished with a Bruker Workstation. $^1H$ and $^{13}C$ NMR chemical shifts (ppm) are referenced to TMS ($CDCl_3$) or TSP ($D_2O$). $^{31}P$ NMR chemical shifts (ppm) refer to a capillary insert of 25% $H_3PO_4$ in

$D_2O$. $^{17}O$ NMR chemical shifts (ppm) are referenced to natural abundance $^{17}O$-water (0.035%) using a capillary insert. $^{15}N$ NMR chemical shifts are referenced to $^{15}N$-urea (at 56.5 ppm relative to $^{15}NH_4Cl$ at pH 7.0) in 0.1 M sodium phosphate (pH 7.0) in a capillary insert.

$^{31}P$ NMR spectra were obtained using an inverse gated decoupling sequence with an acquisition time of 0.4 s and a relaxation delay of 3 s. Under these conditions, the relative resonance intensities represented actual concentrations since saturation and nuclear Overhauser effects were eliminated.

$^{15}N$ NMR spectra of protonated nitrogens were acquired using a refocussed INEPT sequence with proton decoupling during the acquisition. The spectra of non-protonated nitrogens were acquired using an inverse-gated decoupling sequence similar to that used for $^{31}P$ acquisition.

$^{17}O$ NMR spectra were acquired without proton decoupling.

***N',N'*-BIS(2-CHLOROETHYL)PHOSPHORODIAMIDIC-$^{15}N$ ACID PHENYLMETHYL ESTER (2a).** A solution of benzyl alcohol (1.03 mL, 10.0 mmol) in benzene (10 mL) was added dropwise to a suspension of washed (benzene) NaH (530 mg of a 57% oil dispersion, 12.6 mmol) in benzene (10 mL) at 5 °C. Following complete addition, the reaction mixture stirred overnight at room temperature. The resultant sodium benzylate was added *via* syringe to a solution of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (2.58 g, 10.0 mmol) in benzene (10 mL) at 5 °C. After stirring overnight at room temperature, the reaction mixture was filtered, dried ($MgSO_4$), and concentrated to give *N,N*-bis(2-chloroethyl)phosphoramidic chloride phenylmethyl ester [3.00 g, 9.10 mmol, $R_f$ 0.81 (ethyl acetate)].

A glass vessel of $^{15}NH_3$ (250 mL 'break-seal' flask, *ca.* 10 mmol, Aldrich Chemical Co., 98 atom % $^{15}N$) was wired with a septum and was cooled in an ice bath. The glass seal was broken and a solution of triethylamine (1.39 mL, 10.0 mmol) in benzene (10 mL) was introduced into the vessel *via* syringe. This was followed by the similar addition of a solution of crude *N,N*-bis(2-chloroethyl)-phosphoramidic chloride phenylmethyl ester (3.00 g, 9.10 mmol, *vide supra*) in benzene (10 mL). The reaction flask was kept cold (ice) and agitated occasionally

over 3 h. After standing at room temperature for 3 days, the vessel was opened carefully and the reaction mixture was then filtered. The filtrate was concentrated and the resultant material was purified by flash chromatography (4.7 cm x 6.5 in column, ethyl acetate). The product (**2a**) was obtained as an oil which solidified at 5 °C [0.42 g, 14% yield, $R_f$ 0.32 (ethyl acetate), mp 55 - 58 °C]. $^1H$ NMR (360 MHz, $CDCl_3$) $\delta$: 7.45 - 7.30 (m, 5H, aromatic), 5.11 - 4.96 (m, 2H, $CH_2O$), 3.61 (td, $^3J_{HH}$ = 6.5 Hz, $^4J_{HP}$ = 2.0 Hz, 4H, $CH_2Cl$), 3.52 - 3.33 (m, 4H, $CH_2N$), and 2.71 (dd, $^1J_{H-15N}$ = 81 Hz, $^2J_{HP}$ = 4.3 Hz, 2H, $^{15}NH_2$). $^{31}P$ NMR (202.46 MHz, $CDCl_3$) $\delta$ 15.93 (d, $^1J_{31P-15N}$ = 37.4 Hz). $^{15}N$ NMR (50.70 MHz, $CHCl_3$) $\delta$ 17.81 (d, $^{15}NH_2$).

***N',N'*-BIS(2-CHLOROETHYL)PHOSPHORODIAMIDIC-$^{15}N$ ACID CYCLOHEXYLAMMONIUM SALT (1a•CHA).** A mixture of **2a** (350 mg, 1.12 mmol) and Pd/C (10%, 101 mg) in absolute ethanol (10 mL) was hydrogenated at 50 psi for 4 h. The pressure was released and nitrogen was bubbled through the reaction mixture for several minutes. The mixture was diluted with absolute ethanol (10 mL) and to this was added cyclohexylamine (0.260 mL, 2.27 mmol, 2 equiv). After stirring at room temperature for 2.5 h, the suspension was suction filtered through a pad of Celite and the filtrate was concentrated at ambient temperature. The resultant solids (83% crude yield) were washed with ether (1 mL) and they were then dissolved in minimal ethanol. Ether was added to turdidity and the mixture sat at 5 °C for one h. The mother liquor was removed by pipet and the residual solids were washed with ether. The ether washings were added to the mother liquor and this solution was then concentrated at room temperature to give the product (**1a•CHA**) as a white powder (209 mg, 58% yield, mp 112 - 114 °C). $^1H$ NMR (500 MHz, 0.05 M phosphate/$D_2O$, pD 7.4, 5 °C) $\delta$: 3.65 (t, $J$ = 7.0 Hz, 4H, $CH_2Cl$) and 3.37 - 3.28 (m, 4H, $CH_2N$); and for the cyclohexylammonium ion, 3.18 - 3.09 (m, 1H), 2.04 - 1.95 (m, 2H), 1.85 - 1.76 (m, 2H), 1.70 - 1.63 (m, 1H), 1.40 - 1.27 (m, 4H), and 1.24 - 1.12 (m, 1H). $^{31}P$ NMR (202.46 MHz, 0.10 M phosphate, pH 7.0, 4 °C) $\delta$ 13.35 (d, $^1J_{31P-15N}$ = 18 Hz). $^{15}N$ NMR (50.70 MHz, 0.10 M phosphate, pH 7.4, 4 °C) $\delta$ 23.42 (d, $^{15}NH_2$).

**GLYCINE-$^{15}N$ ETHYL ESTER HYDROCHLORIDE.** Gaseous HCl was bubbled (5 min) through a suspension of glycine-$^{15}N$ (3.00 g, 39.4 mmol, Aldrich and Sigma Chemical Companies, 98 atom % $^{15}N$) in absolute ethanol (90 mL). The resultant clear solution was allowed to stir overnight during which time the product precipitated from solution (ethanol was added as needed to facilitate stirring). Residual HCl was evacuated (water aspirator) and the reaction mixture was then concentrated to afford the product as a white powder [5.36 g, 97% yield, mp 141 °C (mp for unlabelled material: 145 - 146 °C)]. $^1H$ NMR (360 MHz, $D_2O$) $\delta$: 4.18 (q, $J$ = 7.2 Hz, 2H, $OCH_2$), 3.79 (s, 2H, $^{15}NCH_2$), and 1.17 (t, $J$ = 7.2 Hz, 3H, $CH_3$).

**DIETHYL IMINODIACETATE-$^{15}N$.** Triethylamine (5.3 mL, 38 mmol) was added to an ice-cooled suspension of glycine-$^{15}N$ ethyl ester hydrochloride (5.4 g, 38 mmol) in $CHCl_3$ (40 mL). The mixture was allowed to stir for several minutes and then a solution of ethylbromoacetate (5.6 mL, 51 mmol, 1.3 equiv) and triethylamine (5.3 mL, 38 mmol) in $CHCl_3$ (40 mL) was added dropwise. The reaction mixture was stirred at room temperature for 3 days and was then filtered and concentrated. The residual material was chromatographed on silica gel (4.7 cm x 41 cm column, $CHCl_3$ - $CH_3OH$, 9:1) giving the product as a yellow oil [4.5 g, 61% yield, $R_f$ 0.78 ($CHCl_3$ - $CH_3OH$, 9:1, $I_2$ repellent TLC spot)]. $^1H$ NMR (360 MHz, $CDCl_3$) $\delta$: 4.20 (q, $J$ = 7.2 Hz, 4H, $OCH_2$), 3.46 (s, 4H, $^{15}NCH_2$), and 1.28 (t, $J$ = 7.2 Hz, 6H, $CH_3$).

**DIETHANOLAMINE-$^{15}N$.** A solution of diethyl iminodiacetate-$^{15}N$ (4.81 g, 25.0 mmol) in THF (25 mL) was added dropwise to an ice-cooled suspension of $LiAlH_4$ (4.51 g, 119 mmol, 4.8 equiv) in THF (100 mL). Upon complete addition, the reaction mixture was stirred at 5 °C (5 min), at room temperature (20 min), and then at reflux (overnight). After the suspension was cooled to room temperature, $H_2O$ (2.0 mL), 15% NaOH (2.0 mL), and another portion of $H_2O$ (6.1 mL) were added slowly and sequentially. The reaction mixture was stirred for 2 h and was then suction filtered. The filtrate was concentrated and the residue was taken up in $CHCl_3$, dried ($MgSO_4$), filtered, and concentrated to provide the product as an oil (0.61

g, 24% yield) which was pure by TLC [$R_f$ 0.25 ($CHCl_3$ - $CH_3OH$, 8:2)]. Soxhlet extraction (THF, 4 days) of the reaction solids gave additional product (1.72 g, 64% yield) which showed some minor impurities by TLC but which was used in subsequent reactions without further purification. Total crude yield: 2.33 g, 88%. $^1H$ NMR (360 MHz, $CDCl_3$) $\delta$: 3.78 - 3.61 (m, 4H, $CH_2O$), 2.81 (t, $J$ = 5.1 Hz, 4H, $^{15}NCH_2$), and 2.30 -1.90 (br s, 3H, $^{15}NH$ and OH).

**BIS(2-CHLOROETHYL)AMINE-$^{15}N$ HYDROCHLORIDE (3).** A solution of thionyl chloride (4.26 mL, 58.3 mmol, 1.8 equiv) in $CHCl_3$ (distilled, 14 mL) was added dropwise to a solution of diethanolamine-$^{15}N$ (1.72 g, 16.2 mmol) in $CHCl_3$ (15 mL). Upon complete addition, the reaction was refluxed for 2 h. The brown mixture was then stored at -20 °C overnight and this afforded light brown crystals (1.47 g, 51% yield) which were used in subsequent reactions without further purification. Addition of ether to the filtrate provided product which was more impure (0.25 g, 9% yield). Total crude yield: 1.72 g, 60%. $^1H$ NMR (360 MHz, $D_2O$) $\delta$: 3.94 - 3.90 (m, 4H, $CH_2Cl$) and 3.53 (t, $J$ = 5.5 Hz, 4H, $^{15}NCH_2$).

**BIS(2-CHLOROETHYL)PHOSPHORAMIDIC-$^{15}N$ DICHLORIDE (4).** Pyridine (57 $\mu$L, 0.70 mmol) was added to a suspension of 3 (0.13 g, 0.70 mmol) in benzene (5 mL) at 5 °C. After stirring 10 min, a second molar equivalent of pyridine (57 $\mu$L, 0.70 mmol) was added followed by phosphorus oxychloride (65 $\mu$L, 0.70 mmol). The mixture was stirred overnight before filtration and concentration. The residual oil was chromatographed on silica gel (product added to dry-packed column, 8 mm x 11 cm, $CHCl_3$) and product 4 was obtained as an oil which solidified at -20 °C [101 mg, 57% yield, $R_f$ 0.64 ($CHCl_3$)]. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$: 3.76 - 3.71 (m, 4H, $CH_2Cl$) and 3.71 - 3.63 (m, 4H, $^{15}NCH_2$). $^{31}P$ NMR (202.46 MHz, $CHCl_3$) $\delta$ 17.6 (d, $^1J_{31P\text{-}15N}$ = 17 Hz).

***N,N*-BIS(2-CHLOROETHYL)PHOSPHORODIAMIDIC-$^{15}N$ ACID PHENYLMETHYL ESTER (2b).** Benzyl alcohol (40 $\mu$L, 0.38 mmol) was added to a suspension of washed (benzene) NaH (20

mg of a 57% oil dispersion, 0.48 mmol) in benzene (2 mL) at 5 °C. The reaction stirred at 5 °C (30 min) and then at room temperature (2 h). The resultant sodium benzylate was then added *via* syringe to an ice-cooled solution of **4** (0.10 g, 0.38 mmol) in benzene (2 mL). Following complete addition, the reaction mixture was stirred at room temperature overnight. It was then cooled (ice bath) and gaseous ammonia was bubbled through the mixture (15 min). The flask was stoppered and stored at room temperature overnight. The mixture was then filtered and concentrated, and the residual material was chromatographed on silica gel (8 mm x 11 cm column, ethyl acetate). Product **2b** was recovered as an oil which solidified at -20 °C [44 mg, 36% yield, $R_f$ 0.39 (ethyl acetate)]. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$: 7.31 (s, 5H, aromatic), 5.01 - 4.89 (m, 2H, $CH_2O$), 3.57 - 3.48 (m, 4H, $CH_2Cl$), 3.41 - 3.27 (m, 4H, $^{15}NCH_2$), and 2.91 - 2.75 (br s, 2H, $^{15}NH_2$). $^{31}P$ NMR (202.46 MHz, $CDCl_3$) $\delta$ 15.9 (d, $^1J_{31P-15N}$ = 36 Hz).

***N,N*-BIS(2-CHLOROETHYL)PHOSPHORODIAMIDIC-$^{15}N$ ACID CYCLOHEXYLAMMONIUM SALT (1b•CHA).** A mixture of **2b** (44 mg, 0.14 mmol) and Pd/C (10%, 21 mg) in absolute ethanol (5 mL) was hydrogenated as described above in the synthesis of **1a•CHA**. Cyclohexylamine (32 $\mu$L, 0.28 mmol, 2 equiv) was added to the reaction mixture and this was stirred for 1.5 h. The crude product (47% crude yield) was purified as described above for **1a•CHA** and product **1b•CHA** was recovered as a powder (13 mg, 30% yield, mp 53 - 55 °C). $^1H$ NMR (500 MHz, 0.05 M phosphate/$D_2O$, pD 7.4, 5 °C) $\delta$: 3.66 (td, $J$ = 7.1, 1.1 Hz, 4H, $CH_2Cl$), 3.33 (dt, $J$ = 10.6, 7.1 Hz, 4H, $^{15}NCH_2$); and for the cyclohexylammonium ion, 3.21 - 3.12 (m, 1H), 2.05 - 1.96 (m, 2H), 1.88 - 1.75 (m, 2H), 1.71 - 1.63 (m, 1H), 1.41 - 1.30 (m, 4H), and 1.13 - 1.23 (m, 1H). $^{31}P$ NMR (202.46 MHz, 0.05 M phosphate/$D_2O$, pD 7.4, 5 °C) $\delta$: 13.36 (d, $^1J_{31P-15N}$ = 30 Hz). $^{15}N$ NMR (50.70 MHz, 0.10 M phosphate, pH 7.4, 4 °C) $\delta$ 31.5 (d, $^{15}NCH_2$).

**BENZYL ALCOHOL-$^{17}O$.** A solution of benzaldehyde (1.02 mL, 10.0 mmol) and water-$^{17}O$ (1.00 g, 55.2 mmol, MSD Isotopes, 10 atom % $^{17}O$) in 0.001 M HCl in THF (6 $\mu$L 1

M HCl in 6 mL THF) was stirred for 2 days at room temperature. THF was removed at reduced pressure and the residual aqueous phase was extracted with ether (2 x 5 mL). The ether layers were dried ($MgSO_4$) and concentrated, and the residual colorless liquid was dissolved in $CH_3OH$ (5 mL). This was added dropwise (*via* syringe) to a suspension of $NaBH_4$ (380 mg, 10.0 mmol, 4 equiv) in $CH_3OH$ (10 mL). After stirring overnight, a TLC analysis of the reaction mixture indicated very little product formation ($R_f$ 0.67 for benzaldehyde and 0.31 for benzyl alcohol in $CHCl_3$). More $NaBH_4$ (380 mg, 10 mmol) was added, causing the reaction mixture to reflux. After stirring 2 h, TLC showed no unreacted starting material. The mixture was neutralized (pH paper) with 1 M HCl and $CH_3OH$ was then removed at reduced pressure. The aqueous layer was extracted with $CH_2Cl_2$ (2 x 20 mL) and the organic layers were dried ($MgSO_4$) and concentrated to give crude benzyl alcohol-$^{17}O$ (613 mg, 57% yield). The product was identified by TLC comparison with unlabelled benzyl alcohol ($R_f$ 0.30, $CHCl_3$) and used without further purification.

***N,N*-BIS(2-CHLOROETHYL)PHOSPHORODIAMIDIC ACID PHENYLMETHYL ESTER-$^{17}O$ (2c).** A hexane solution of *n*-butyllithium (3.54 mL of 1.6 M, 5.66 mmol) was added dropwise *via* syringe to a solution of crude benzyl alcohol-$^{17}O$ (613 mg, 5.66 mmol) in THF (10 mL) at -23 °C. The resultant white suspension was stirred at this temperature for 1 h and was then added dropwise (syringe) to a solution of bis(2-chloroethyl)phosphoramidic dichloride (1.47 g, 5.66 mmol) in THF (10 mL) at -23 °C. The reaction mixture was stirred at this low temperature for 1.5 h and then at 5 °C for 0.5 h. Gaseous ammonia was then bubbled (5 min) through the ice-cooled mixture after which the flask was stoppered and stored overnight at room temperature. The suspension was filtered, concentrated, and chromatographed on silica gel (2 cm x 18 cm column, ethyl acetate). Product **2c** was recovered as a colorless oil which solidified to a white, wax-like solid upon standing at -20 °C [533 mg, 30% yield (assumes use of 5.66 mmol benzyl alcohol-$^{17}O$), $R_f$ 0.41 (ethyl acetate)]. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 7.41 - 7.30 (m, 5H, aromatic), 5.10 - 4.97 (m, 2H, $CH_2O$), 3.70 - 3.52 (m, 4H, $CH_2Cl$), 3.52 - 3.35 (m, 4H, $CH_2N$), and 2.95 -

2.80 (m, 2H, $NH_2$). $^{13}C$ NMR (126 MHz, $CDCl_3$) $\delta$ 136.4, 128.6, 128.4, and 127.8 (aromatic), 67.2 [d, $^2J_{13C-31P}$ = 4.1 Hz, $^{16}O$-$CH_2$ (91% of total benzylic carbon intensity)], 65.3 [d, $^2J_{13C-31P}$ = 6.1 Hz, $^{17}O$-$CH_2$ (9% of total benzylic carbon intensity)], 49.2 (d, $^2J_{13C-31P}$ = 4.7 Hz, $CH_2N$), and 42.5 ($CH_2Cl$). $^{31}P$ NMR (202 MHz, $CDCl_3$) $\delta$ 16.2 [$^{16}O$-$^{31}P$ (91% of phosphorus content)] and 15.8 [$^{17}O$-$^{31}P$ (9% of phosphorus content)]. $^{17}O$ NMR (68 MHz, $CDCl_3$) $\delta$ 64.1.

***N,N*-BIS(2-CHLOROETHYL)PHOSPHORODIAMIDIC ACID-$^{17}O$ CYCLOHEXYLAMMONIUM SALT (1c•CHA).** A mixture of **2c** (0.26 g, 0.84 mmol) and Pd/C (10%, 0.11 g) in absolute ethanol (10 mL) was hydrogenated at 50 psi for 3 h. The pressure was released and nitrogen was bubbled through the reaction mixture for several minutes. Absolute ethanol (10 mL) and cyclohexylamine (0.19 mL, 1.7 mmol, 2 equiv) were added and the mixture was then stirred for 1 h. The suspension was filtered and the filtrate was concentrated at ambient temperature. The residual solids (135 mg, 50% crude yield) were recrystallized as described above for **1a•CHA**. Following one recrystallization, a white microcrystalline solid enriched (*ca.* 50%) in product **1c•CHA** was obtained (56 mg). $^{31}P$ NMR (202.46 MHz, 0.1 M NaCl, pH 7.0, 4 °C) $\delta$ 13.10 ($^{16}O$-$^{31}P$) and 13.07 ($^{17}O$-$^{31}P$). $^{17}O$ NMR (68 MHz, 0.1 M NaCl, pH 6.9, 4 °C) $\delta$ 98.6. [For the unidentified impurity in samples of **1a•CHA**: $^{31}P$ NMR $\delta$ 9.46 ($^{16}O$-$^{31}P$) and 9.43 ($^{17}O$-$^{31}P$); and $^{17}O$ NMR $\delta$ 76.8.].

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