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Research Article

Synthesis of (^{3}H,^{33}P)-phosphoramide and -isophosphoramide mustards and metabolites (^{3}H)-chloroethylaziridine and -aziridine for studies of DNA alkylation

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Abstract: Reduction of diethyliminodiacetate with [^{3}H]-$LiAlH_4$ and then reaction with $SOCl_2$ gave bis(2-chloro-2-[^{3}H]-ethyl)amine hydrochloride. This compound, together with [^{33}P]-phosphorus oxychloride, provided for the synthesis of [^{3}H,^{33}P]-phosphoramide mustard (as its cyclohexylammonium salt) in three steps over 2 days. Similarly, 2-[^{3}H]-ethanolamine was reacted with $SOCl_2$ to give 2-chloro-2-[^{3}H]-ethylamine hydrochloride which, along with [^{33}P]-$POCl_3$, was used to synthesize [^{3}H,^{33}P]-isophosphoramide mustard in two steps over 1 day. ^{1}H NMR studies were carried out to determine optimal times for *in situ* formation and storage of chloroethylaziridine and aziridine. A solution of 10 mM bis(2-chloroethyl)amine hydrochloride in 0.1 M phosphate/D_2O, pD 7.9 at 37°C for 3 h gave chloroethylaziridine without contamination by starting material or hydrolysis products. For aziridine, the disappearance of 0.2 M 2-chloroethylamine hydrochloride in 2 M NaOD/D_2O at pD 14, 37°C, gave $k = 0.00455$ min^{-1} ($R^2 = 1.000$) and $\tau_{1/2} = 2.55$ h; no hydrolysis product was observed over the course of the NMR experiment (4 h). It was concluded that ~13 h (5 half-lives) of reaction time would yield a solution of aziridine which was relatively free of contaminants. Using these reaction conditions, ^{3}H-labeled chloroethylamines were used to synthesize 1-(2-chloro-2-[^{3}H]-ethyl)-2-[^{3}H]-aziridine and 2-[^{3}H]-aziridine *in situ*. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: phosphoramide mustard; isophosphoramide mustard; radiolabel; chloroethylaziridine; aziridine

Introduction

The phosphoramide mustard metabolites from the anticancer drugs cyclophosphamide and ifosfamide are generally believed to be the DNA crosslinking agents of therapeutic consequence; however, contributions to DNA alkylations from other metabolites must be considered as well.[1–3] In published[1] and unpublished work, we have shown that phosphoramide mustard (PM, **1**) and isophosphoramide mustard (IPM, **2**) form aziridinyl intermediates which partition between two pathways: alkylation and P–N bond hydrolysis (Schemes 1 and 2). In the absence of a strong nucleophile, P–N bond scission accounts for up to 90% of the observed products. Major hydrolytic products include chloroethylaziridine (CEZ, **3**) from PM and aziridine (**4**) from IPM. While CEZ is a bisalkylating agent and could crosslink DNA, aziridine is only a monoalkylator but is of interest as a possible source of point mutations.

A goal of this project was to synthesize radiolabeled phosphoramide mustards which could be used to study the contribution of each alkylating agent to crosslinking and/or monoalkylation of DNA.[4] The synthesis of PM and IPM, each radiolabeled with ^{33}P and ^{3}H, would allow for a determination of the extent of DNA alkylation due to (1) PM versus CEZ formed *in situ* from PM and (2) IPM versus aziridine formed *in situ* from IPM. DNA crosslinks formed by intact PM would be incorporated with ^{3}H and ^{33}P while crosslinks derived from CEZ would be labeled with only ^{3}H. For IPM, crosslinks would have to be formed from the intact phosphoramide mustard; monoalkylations of single DNA strands, however, would have different activities of ^{33}P and ^{3}H depending on the incidence of reaction with IPM or aziridine. ^{3}H-Labeled CEZ and

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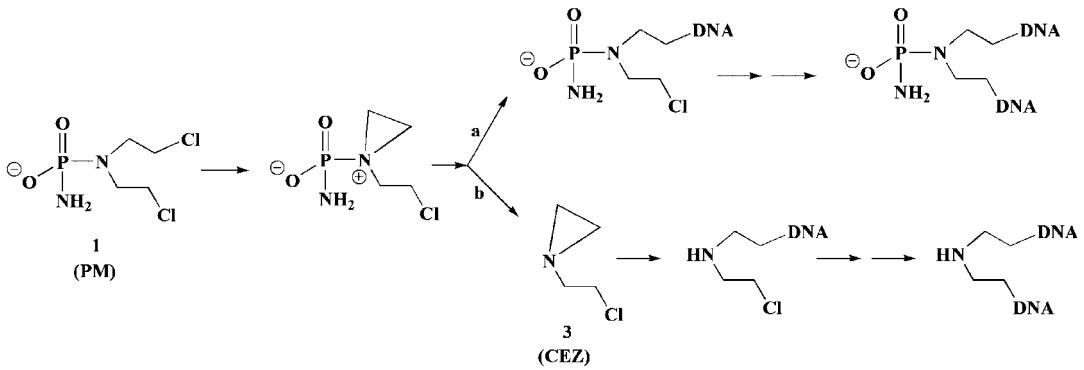
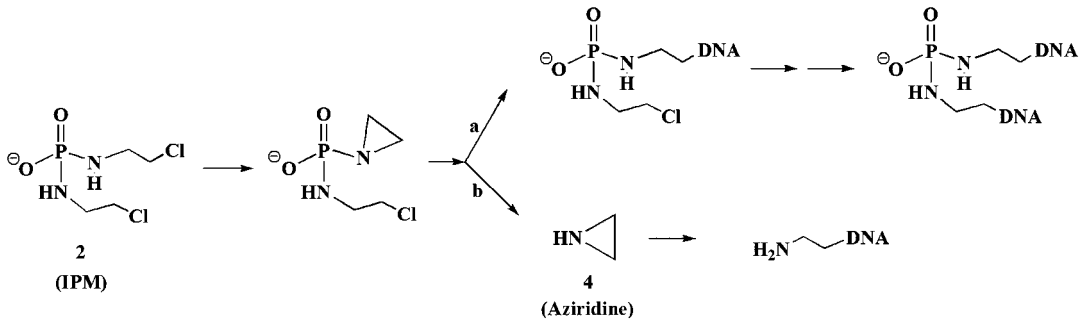


Scheme 1 Phosphoramide mustard (**1**): alkylation (a) versus P–N bond hydrolysis (b).



Scheme 2 Isophosphoramide mustard (**2**): alkylation (a) versus P–N bond hydrolysis (b).

aziridine were also synthesized for studies of their independent DNA alkylating activities.

Results and discussion

The syntheses of [^{3}H,^{33}P]-PM and -IPM are shown in Schemes 3 and 4, respectively. These pathways incorporate the major elements of published syntheses for ^{15}N, ^{17}O/^{18}O or ^{2}H-labeled phosphoramide mustards with some modifications to accommodate the use of radioactive materials.[5–8] Given the expense of the radiolabeled materials, the short half-life of ^{33}P (~25 days) and the isolation difficulties associated with a number of intermediates and products, various kinetic experiments were done with unlabeled materials so as to maximize yield and efficiency.

In the literature, many variations in solvent, base and reaction time have been used for the synthesis of **7** from $POCl_3$ and bis(2-chloroethyl)amine hydrochloride (**6**). After determining yields using unlabeled materials and different base/solvent combinations, pyridine and CH_2Cl_2 were selected for use in the radiosynthesis of **7**: Et_3N with THF (39% yield), C_6H_6 (50% yield) or CH_2Cl_2 (81% yield); pyridine with THF (7% yield), C_6H_6 (37% yield) or CH_2Cl_2 (87% yield). To determine the optimal reaction time for the synthesis of **7**, product formation was followed by ^{31}P NMR using 0.14 M bis(2-chloroethyl)amine hydrochloride (**6**) in CD_2Cl_2 with one molar equivalent of $POCl_3$ and two of pyridine. Spectra were taken at 9 time points over 13 h at a probe temperature of 20°C. Decrease of the resonance for $POCl_3$ was concomitant with an increase of a signal for **7**. A second-order kinetic plot for the disappearance of $POCl_3$ gave $\tau_{1/2} = 86$ min ($k = 0.173\ M^{-1}\ min^{-1}$; R^2 0.968); thus, it was estimated that product formation would near completion (>98%) after 8–9 h (6 half-lives).

CEZ (**3**) and aziridine (**4**) are volatile compounds and no attempts were made to isolate them. Rather, studies using unlabeled starting materials were carried out to determine optimal times for formation and storage in solution. For CEZ, a solution of 10 mM bis(2-chloroethyl)amine hydrochloride (**6**) in 0.1 M phosphate/D_2O, pD 7.9 (adjusted with 40% NaOD/D_2O) was heated at 37°C for 3 h. The pD was adjusted to 7 with conc HNO_3 and the solution was analyzed by ^{1}H NMR.[1] (*Note:* Loss of chloride from a 2-chloroethylamine to give an aziridinyl moiety is a reversible reaction; therefore, use of HCl for pH adjustments is to be avoided in kinetic experiments.) Only the desired product was observed and it was concluded that these

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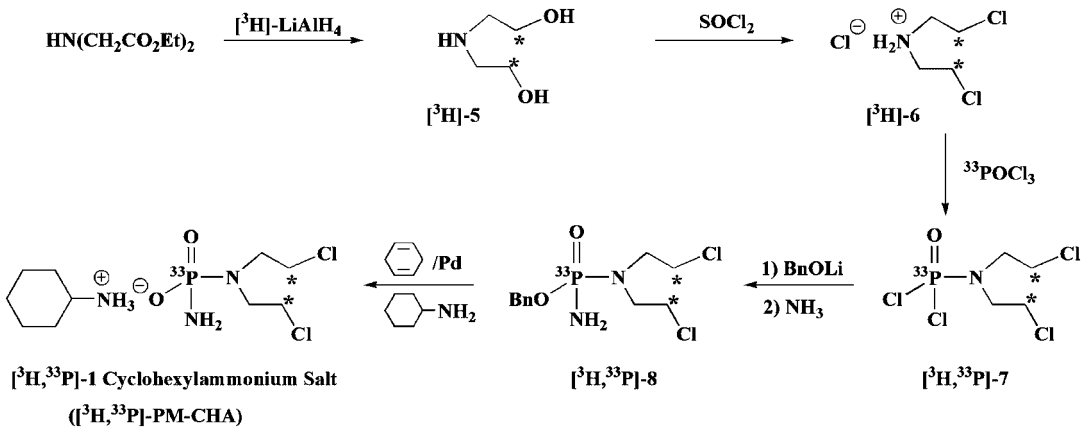
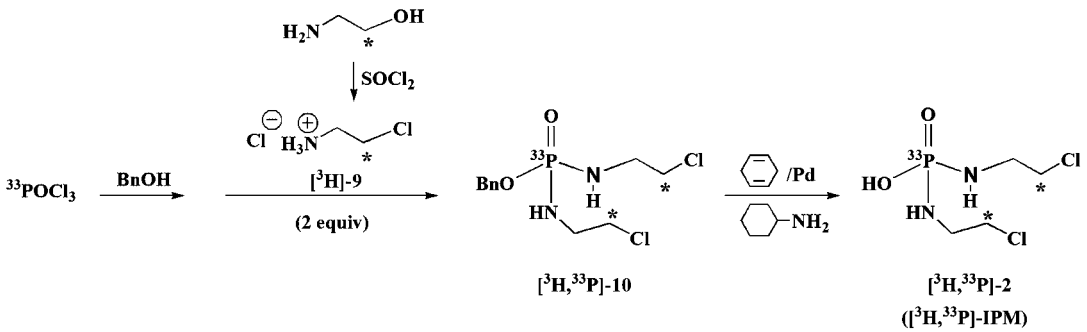


Scheme 3 Synthesis of [^{3}H,^{33}P]-phosphoramide mustard ([^{3}H,^{33}P]-**1**).



Scheme 4 Synthesis of [^{3}H,^{33}P]-isophosphoramide mustard ([^{3}H,^{33}P]-**2**).

conditions (3 h, 37°C, pD 7.9) would provide buffered solutions of CEZ without contamination by starting material or degradation (hydrolysis) products. These solutions of CEZ were predicted to be reasonably stable at low temperature (less than 5% decomposition after about a week at −23°C assuming the limit of NMR detection to be 5% and a two-fold rate deceleration for every drop of 10°C). The stability of CEZ at pD 7.9 (pH ~ 8.3)[9] was not surprising given that the pK_a of CEZ is 6.62 and the half-life of CEZ has been reported to be 20 h at pH 7.4, 37°C.[1,3]

Favorable conditions of pH for aziridine formation were determined using samples of 10 mM 2-chloroethylamine hydrochloride (**9**) in 0.1 M phosphate/D_2O at pD values of 8, 10, 12, and 14 (pD adjusted using 40% NaOD/D_2O). The samples were heated at 37°C for 3 h and then each was adjusted with conc HNO_3 to a pD of 7. ^{1}H NMR analysis of each solution showed that the ratio of 2-chloroethylamine to aziridine was: 90:10 at pD 8; 68:32 at pD 12; 62:38 at pD 10; and 46:54 at pD 14. Hydrolysis product ethanolamine was not observed in any sample. It was determined from this data that the synthesis of [^{3}H]-aziridine would be carried out at pH 14. For considerations of reaction time, a solution of 0.2 M 2-chloroethylamine hydrochloride (**9**) in 2 M NaOD/D_2O at pD 14 was monitored by ^{1}H NMR at a probe temperature of 37°C. Spectra were taken at 10 min intervals over 4 h; during this time, the formation of aziridine was observed but no hydrolysis product (ethanolamine) was detected. A first-order kinetic plot for the disappearance of 2-chloroethylamine gave $k = 0.00455\,\text{min}^{-1}$ ($R^2 = 1.000$) and $\tau_{1/2} = 2.55$ h; thus, aziridine formation would near completion (>96%) after ~13 h (5 half-lives). Given this kinetic data and the fact that the pK_a of aziridine is 8.01, it was anticipated that solutions of aziridine at pH 14 would have good stability when stored at low temperature (e.g. less than 5% decomposition after about a week at −23°C assuming the limit of NMR detection to be 5% and a two-fold rate deceleration for every drop of 10°C).

Experimental

In general, small-scale reaction flasks (vials) were flushed with nitrogen and then sealed with a septum.

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Reagents were added via syringe through the septum. Reactions carried out at 5°C used an ice/water bath. Generally, chemicals were purchased from Aldrich, Fisher, or VWR. Reagents and solvents were freshly distilled. Chromatography used Merck 230–400 mesh silica gel. Analytical TLC plates were from Merck (250 microns). NMR spectra were recorded on a Varian Inova-400 or a GE QE-300 spectrometer. Measurements of specific activity for synthetic intermediates with single labels were made using ScintiLene scintillation cocktail (Fisher) and a Packard TRICARB 1900 CA liquid scintillation counter. Compounds with both ^{3}H and ^{33}P were dissolved in 4a20 scintillation fluor (Research Products International) and counted on an LKB RackBeta liquid scintillation counter using a dual label program; corrections were made for spillover. No corrections were made for radioactive decay of ^{33}P. The specific activities given for labeled materials obtained commercially were the minimum values guaranteed by the supplier; activities were not independently measured.

2,2′-(^{3}H)-diethanolamine ((^{3}H)-5)

With minor modifications to the synthesis of diethanolamine-^{15}N,[7] diethyliminodiacetate (87 µl, 0.5 mmol) in THF (1 ml) was added to [^{3}H]-$LiAlH_4$ in THF (1 M, 1 ml, 1.0 mmol, 2 eq., 7 mCi/mmol, American Radiolabeled Chemicals, Inc.) at 5°C. After being stirred overnight at room temperature, the reaction mixture was cooled to 5°C and water–THF (1:1, 0.8 ml) was added slowly. The entire reaction mixture was extracted using a Soxhlet (5 h, ~15 ml THF); solvent was then evaporated using a stream of N_2 and a hotplate set on the lowest setting. The residue was co-evaporated with 2 × 10 ml CH_3CN to give the product (yellow oil) which was used without further purification.

Bis(2-chloro-2-(^{3}H)-ethyl)amine hydrochloride ((^{3}H)-6)

According to the synthesis of 2-chloroethylamine-^{15}N hydrochloride,[6] thionyl chloride (0.77 ml, 10.6 mmol) was added to crude [^{3}H]-**5** in CH_3CN (2 ml) and the reaction mixture was stirred for 36 h. Product was precipitated by adding the reaction mixture to ether (50 ml). Following centrifugation and washing with ether (3 × 10 ml with centrifugation and removal of supernatant after each wash), the product was dried under vacuum (4–5 h) and isolated as a tan powder (54 mg, 0.3 mmol, 60% net yield over two steps from diethyliminodiacetate, specific activity 17.6 mCi/mmol).

N,N-Bis(2-chloro-2-(^{3}H)-ethyl)phosphoramidic-(^{33}*P*) dichloride ((^{3}H,^{33}P)-7)

Based on the synthesis of ^{15}N-labeled material,[7] pyridine (12.2 µl, 0.15 mmol) was added to a suspension of [^{3}H]-**6** (26 mg, 0.15 mmol, 17.6 mCi/mmol) in CH_2Cl_2 (1.1 ml) at 5°C. After several minutes, [^{33}P]-$POCl_3$ (13.8 µl, 0.15 mmol, 7.3 mCi/mmol, custom synthesis by American Radiolabeled Chemicals, Inc.) was added followed by pyridine (12.2 µl, 0.15 mmol). The mixture was stirred at room temperature overnight and then concentrated under a stream of N_2. The residue was flash chromatographed using CH_2Cl_2 and a Pasteur pipette filled with silica gel. The recovered yellow oil was chromatographed again to give the product as a pale yellow oil [11 mg, 0.04 mmol, 27% yield, R_f 0.6 ($CHCl_3$)].

N,N-Bis(2-chloro-2-(^{3}H)-ethyl)phosphorodiamidic-(^{33}*P*) acid phenylmethyl ester ((^{3}H,^{33}P)-8)

The product was prepared according to the synthesis of ^{17}O labeled material.[7] A freshly prepared solution of 0.58 M lithium benzyloxide (74 µl, 0.042 mmol) in THF[7] was added dropwise to a solution of [^{3}H,^{33}P]-**7** (11 mg, 0.04 mmol) in THF (54 µl) at −29°C (*O*-xylene/liq N_2). The mixture was stirred for 45 min. NH_3 was bubbled through the mixture for 5 min and the flask was tightly sealed with a septum and parafilm (NH_3 condenses at low temp). After overnight at room temperature, the flask was opened carefully to allow for the escape of gaseous NH_3 and the reaction mixture was then concentrated under a stream of N_2. The residue was flash chromatographed through a Pasteur pipette filled with silica gel. Impurities were eluted using *n*-hexane–EtOAc 1:1 followed by *n*-hexane–EtOAc 1:3. Product was eluted using EtOAc (R_f 0.4). Concentration gave the product as a colorless oil (7.3 mg, 0.023 mmol, 55% yield).

N,N-Bis(2-chloro-2-(^{3}H)-ethyl)phosphorodiamidic-(^{33}*P*) acid cyclohexylammonium salt ((^{3}H,^{33}P)-PM-CHA; (^{3}H,^{33}P)-1-CHA)

By analogy to the catalytic transfer hydrogenation of the phenylmethyl ester of deuterated isophosphoramide mustard,[5] a 1 cm × 3 cm column of palladium black was prepared. The column was conditioned by passing a solution of 25% 1,4-cyclohexadiene in ethanol through it at a rate of one drop per second until the column became hot enough to boil the ethanol. The column was cooled by quickly flushing it with a solution of 6% 1,4-cyclohexadiene in ethanol.

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A solution of [^{3}H,^{33}P]-**8** (7.3 mg, 0.023 mmol) and cyclohexylamine (5.9 μl, 0.05 mmol) in 1 ml 6% 1,4-cyclohexadiene in ethanol was added to the column at a flow rate of 1 drop per 3 s. Approximately 1 ml of 6% 1,4-cyclohexadiene in ethanol was used to elute product. Volatiles were evaporated under a stream of N_2 and the residue was washed with 2 ml ether to give product as a white solid. Staring material was evident (TLC) in the ether wash; therefore, the ether solution was concentrated (N_2) and the residue was taken up in 1 ml 6% 1,4-cyclohexadiene in ethanol, cyclohexylamine (5.9 μl, 0.05 mmol) was added, and the solution was again passed through the Pd column and product collected as above. The combined yield was 78% (5.6 mg, 0.018 mmol, specific activity ^{33}P = 7.9 mCi/mmol and ^{3}H = 17.8 mCi/mmol).

2-Chloro-2-(^{3}H)-ethylamine hydrochloride ((^{3}H)-9)

Thionyl chloride (1.05 ml, 14.4 mmol) was added to a solution of 2-[^{3}H]-ethanolamine (1.43 mmol, 86 μl, 3.5 mCi/mmol, American Radiolabeled Chemicals, Inc.) in CH_3CN (5.4 ml) and the mixture was stirred for 48 h. As described above for [^{3}H]-**6**, product was precipitated using ether (150 ml) and isolated as an off-white solid (99 mg, 0.85 mmol, 59% yield, specific activity 3.8 mCi/mmol).

N,N′-Bis(2-chloro-2-(^{3}H)-ethyl)phosphorodiamidic-(^{33}*P*) acid phenylmethyl ester ((^{3}H,^{33}P)-10)

Product was synthesized as described for ^{17}O-labeled material[8] using [^{33}P]-$POCl_3$ (13.7 μl, 0.15 mmol, 7.3 mCi/mmol, custom synthesis by American Radiolabeled Chemicals, Inc.) in $CHCl_3$ (0.75 ml), benzyl alcohol (15.3 μl, 0.15 mmol) in $CHCl_3$ (0.15 ml) and triethylamine (22 μl, 0.16 mmol). The mixture was stirred at −29°C (*O*-xylene and liq N_2 slush) for 1 h. Compound [^{3}H]-**9** (35.5 mg, 0.31 mmol, 3.8 mCi/mmol) was added in one portion as a solid, followed by triethylamine (91 μl, 0.65 mmol). The reaction was stirred at −29°C (10 min), then at −15°C (ethylene glycol/dry ice; 5 min) and finally at room temperature (2 h). The reaction mixture was washed sequentially with water (10 ml), 10% citric acid (10 ml) and saturated $NaHCO_3$ (10 ml). The $CHCl_3$ layer was dried ($MgSO_4$), filtered and evaporated under a stream of dry N_2. The residue was flash chromatographed using a Pasteur pipette filled with silica gel. Impurities were first eluted with EtOAc; then EtOAc–ethanol (95:5) was used to elute product (R_f 0.6) in 37% yield (15.7 mg, 0.056 mmol).

N,N′-Bis(2-chloro-2-(^{3}H)-ethyl)phosphorodiamidic-(^{33}*P*) acid ((^{3}H,^{33}P)-IPM;(^{3}H,^{33}P)-2)

As described for the synthesis of deuterated **2**,[5] a solution of [^{3}H,^{33}P]-**10** (15.7 mg, 0.056 mmol) in 2 ml 6% 1,4-cyclohexadiene in ethanol was passed through a 1 cm × 3 cm column of palladium black. The column was eluted with 6% 1,4-cyclohexadiene in ethanol at a rate of 1 drop every 3 s and then was flushed with another ml of 6% 1,4-cyclohexadiene in ethanol. Concentration and washing with ether gave the product as a white solid (9.4 mg, 0.043 mmol, 77% yield, specific activity ^{33}P = 7.8 mCi/mmol and ^{3}H = 10.6 mCi/mmol).

1-(2-Chloro-2-(^{3}H)-ethyl)-2-(^{3}H)-aziridine ((^{3}H)-CEZ; (^{3}H)-3)

Compound [^{3}H]-**6** [21.4 mg, 0.12 mmol, mix of material from different syntheses (specific activities of 6.6 and 17.6 mCi/mmol)] was dissolved in 0.1 M phosphate buffer, pH 7.9 (5.90 ml) and then 1.04 M NaOH (100 μl) was used to adjust the pH back to 7.9. The mixture was heated at 37°C for 3 h and then, after cooling to room temp, the pH was adjusted to 7.4 with conc HNO_3 (5 μl). No attempt was made to isolate the volatile product, rather it was stored (−140°C) in solution: 20 mM **3** in 0.1 M phosphate, pH 7.4, specific activity 11.2 mCi/mmol.

2-(^{3}H)-aziridine (4)

A solution of [^{3}H]-**9** [14.3 mg, 0.12 mmol, mix of material from different syntheses (specific activities of 3.8 and 7.9 mCi/mmol)] in 1 M NaOH (3.06 ml) was heated at 37°C for 13 h. After cooling to room temperature, 3.06 ml of 1 M HNO_3 in 0.2 M phosphate buffer (phosphate pH = 7.4 before addition of HNO_3) was added. No attempt was made to isolate the volatile product, rather it was stored short term in solution at −140°C until used 'as is' for crosslinking studies: 20 mM **4** in 0.1 M phosphate, pH 7.4 (specific activity 6.1 mCi/mmol).

Conclusion

[^{3}H,^{33}P]-PM and [^{3}H,^{33}P]-IPM were synthesized using pathways that were composites of those used previously for ^{15}N, ^{17}O/^{18}O and ^{2}H-labeled phosphoramide mustards. For [^{3}H,^{33}P]-PM, the nitrogen mustard [^{3}H]-**6** was prepared first and then, immediately upon receipt of [^{33}P]-$POCl_3$, these materials were used to produce the final product within 2 days. Similarly, [^{33}P]-$POCl_3$ was used with pre-prepared [^{3}H]-**9** to give [^{3}H,^{33}P]-IPM within one day. *In situ* syntheses of [^{3}H]-CEZ and [^{3}H]-aziridine were optimized not for considerations of radioisotope stability but rather in consideration of their reactivities

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as alkylating agents. Preliminary data for the alkylation of each of these compounds with oligonucleotides has been published noting the following correction: the statement that CEZ was a poor DNA-crosslinking agent was subsequently found to be inaccurate.[4]

Acknowledgements

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