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SYNTHESIS OF ^{17}O (and ^{18}O) LABELLED ISOPHOSPHORAMIDE MUSTARD

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SUMMARY

The ifosfamide metabolite isophosphoramide mustard (IPM) was synthesized with isotopic enrichment at oxygen. Labelled benzaldehyde was made by exchange with isotopically enriched water (13.4 atom % ^{17}O, 25.5 atom % ^{18}O) and this was then reduced with sodium borohydride to give labelled benzyl alcohol (72% yield). Triethylamine and labelled benzyl alcohol were added to $POCl_3/CHCl_3$ at -23 °C. Subsequent addition of 2-chloroethylamine hydrochloride (2 equiv) and then triethylamine (4.4 equiv) (-15 °C) provided $^{17}O/^{18}O$ enriched *N,N'*-bis-(2-chloroethyl)phosphorodiamidic acid phenylmethyl ester [61%, $BzOP(O)(NHCH_2CH_2Cl)_2$]. Catalytic (10% Pd/C) hydrogenation of this ester at atmospheric pressure gave IPM (22%). GC/MS was used to determine mole percent enrichments of 9.3% ^{17}O and 17.9% ^{18}O for IPM (single labelling within one molecule).

Key words. ^{17}O-isophosphoramide mustard; benzyl alcohol-^{17}O; *N,N'*-bis-(2-chloroethyl)phosphorodiamidic-^{17}O acid phenylmethyl ester; ^{18}O-isophosphoramide mustard.

INTRODUCTION

Ifosfamide (*Ifex*) is one of the most clinically useful analogs of the anticancer drug cyclophosphamide (*Cytoxan*).[1-4] These drugs undergo similar metabolic transformations, ultimately generating the DNA bisalkylating agents

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isophosphoramide mustard [IPM, $HOP(O)(NHCH_2CH_2Cl)_2$] and phosphoramide mustard [PM, $HOP(O)(NH_2)N(CH_2CH_2Cl)_2$], respectively.[2,3] While these metabolites share the essentials of the same alkylation mechanism,[5-7] IPM and PM exhibit significantly different reactivities.[5,6]

The alkylating activities of IPM and PM must be influenced by the protonation states of the nitrogen and oxygen centers in these molecules. ^{15}N and ^{17}O isotopic labelling and NMR spectroscopy can be used to determine the discrete pK_a values associated with each of these metabolites. Such synthetic and NMR studies have been accomplished for PM.[8,9] Pursuant to companion NMR studies of IPM, we have reported the synthesis of ^{15}N labelled IPM;[10] we now report the synthesis of IPM with ^{17}O enrichment at oxygen [*N,N'*-bis(2-chloroethyl)phosphorodiamidic acid-^{17}O (^{17}O-IPM, 1)].

As a side note, ^{18}O-IPM was produced concurrently with ^{17}O-IPM as a result of the fact that the original isotope source (water) was enriched in both ^{17}O and ^{18}O.

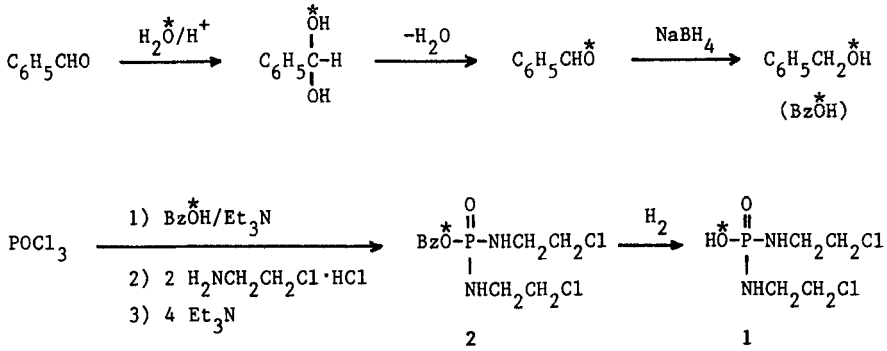
DISCUSSION

Using previous syntheses of IPM and PM as models, ^{17}O-IPM was synthesized as shown in the Scheme.[8,11-13] As in the synthesis of ^{17}O-PM,[8] benzyl alcohol-^{17}O was envisioned as a convenient vehicle by which ^{17}O might be incorporated into IPM. An exchange reaction between benzaldehyde and water which was enriched in ^{17}O (13.4 atom %) produced labelled aldehyde. This product was then reduced to benzyl alcohol-^{17}O, as previously reported.[8] Since the isotope source (water) was also enriched in ^{18}O (25.5 atom %), each compound synthesized was enriched in a mixture of ^{17}O and ^{18}O (single label per molecule); however, reference in this paper will be made primarily to ^{17}O labelled materials since these were of greatest interest for NMR purposes.

As outlined in the Scheme, benzyl alcohol-^{17}O was reacted with phosphorus oxychloride and then 2-chloroethylamine hydrochloride to give benzyl phosphorodiamidate 2. The hydrogenation of 2 provided ^{17}O-IPM (1). The reaction time required for the atmospheric hydrogenation of labelled 2 (60 min) was comparable to that for unlabelled material (40 min) under similar conditions. Thus, there was no dramatic isotope effect such as that which was observed in the production

of $^{15}N_2$-IPM by a related hydrogenation reaction.[10] Previous syntheses of PM and IPM using medium pressure hydrogenation reactions also gave an unidentified impurity in relatively high quantities.[8,10] This impurity was not generated in the atmospheric hydrogenation reaction reported herein.

Scheme



* = ^{17}O (or ^{18}O)

Reliable quantification of isotope enrichment in 1 and 2 could not be accomplished by ^1H or ^{31}P NMR due to overlapping signals under the conditions of spectral acquisition. GC/MS was used, therefore, to determine that phosphorodiamidate 2 was enriched in ^{17}O by 8.4 ± 0.2 mole % (and in ^{18}O by 18.4 ± 0.1 mole %). Similarly, the incorporation of ^{17}O in IPM was found to be 9.3 ± 0.4 mole % (and ^{18}O was 17.9 ± 0.4 mole %). The errors were expressed as average deviations within the limits of the instrumentation, the values determined for ^{17}O enrichment of IPM and its benzyl ester precursor were the same. As expected, MS analysis ruled out multiple labelling within one molecule; however, it was anticipated that in IPM the ^{17}O isotope was scrambled between both oxygen centers (not shown in the Scheme for simplicity).

It should also be noted that the synthesis of 2 proved to be very sensitive to conditions. With benzyl alcohol, phosphorus oxychloride and 2-chloroethylamine hydrochloride as starting materials, different bases (pyridine, triethylamine, sodium hydride), solvents (benzene, CH_2Cl_2, $CHCl_3$), temperatures (4 °C to refluxing $CHCl_3$), reaction times (hours to days), and order of addition of reagents were tried in various combinations. None of these attempts produced 2

in useful quantities. Keys to the successful synthesis of 2 were shorter reaction times and lower temperatures, as reported in the experimental section.

The synthesis of 2 via an aziridinyl phosphorodiamidate intermediate was briefly investigated using a reported method for unlabelled material.[11] Difficulties were encountered in repeating this synthesis and it was determined (personal communication from the authors) that preparations of the intermediate benzyl phosphorodichloridate [BzOP(*O*)Cl_2] were more successful when benzyl alcohol was added *to* a solution of $POCl_3$ as opposed to the reverse manner stated in the publication. This procedure was abandoned, however, in favor of the synthesis reported herein because the latter involved fewer steps and it also obviated the need for aziridine as a reagent.

CONCLUSION

The synthesis of ^{17}O labelled IPM was surprisingly difficult, primarily because the generation of its precursor, benzyl ester 2, was very sensitive to (*inter alia*) temperature, reaction time, and sequence of addition of reactants. Once favorable conditions for the synthesis were found, however, 2 could be produced with consistency and in good yields. The net yield of isotopically enriched IPM over 4 steps was 10%.

EXPERIMENTAL SECTION

Reagent grade chloroform, phosphorus oxychloride, and triethylamine were dried and/or distilled before use. Isotopically enriched water (26.7 atom % ^{17}O, 51.0 atom % ^{18}O) was obtained from Isotec, Inc. (Miamisburg, OH). All other solvents and reagents were generally obtained from Aldrich Chemical Company or Fisher Scientific Company.

Analytical TLC employed 2.5 cm x 10 cm plates coated with a 250 μm layer of silica gel GF (Analtech); I_2 and a 254-nm UV lamp were used for component visualization. Chromatography columns used silica gel (60 - 200 mesh) from J.T. Baker Chemicals.

NMR spectra were obtained on a Bruker AMX300 spectrometer. ^1H NMR chemical shifts (ppm) were referenced to TMS ($CDCl_3$) or TSP (D_2O). ^{31}P NMR chemical shifts (ppm) were referenced to a capillary insert of 1% H_3PO_4 in D_2O.

Benzyl Alcohol-^{17}O. Water-^{17}O (0.50 g, 27 mmol, 26.7 atom % ^{17}O) was diluted with distilled, deionized water (0.50 g, 28 mmol). Following a literature procedure,[8] this ^{17}O enriched water (1.0 g, 55 mmol, 13.4 atom % ^{17}O) was used in an acid catalyzed exchange reaction with benzaldehyde (10 mmol) to give benzaldehyde-^{17}O. The aldehyde was then reduced with $NaBH_4$ to provide benzyl alcohol-^{17}O [0.78 g, 7.2 mmol, 72% yield, R_f 0.2 ($CHCl_3$)] which was used without further purification.

N,N'-Bis(2-chloroethyl)phosphorodiamidic-^{17}O Acid Phenylmethyl Ester (2). Under a nitrogen atmosphere, a solution of $POCl_3$ (0.67 mL, 7.2 mmol) in $CHCl_3$ (30 mL) was cooled to -23 °C (CCl_4/dry ice). Using an addition funnel, a solution of benzyl alcohol-^{17}O (0.78 g, 7.2 mmol) and triethylamine (1.1 mL, 7.9 mmol) in $CHCl_3$ (7 mL) was then added dropwise. Following complete addition, the reaction mixture was stirred for 0.5 h at -23 °C. Powdered 2-chloroethylamine hydrochloride (1.67 g, 14.4 mmol) was then added slowly; following complete addition, the bath was changed to ethylene glycol/dry ice (-15 °C). With vigorous stirring of the reaction mixture, triethylamine (4.4 mL, 31.7 mmol) was slowly added by syringe. Following this addition, the bath was removed and the reaction mixture was stirred at room temperature for 1 h. The reaction solution was washed with water (3 x 7 mL) and the organic layer was dried ($MgSO_4$), filtered, and concentrated at room temperature. The residual oil was chromatographed (2 x 23 cm column, CH_2Cl_2 - CH_3OH, 19:1) to give the product (1.29 g, 4.1 mmol, R_f 0.38) as a colorless oil. Impure fractions were combined and rechromatographed to provide additional product (0.09 g, 0.3 mmol). The total yield was 61%. ^1H NMR (300 MHz, $CDCl_3$) δ 7.60 - 7.28 (m, 5H, aromatic), 5.22 - 4.95 (overlapping doublets, 2H, CH_2O), 3.54 (apparent t, J = 4.9 Hz, 4H, two CH_2Cl), and 3.49 - 3.15 (m, 6H, two $NHCH_2$). ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 14.9.

N,N'-Bis(2-chloroethyl)phosphorodiamidic-^{17}O Acid (1). A 3-neck 100 mL round bottom flask was fit with a gas inlet (with stopcock), a glass stopper, and a septum. To this flask was added a solution of the above benzyl ester 2 (1.29 g, 4.1 mmol) in methanol (32 mL). While this solution was stirring, 10% Pd/C (80 mg) was added to it. The mixture was degassed over several minutes by attaching the

gas inlet to a water aspirator. The mixture was then cooled with a NaCl/ice bath (*ca.* -7 to 0 °C). An atmosphere of hydrogen was introduced to the reaction flask through the septum by using a balloon filled with hydrogen and fit with a needle. The hydrogen-filled balloon was replaced as necessary. The progress of the reaction was monitored by TLC; after 1 h the benzyl ester was no longer visible (R_f 0.38, CH_2Cl_2 - CH_3OH, 19:1). The reaction mixture was allowed to stir an additional 15 min before filtration and then concentration at room temperature to approximately 25% of the original volume. The solution was stored at -20 °C overnight and the resultant crystals (176 mg) were collected and washed with ether. The mother liquor was concentrated to about 6 mL and stored again at -20 °C. This resulted in the crystallization of additional product (26 mg). No unreacted benzyl ester starting material was detected (TLC) in the mother liquor. Labelled IPM was thus obtained in 22% yield (202 mg, 0.9 mmol). ^{1}H NMR (300 MHz, D_2O) δ 3.65 (apparent t, J = 5.4 Hz, 4H, two CH_2Cl) and 3.22 - 3.11 (m, 4H, two NCH_2). ^{31}P NMR (121.5 MHz, DMSO-d_6) δ 12.6.

Mass Spectral Analyses. The mole percent enrichments of IPM (1) were determined on a Hewlett Packard 5970 Mass Selective Detector. Samples for GC/MS were prepared by reacting 1 (10 μg) with *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (100 μL, Pierce, USA) in acetonitrile (400 μL) for 15 min at 80 °C. Samples (1 μL) were then injected onto a 20 m x 0.25 μm DB-5 capillary column (J & W Scientific) using helium at 2 psi as the carrier gas. The GC conditions included: the oven at 165 °C for 1 min and then raised to 250 °C at a rate of 4 °C/min; the injector at 275 °C; and the transfer line at 290 °C. A peak at 10 min was identified as a di-(*t*-butyldimethylsilyl) derivative of IPM with a characteristic loss M-57 at m/z 391 (loss of *t*-butyl). A peak at 8.4 min was also identified as a di-(*t*-butyldimethylsilyl) derivative of IPM and this exhibited a major ion at m/z 355 (loss of HCl and *t*-butyl). Ion abundances of m/z 391 through m/z 398 as well as m/z 355 through m/z 362 were used to determine average (8 injections) mole percent enrichments (with average deviations) of 9.3 ± 0.4% ^{17}O and 17.9 ± 0.4% ^{18}O (and 75.4 ± 0.3% ^{16}O).

Benzyl ester 2 was derivitized and analyzed in a similar manner. A peak at 13.7 min was identified as a mono-*t*-butyldimethylsilyl derivative and this

exhibited a major ion at m/z 331 (loss of HCl and *t*-butyl). Ion abundances of m/z 331 through m/z 336 were used to determine average (10 injections) mole percent enrichments (with average deviations) of 8.4 ± 0.2% ^{17}O and 18.4 ± 0.1% ^{18}O (and 73.7 ± 0.2% ^{16}O).

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