SYNTHESIS OF CYCLOPHOSPHAMIDE-4,4,5,5-d_4

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SUMMARY

3-Hydroxypropionitrile was subjected to a base-catalyzed exchange reaction in D_2O which provided 2,2-dideuterio-3-deuteroxypropionitrile ($DOCH_2CD_2CN$) in 70% yield. Reduction of the nitrile with $LiAlD_4$ gave 3-amino-2,2,3,3-tetradeuteriopropan-1-ol ($HOCH_2CD_2CD_2NH_2$) in a crude yield of 71%. Reaction of this intermediate with *N*,*N*-bis(2-chloroethyl)phosphoramidic dichloride [$Cl_2P(O)N(CH_2CH_2Cl)_2$] followed by the combination of those chromatography fractions which contained only pure material gave cyclophosphamide-4,4,5,5-d_4 as a white oil in 13% yield. A portion of this oil was converted to the monohydrate by the addition of water (1.1 equivalents) and crystallization from ether/petroleum ether (62% yield). For the hydrate, MS analyses gave an average mole percent enrichment (with average deviation over 5 determinations) of 89.1 ± 0.5% d_4.

Key words. 2,2-dideuterio-3-deuteroxypropionitrile; 3-amino-2,2,3,3-tetradeuteriopropan-1-ol; cyclophosphamide-4,4,5,5-d_4; cyclophosphamide-4,4,5,5-d_4 monohydrate.

INTRODUCTION

The anticancer drug cyclophosphamide (CP) is one of the most clinically effective alkylating agents.[1] As such, there is continued interest in enhancing the sensitivity of the quantitative analyses of this prodrug and its metabolites. We have recently developed a new method based on GC/MS techniques which quantifies relevant CP metabolites in human plasma;[2,3] we are currently extending this method to the quantification of CP itself.

One of the attractive features of quantifications by MS is the ability to use internal standards which differ from the analyte only by isotopic enrichment.

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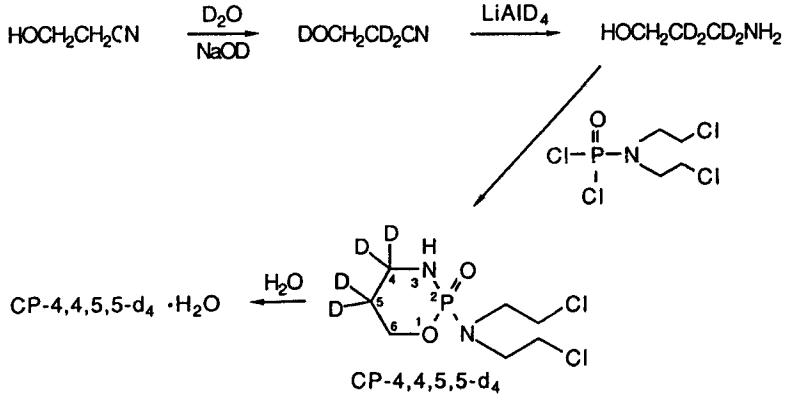
For the MS analysis of unmetabolized CP (where kinetic isotope effects were not a concern), tetradeuterated analog CP-4,4,5,5-d_4 monohydrate (Merck and Cambridge Isotopes) was incorporated into the analytical protocol. When commercial supplies of the labelled material were depleted, a synthesis for CP-4,4,5,5-d_4 and its monohydrate was needed, as reported herein.

DISCUSSION

Strategies for the incorporation of isotopes into the cyclic moiety of CP generally involve the synthesis of an appropriately labelled amino propanol. For the ultimate target CP-4,4,5,5-d_4, the desired intermediate was 3-amino-2,2,3,3-tetradeuteriopropan-1-ol. Literature procedures for the synthesis of other deuterium labelled 3-amino-1-propanols and, ultimately, other labelled CP analogs were modified and adapted to our needs and resulted in the shown scheme.[4-6]



The conversion of the deuterated CP to its hydrate was the most problematic step in this sequence. We have observed that the crystalline hydrate is considerably more stable than the non-hydrated, neat oil. On the other hand, dilute solutions of CP-4,4,5,5-d_4 monohydrate in CH_3OH (e.g., 1 mg/mL, 3.5 mM) have been stored for a year at -20 °C with no apparent loss as a standard for GC/MS procedures. Stopping at the oil stage would, therefore, be acceptable if the oil were then solvated. On the other hand, working with a standard which is a solid rather than an oil generally allows for a greater confidence level in

weight and purity assessments. Unsuccessful attempts at crystallization included the use of aqueous NaOH (pH 12-13),[7] water,[4,8] water/methanol, and water/acetone. While not always consistent in its success, the ether/petroleum ether method described in the experimental provided CP-4,4,5,5-d_4 monohydrate as a white powder (62% recovery). Stable isotope data provided by MS analyses gave an average mole percent enrichment of 89% d_4. Most importantly with respect to its use as an internal standard, the material was free of unlabelled CP (0.0% d_0).

EXPERIMENTAL

Tetrahydrofuran (THF) and triethylamine were dried and distilled. All reactions were carried out under nitrogen; those done at 5 °C refer to ice bath conditions. All solvents were reagent grade; chemicals were generally purchased 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 was used for component visualization. Silica gel from EM Reagents (< 230 mesh) was used for flash chromatography. Melting points were obtained on a Fisher-Johns Melting Point Apparatus and are uncorrected.

500 MHz NMR spectra were obtained on an 11.8 T Bruker MSL500 spectrometer; ^{1}H NMR chemical shifts (ppm) are referenced to TMS ($CDCl_3$). Mole percent enrichments were determined on a Hewlett Packard 5970 Mass Selective Detector.

2,2-Dideuterio-3-deuteroxypropionitrile. With minor modifications to reported procedures,[5] a solution of 3-hydroxypropionitrile (0.30 mol, 20.5 mL), 40% w/w NaOD/D_2O (0.2 mL; ≥ 98 atom % D; MSD Isotopes), and D_2O (12 mol, 240 mL, 99.9 atom % D, Aldrich) was refluxed under N_2 for 45 min. After cooling to room temperature, the solution was neutralized (pH paper) with 20% DCl/D_2O (5 drops; ≥ 99 atom % D; Aldrich) and excess D_2O was then removed by distillation at reduced pressure (water aspirator). The pot residue was then vacuum distilled and the product was collected as a colorless liquid in 70% yield [0.21 mmol, 15.3 g, b.p. 149 °C at *ca.* 4 mm, R_f 0.6 in $CHCl_3$-CH_3OH (9:1)]. ^{1}H NMR ($CDCl_3$) δ 3.82 (s, CH_2O); a shoulder (singlet) at δ 3.83 was ascribed to some $\underline{H}OCH_2CD_2CN$.

3-Amino-2,2,3,3-tetradeuteriopropan-1-ol. With modifications to a synthesis using $LiAlH_4$,[6] a solution of 2,2-dideuterio-3-deuteroxypropionitrile (55 mmol, 4.1 g) in THF (50 mL) was added dropwise to a stirring suspension of $LiAlD_4$ (119 mmol, 5.0 g, 98 atom % D, Aldrich) in THF (100 mL). After heating at reflux overnight, the reaction mixture was hydrolyzed at room temperature by the slow, sequential addition of water (2.4 mL), 15% NaOH (2.4 mL) and more water (7.2 mL). The mixture stirred 4 days (as a convenience not a necessity) and was then suction filtered. The filtrate was dried ($MgSO_4$), filtered and concentrated on a rotary evaporator. The residual oil was dissolved in CH_2Cl_2, and then dried ($MgSO_4$), filtered and concentrated again giving the product as an oil which was used without further purification [35 mmol, 2.8 g, 64%, R_f 0.2 in $CHCl_3$-CH_3OH (9:1)]. The filter cake was subjected to a Soxhlet extraction over 2 days using THF; this provided an additional 7% recovery of crude product (4 mmol, 0.3 g). ^1H NMR ($CDCl_3$) δ 3.78 (s, 2H, CH_2O) and 2.80 - 1.80 (broad s, 3H, OH and NH_2).

Cyclophosphamide-4,4,5,5-d_4. Using selected features of reported procedures for the synthesis of cyclophosphamide (labelled and unlabelled),[4,7] a solution of 3-amino-2,2,3,3-tetradeuteriopropan-1-ol (15 mmol, 1.19 g) and Et_3N (30 mmol, 4.18 mL) in ethyl acetate (15 mL) was added dropwise to a solution of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride[9] [$POCl_2N(CH_2CH_2Cl)_2$, 15 mmol, 3.88 g] in ethyl acetate (15 mL) at 5 °C. After being stirred at room temperature for 2 days, water (25 mL) was added. The ethyl acetate layer was removed and the remaining aqueous layer was extracted with CH_2Cl_2 (3 x 25 mL). All organic layers were combined, dried ($MgSO_4$), filtered, and concentrated at reduced pressure.

The crude material was split into 3 portions and each was flash chromatographed on silica gel [< 230 mesh, 30 g, 3.5 cm x 8.5 cm column, CH_2Cl_2-CH_3OH (98:2) eluant]. Fractions (5 mL each) containing pure product [R_f 0.2 in CH_2Cl_2-CH_3OH (98:2); R_f 0.6 in CH_2Cl_2-CH_3OH (9:1)] were combined and concentrated to give a white oil. The total yield was 13% (0.43 g, 2 mmol). Fractions with less pure product were obtained and could have been re-chromatographed.

Cyclophosphamide-4,4,5,5-d_4 Monohydrate. Water (0.72 mmol, 13 μL, 10% excess) was added to a portion of the purified cyclophosphamide-d_4 (172 mg, 0.65

mmol). The mixture was agitated and then ether (2 mL) was added. After overnight at -20 °C, no crystals were obvious; low boiling (30 - 60 °C) petroleum ether was added until precipitation occurred. The mixture was again stored overnight at -20 °C and the product was collected as a white powder (0.40 mmol, 114 mg, 62% yield, m.p. 39-42 °C; lit.[4] m.p. for CP-4,4,5,5,6,6-d_6, 40-41 °C). ^{1}H NMR ($CDCl_3$) δ 4.46 - 4.41 (m, 1H, C_6-H), 4.30 - 4.22 (m, 1H, C_6-H), 3.63 (t, $^3J_{HH}$ = 7 Hz, 4H, two CH_2Cl), 3.52 - 3.35 (m, 4H, two NCH_2), 2.55 (broad s, 1H, NH), and 1.73 (broad s, 2H, water of hydration). As expected, resonances for protons at C_4 (δ 2.0 - 1.8) and C_5 (δ 3.5 - 3.3) were undetected.[7]

Mass Spectral Analyses. Ion abundances of m/z 224 through m/z 230 (CP-d_4 minus HCl) were used to determine the following mole percent enrichments (the average of 5 injections ± average deviations). For the synthetic monohydrate: CP-d_0, 0.0 ± 0.0%; CP-d_1, 0.0 ± 0.0%; CP-d_2, 2.0 ± 0.1%; CP-d_3, 10.9 ± 0.3%; and CP-d_4, 89.1 ± 0.5%. For commercially labelled material (Merck and Cambridge Isoptopes): CP-d_0, 0.1 ± 0.0%; CP-d_1, 0.2 ± 0.0%; CP-d_2, 1.3 ± 0.0%; CP-d_3, 5.3 ± 0.1%; and CP-d_4, 95.1 ± 0.1%. For unlabelled cyclophosphamide monohydrate (as reference; Aldrich): CP-d_0, 99.6 ± 0.3%; and CP-d_1, 0.7 ± 0.1%.

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