THE SYNTHESIS OF SPIROMUSTINE-d_8.
A GENERAL APPROACH TO OCTADEUTERATED NITROGEN MUSTARDS.

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SUMMARY

The synthesis of deuterium labelled spiromustine, a potential new antitumor agent designed specifically for neoplasms of the central nervous system, is described. Eight deuterium atoms have been incorporated specifically into the nitrogen mustard moiety to provide a deuterated internal standard suitable for selected ion monitoring by GC/MS. Starting with ethylene glycol-d_6, diethanolamine-d_8 is produced efficiently in good yield with no deuterium exchange via a 2-imino-1,3-oxazolidine intermediate. The resultant diethanolamine-d_8 may then be used to synthesize spiromustine-d_8 or other octadeuterated nitrogen mustards.

Keywords: spiromustine-d_8, diethanolamine-d_8, ethylene glycol-d_6, octadeuterated nitrogen mustards, antitumor agents, spirohydantoin mustard.

INTRODUCTION

Spiromustine (NSC 172112, 3-[2-[bis(2'-chloroethyl)amino]ethyl]-5,5-pentamethylene hydantoin, 1a) is a potential antitumor agent that has been designed specifically for use against central nervous system (CNS) tumors (1). This compound combines a substituted hydantoin carrier with a nitrogen mustard moiety to produce a molecule with alkylating antitumor properties

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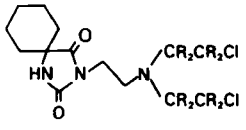
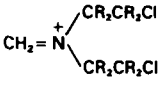
and a partition coefficient equal to that of the well-known anticonvulsant, phenytoin. It is hypothesized that these features provide an antitumor agent that can cross the blood-brain barrier and thus have activity against CNS neoplasms. This indeed appears to be the case, since spiromustine is active against several murine intracerebral tumor systems producing multiple long-term survivors in intracerebral ependymoblastoma (2). Significant antitumor activity is also observed against murine L1210 and P388 leukemia, B16 melanoma, colon 26 tumor and MX-1 mammary human tumor xenograft. For these reasons, the National Cancer Institute has developed spiromustine to clinical trial (3).



1a: R = H
1b: R = D



2a: R = H
2b: R = D

A sensitive and specific analytical method is desired to measure 1a in biological samples in order to carry out pharmacokinetic studies in conjunction with currently conducted clinical trials. Several factors must be considered in developing such an assay. As is often the case for nitrogen mustards, spiromustine is a very reactive compound and is quite unstable in aqueous solutions. Its half-life in 10% dimethylacetamide-90% water at 25° and pH 6 is only 14.1 min (4). Thus considerable metabolism might be expected in vivo and further decomposition is possible after collection of biological samples. It is also anticipated that the total administered dose of spiromustine will only be a few milligrams in the initial trials. Preclinical pharmacology (5,6) and toxicology (2) studies have indicated that plasma levels of parent drug are likely to be much less than 1 μg/ml for most of the period of drug exposure. Furthermore, penetration into other body spaces (e.g. the CNS) is likely to occur after peak plasma

levels have been reached and to be strongly influenced by plasma-protein drug binding, so drug levels in cerebrospinal fluid and tissue will probably be much lower than in plasma. Thus, because of the chemical properties of 1a and the requirement for maximum possible sensitivity, the only feasible method for the reliable measurement of this compound in biological fluids and tissues is selected ion monitoring during gas chromatography-mass spectrometry (GC/MS) analysis. Crucial to this type of assay is selection of a proper internal standard.

It is generally acknowledged that the best internal standard for mass spectrometric quantitation is the stable isotopic analog of the compound under study (7). This is because these labelled analogs should behave identically under all chemical and physical processes such as extraction, derivatization and mass spectrometric ionization (8). In view of this, we were interested in synthesizing a deuterated analog of spiromustine for use as an internal standard. The most intense peak by far in the electron impact mass spectrum of 1a is due to the imminium ion 2a. Because this ion contains two chlorine atoms, the mass spectrum consists of an isotopic cluster in a 9:6:1 ratio separated by 2 amu intervals; isotopic contributions from ^{13}C, ^{2}H and ^{15}N are superimposed on this cluster (Table 1, compound 1a). Tetradeuterated nitrogen mustards have been reported (7), but an octadeuterated compound was felt to be more advantageous for the following reason. In a selected ion monitoring analysis, it is generally advisable to monitor at least two ions for both the compound of interest and the internal standard to insure specificity and to check for interferences. In the case of 1a, in order to achieve maximum sensivity, these would be the ions at m/z 154 and 156. Use of octadeuterated 1b as an internal standard would eliminate the overlap at m/z 158 expected with a tetradeuterated analog and the need to make corrections for the different isobaric species, since the corresponding ions (2b) at m/z 162 and 164 are shifted by 8, rather than by 4 amu.

Table 1

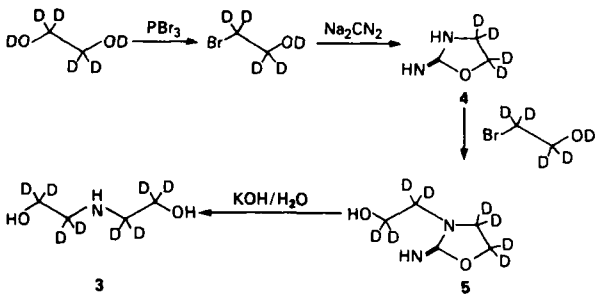
Partial electron impact mass spectra of spiromustine

Ion	1a (R=H)		1b (R=D)	
	m/z	rel. int (%)	m/z	rel. int. (%)
2 ($^{35}Cl_2$)	154	100	162	100
2 ($^{35}Cl^{37}Cl$)	156	64	164	64
2 ($^{37}Cl_2$)	158	10	166	10
$C_{10}H_{15}N_2O_2$	195	3.6	195	3.5
M-CR_2Cl	286	5.4	292	4.7
M-RCl	299	3.0	306	3.0

In the case of spiromustine, the bis(chloroethyl)amino moiety to be labelled originates from diethanolamine (1). Thus the initial goal is a straightforward synthesis of diethanolamine-d_8 (3). This report describes the microscale preparation of 3 in high isotopic purity using relatively inexpensive starting materials and its subsequent application to the synthesis of spiromustine-d_8. It should also be noted that 3 can provide a route to other octadeuterated nitrogen mustards, for example cyclophosphamide (9,10) and nitrogen mustard itself.

RESULTS AND DISCUSSION

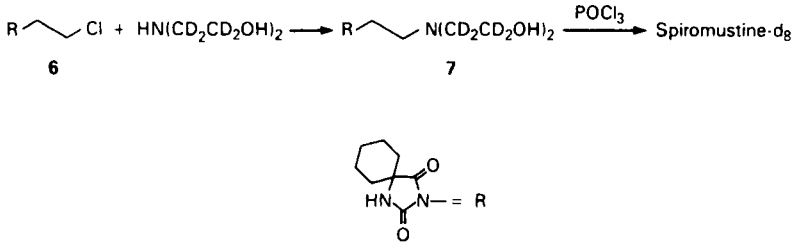
Diethanolamine is usually prepared from ethylene oxide and ammonia, affording both ethanolamine and triethanolamine as side products (11). Separation from these amino alcohols then requires fractional distillation. This procedure was unsuitable in the present case because of the high cost of tetradeuteroethylene oxide, the need for special gas handling equipment, the microscale nature of the synthesis, and the low yield of desired product. Accordingly, an alternate route to diethanolamine-d_8 was developed via the 2-imino-1,3-oxazolidines shown in Scheme 1 (12). Here the deuterated precursor is the much less expensive ethylene glycol-d_6 which is used to make 2-bromoethanol-d_5 (13). Since even this material is not cheap, all steps in this approach were first optimized using unlabelled materials.



Scheme 1

Reaction of the basic cyanamide anion with the halohydrin first forms ethylene oxide *in situ* (14) and then undergoes reaction to form the 2-imino-1,3-oxazolidines 4 and 5. The direct formation of 5 in a one pot reaction was not as straightforward as anticipated from the literature (12) and required extensive modification of reaction conditions. Accordingly, unlabelled reference samples of 4 and 5 were synthesized by alternate routes (15,16) to confirm structures and determine absolute yields. The problems with this reaction appear to be caused by the higher temperatures required for the formation of 5 from 4 and the concomitant loss of the very volatile ethylene oxide which is being generated *in situ*. The possibility of a side reaction between ethylene oxide and the 2-imino functionality of 4 also exists. The effect of changing pH, temperature, solvents, and proportions of reactants was investigated before the reaction was finally optimized as a one-pot procedure conducted in 50% dioxane:water where 2-bromoethanol was added at 0° with very slow warming to room temperature. A 44% yield of 5 could be realized before isolation. The efficient isolation of this product was then achieved by methylene chloride extraction from alkaline solution followed by cation exchange chromatography (82% recovery).

Saponification of oxazolidine 5 required very vigorous conditions. Treatment of 5 with 11.6 N potassium hydroxide for 3 h at reflux resulted in a 75% yield of diethanolamine after cation exchange chromatography. Thus a 16% yield of diethanolamine-d_8 was achieved based on the ethylene glycol-d_6 starting material.



Scheme 2

The condensation of diethanolamine-d_8 with spirohydantoin 6 (Scheme 2) was carried out on only an equimolar basis because of the need to conserve the labelled material. Accordingly, the yield of 7 was only about one-half of that reported with a 3-fold excess of diethanolamine (1). Attempts to optimize this portion of the synthetic sequence were unsuccessful except for addition of 0.2 equivalents of 18-crown-6-ether as an alkali metal ion solubilizer (17). Treatment of the dihydroxy compound 7 with phosphorus oxychloride then produced spiromustine-d_8 in 54% yield. Thus an overall maximum yield of 2.1% is possible for this sequence of reactions based on ethylene glycol starting material.

Mass spectral analysis (Table 1, compound 1b) showed that the synthesized spiromustine-d_8 consisted of 97% d_8 and 3% d_7 species, indicating that no exchange had occurred during the synthesis even though deuterated reagents and solvents were not used. The deuterium content of 1b was exactly the same as that of starting material. Equally important, no overlap of the isotopic clusters due to fragment ions 2a and 2b were observed in the mass spectra of 1a and 1b. Thus, even though the overall yield was small, more than enough high quality spiromustine-d_8 was obtained for use as an internal standard in the proposed GC/MS assay from relatively inexpensive starting materials.

EXPERIMENTAL

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on a

Varian HR-220 spectrometer in D_2O with 3-(trimethylsilyl)-1-propane-sulfonic acid (TSP) as an internal reference. Electron impact mass spectra were obtained on a VG Analytical 7070E double focussing mass spectrometer via a direct insertion probe that was linearly temperature programmed from ambient to 300°. Spectra were acquired at a scan speed of 2s/decade and a resolution of 2000 while using a VG Analytical 2035 data system. Compound purity was determined by gas chromatography (GC). A Varian Aerograph 2740 gas chromatograph equipped with a flame ionization detector was temperature programmed as indicated and separations were carried out on the appproprIate 2 mm id x 1.83 m glass columns packed for on-column injection. Both the injector and detector were maintained at 250° while a helium carrier gas flow of 30 ml/min was employed. Thin layer chromatography (TLC) was performed on Analtech silica gel GHLH plates of 250μ thickness, and the spots were visualized by iodine. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Ethylene glycol-d_6, 99 atom per cent D, was purchased from Stohler Isotopes. 2-Bromoethanol was obtained from Aldrich Chemical Co. and redistilled before use. HPLC-grade dimethylformamide (DMF) from Burdick and Jackson was twice distilled before use. Analytical grade Dowex 50W-X8 cation exchange resin was sequentially pretreated with 1N NaOH and 1N HCL before being converted to the ammonium form with 1N $(NH_4)_2SO_4$.

2-Bromoethanol-d_5. The method of Chaudhuri et al. (13) was employed starting with 5 g (73.5 mmol) of ethylene glycol-d_6 and 2.6 ml (27.7 mmol) of phosphorus tribromide. Fractional distillation of the resulting reaction mixture at 30 mm Hg gave some ethylene dibromide (38-40°) and 5.1 g 2-bromoethanol-d_5 (58-62°, 51% yield). GC analysis on a column of 10% Carbowax 20M on 80/100 mesh Supelcoport, temperature programmed from 65 to 110° at 2°/min, indicated a purity of 94% for 2-bromoethanol (ethylene dibromide, t_R = 5.0 min; 2-bromoethanol, t_R = 14.7 min).

2-Imino-3-(2'-hydroxyethyl)-1,3-oxazolidine (5, unlabelled reference). The procedure of Matveev (16) was used with a modified reaction workup since a smaller scale preparation starting with only 5 g (47 mmol) diethanolamine

was carried out. After the product residue was extracted with 25 ml p-dioxane to remove unreacted diethanolamine, the lower non-dioxane soluble phase was dissolved in 25 ml methanol. One-half of this solution was evaporated to dryness and the residue was dissolved in 30 ml cold 5 N NaOH saturated with NaCl before being extracted with 5 x 130 ml CH_2Cl_2. The organic extracts were combined, dried over K_2CO_3, and then evaporated to dryness. The resulting residue was further dried in vacuo overnight to give 1.29 g of a white crystalline product in contrast to the previously reported oil (12,16). An identical workup of the other half of the solution gave an additional 0.98 g of product (37% total yield). A 0.5 g portion of this product was twice recrystallized from isopropanol, m.p. 92°.

Anal. Calcd for $C_5H_{10}N_2O_2$: C, 46.14; H, 7.74; N, 21.52

Found: C, 46.00; H, 7.75; N, 21.33.

TLC: R_f = 0.47, $CHCl_3$: MeOH: conc NH_4OH (75:25:7, v/v).

^{1}H-NMR (D_2O: δ 4.30 (t, 2, J=7 Hz, H-5), 3.70 (t, 2, J=5 Hz, H-2'), 3.59 (t, 2, J=7 Hz, H-4), 3.32 (t, 2, J=5 Hz, H-1').

Mass Spectrum: m/z (relative intensity) 130 (M^+, 5.2), 100 (32), 99 (19), 86 (59), 85 (28), 56 (100).

2-Imino-3-(2'hydroxyethyl)-1,3-oxazolidine-d_8 (5). To a flask immersed in an ice-water bath and containing 5.3 ml water was added 1.6 g (18.6 mmol) ground disodium cyanamide. To this stirred solution 5.3 ml p-dioxane was added as a cosolvent followed by 1.45 ml (20 mmol) 2-bromoethanol-d_5. The ice bath was allowed to remain in place so that the reaction mixture would only warm up to room temperature very slowly. After 24 h, the reaction mixture was cooled to 0° in an ice bath and a second portion of 1.45 ml 2-bromoethanol-d_5 was added. The same warming procedure as above was followed and the reaction mixture was maintained at room temperature for 40 h. The reaction mixture was then concentrated to approximately one-third of its volume, dissolved in 50 ml 5N NaOH saturated with NaCl, and extracted with 4 x 250 ml CH_2Cl_2. After combination, the organic extract was dried

over K_2CO_3, filtered, evaporated to dryness, and then dried overnight *in vacuo* to give 1.86 g of an oily product. Analysis by TLC using $CHCl_3$: MeOH: conc NH_4OH (18:6:1, v/v) indicated two spots corresponding to 4 and 5. The product was applied to a 22 x 260 mm Dowex 50W-X8 cation exchange column in the NH_4^+ form at a flow rate of 1.2 ml/min. The column was then rapidly rinsed with 200 ml water and the oxazolidines were eluted at a flow rate of 1.2 ml/min with 1000 ml of a linear gradient from 0.5 to 2.0 N NH_4OH. After an initial 200 ml fraction was collected, 30 ml portions were collected, evaporated and analyzed by TLC. A minor quantity of 4 was found in fractions 4 and 5 with fractions 7-13 containing the main product 5. These fractions were combined, evaporated to residue, and dried *in vacuo* to yield 0.89 g of crystalline compound 5 (34% yield).

Diethanolamine-d_8 (3). Compound 5 (0.84 g, 5.7 mmol) was refluxed for 3 h with 13 ml 11.6 N KOH. This solution was then stored overnight at 5° before being diluted with 130 ml water and acidified with 30 ml 6N HCl. Cation exchange chromatography using the same procedure as described above gave diethanolamine eluting in the first 200 ml of the NH_4OH gradient. Evaporation of this fraction to dryness followed by further drying *in vacuo* for 1 h gave 644 mg of compound 3 (92% yield). GC analysis on a column packed with 60/80 mesh Tenax GC temperature programmed from 130-125° at 8°/min indicated a single major peak with the same t_R as a diethanolamine standard (10.8 min).

Mass Spectrum: m/z (relative intensity) 113 (M^+, 0.8), 112 (M-H, 0.3), 111 (M-D, 0.7), 80 (100), 62 (56), 45 (40).

3-[2-[bis(2'-hydroxyethyl)amino]-5,5-pentamethylenehydantoin-d_8 (7). Compound 6, 1.07 g (4.6 mmol); ground potassium iodide, 768 mg (4.6 mmol); diethanolamine-d_8, 457 mg (3.8 mmol) and 18-crown-6-ether, 204 mg (0.8 mmol) were dissolved in 22 ml DMF and stirred for 15 h at 100°. The DMF was removed under reduced pressure, the residue treated with saturated $NaHCO_3$ solution and water, and this mixture twice extracted with ethyl acetate. The organic phase was washed with water, dried over Na_2SO_4, filtered and

evaporated. After drying overnight in vacuo, a crystalline sample weighing 981 mg was obtained. Recrystallization from toluene gave 477 mg of compound 7. GC analysis on a column packed with 3% OV-17 on Supelcoport 120/140 mesh and temperature programmed from 200 - 240° at 2°/min and comparison to a silylated reference sample of non-labelled 7 (4) indicated a yield of 25% and some contamination with compound 6.

Spiromustine-d_8 (1b). Phosphorus oxychloride (3.75 ml) and 450 mg recrystallized 7 (1.5 mmol) were heated at 80° for 1.5 h. The reagent was removed at reduced pressure (30 mm Hg), 1.5 ml conc. HCl was added, and the flask was gently heated with a hot air blower until the oily residue dissolved. This solution was added to cold saturated NaOAc, producing a white precipitate which was twice extracted with ethyl acetate. The organic fractions were combined, washed with water, dried over Na_2SO_4, filtered and evaporated to dryness. The crystalline residue was dried overnight in vacuo and then twice recrystallized from 2-propanol to give 141 mg of pure spiromustine-d_8, m.p. 123° (lit. (1) 125-126°). GC analysis using the same conditions described for compound 7 indicated a single peak with t_R = 21.5 min.

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