SYNTHESIS OF 5', 5'-DIDEUTERATED NUCLEOSIDE AND NUCLEOTIDE ANABOLITES OF 6-MERCAPTOPURINE

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SUMMARY

6-Mercaptopurine riboside, 6-methylmercaptopurine riboside, 6-mercaptopurine riboside-5'-phosphate and 6-methylmercaptopurine riboside-5'-phosphate were synthesized with 2 deuteriums in the ribose moiety at the 5'-position so as to act as internal standards for the quantitation of these anabolites of 6-mercaptopurine from biological media by gas chromatography-selected ion monitoring mass spectrometry with chemical ionization.

Key Words: 6-Mercaptopurine Nucleosides, Nucleotides; Deuterium; Mass Spectrometry.

INTRODUCTION

6-Mercaptopurine (6-MP) or 6-thiopurine is a hypoxanthine analog which is widely used as an antineoplastic (1) and immunosuppressive agent (2). 6-MP is taken up by cells and converted by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) directly into 6-mercaptopurine riboside-5'-phosphate (MPRP), a potent inhibitor of nucleic acid synthesis. MPRP can then be methylated at the sulfur to give 6-methyl mercaptopurine riboside-5'-phosphate (MMPRP), another active metabolite (anabolite). These monophosphates may be dephosphorylated *in vivo* to give the corresponding ribosides, 6-mercaptopurine riboside (MPR) and

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6-methyl mercaptopurine riboside (MMPR). This enzymatic dephosphorylation may be important in the development of clinical resistance to 6-MP (3). Further transformation of the phosphates with subsequent incorporation into DNA (as 6-mercaptoguanosine phosphate) also occurs, and indeed it has been suggested that this may be the crucial requirement for the biological activities of these purine analogs (4,5).

The elucidation of the metaboism of 6-MP requires sensitive methods for the separation and quantitative determination of its various anabolites. Most of the initial work utilized paper chromatography for qualitative identification of metabolites (6,7). In 1972, Nelson et al. (8) described the separation of 6-MP ribonucleosides and ribonucleotides by high pressure liquid chromatography (HPLC), using a modified procedure of Caldwell (9). Breter has recently (10) improved the HPLC detection limit for standard solutions of thiopurines using a variable wavelength UV detector. A highly sensitive and specific HPLC method with fluorimetric detection has also recently been reported by Tidd and Dedher (11) for measurement of 6-thioguanine nucleotide metabolites of 6-MP and 6-thioguanine.

We are developing a gas chromatographic-mass spectrometric (GC-MS) assay for MPR, MMPR, MPRP and MMPRP to quantitate these compounds in vivo. The ribosides are assayed as their permethylated derivatives (12) by GC-MS utilizing chemical ionization (CI) and selected ion monitoring (SIM) (13). The monophosphates are initially separated from the ribosides by column chromatography on XAD-4 resin, enzymatically dephosphorylated and subsequently analyzed as the permethylated ribosides by GC-MS (14). XAD-4 resin separates MPR from MMPR or they are distinguished by permethylation with CD_3I.

In order to more effectively quantitate these nucleosides and nucleotides from biological media by GC-MS with SIM (15) we have synthesized deuterated analogs of these compounds doubly labeled in the 5'-position of the ribose moiety, to use as internal standards.

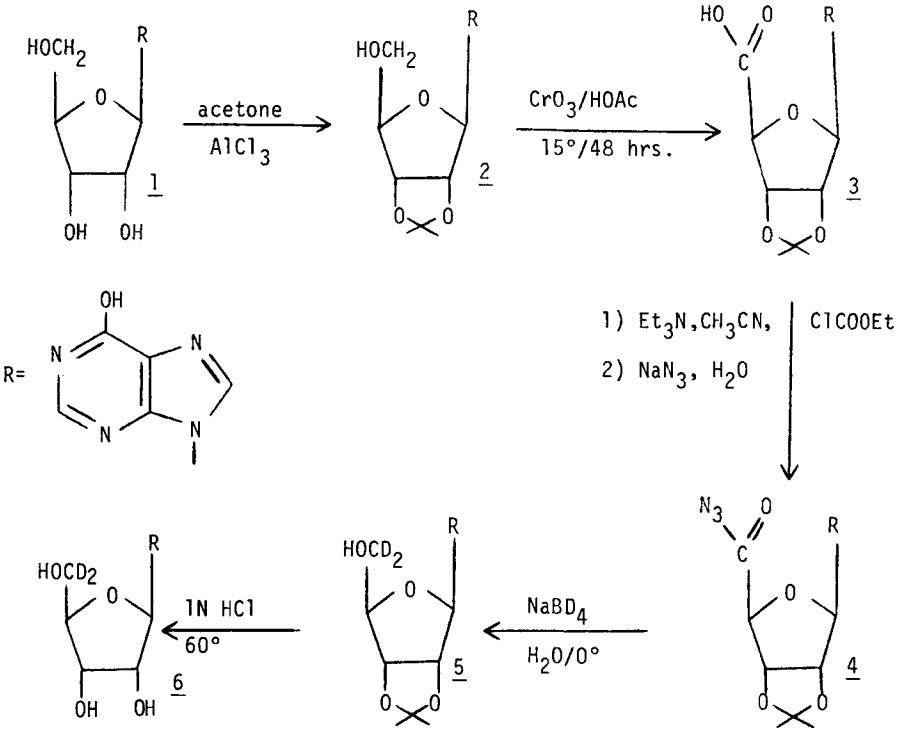
DISCUSSION

Specific deuterium labeling of purine nucleosides can theoretically be accomplished in the purine ring system or in the ribose moiety. The purine ring contains only one nonexchangeable proton at carbon-2 (16). The ribose moiety on the other hand offers six nonexchangeable sites for possible labeling. Schmidt et al. in 1968 (17) synthesized 5',5'-d_2-adenosine from adenosine in an overall yield of 54%. It therefore seemed feasible to utilize an analogous synthetic scheme for incorporation of two deuteriums at the 5' carbon of inosine, with subsequent conversion of inosine to MPR, MMPR, MPRP and MMPRP by known methods (18,19).

Scheme 1 depicts the actual modified route we developed for the incorporation of deuterium into inosine. Isopropylidene inosine (2) is selectively oxidized (20) with chromic acid to give 2', 3'-isopropylidene inosinic acid (3) which, after conversion to the acid azide (4) is reduced back to the primary alcohol (5) with sodium borodeuteride in water.

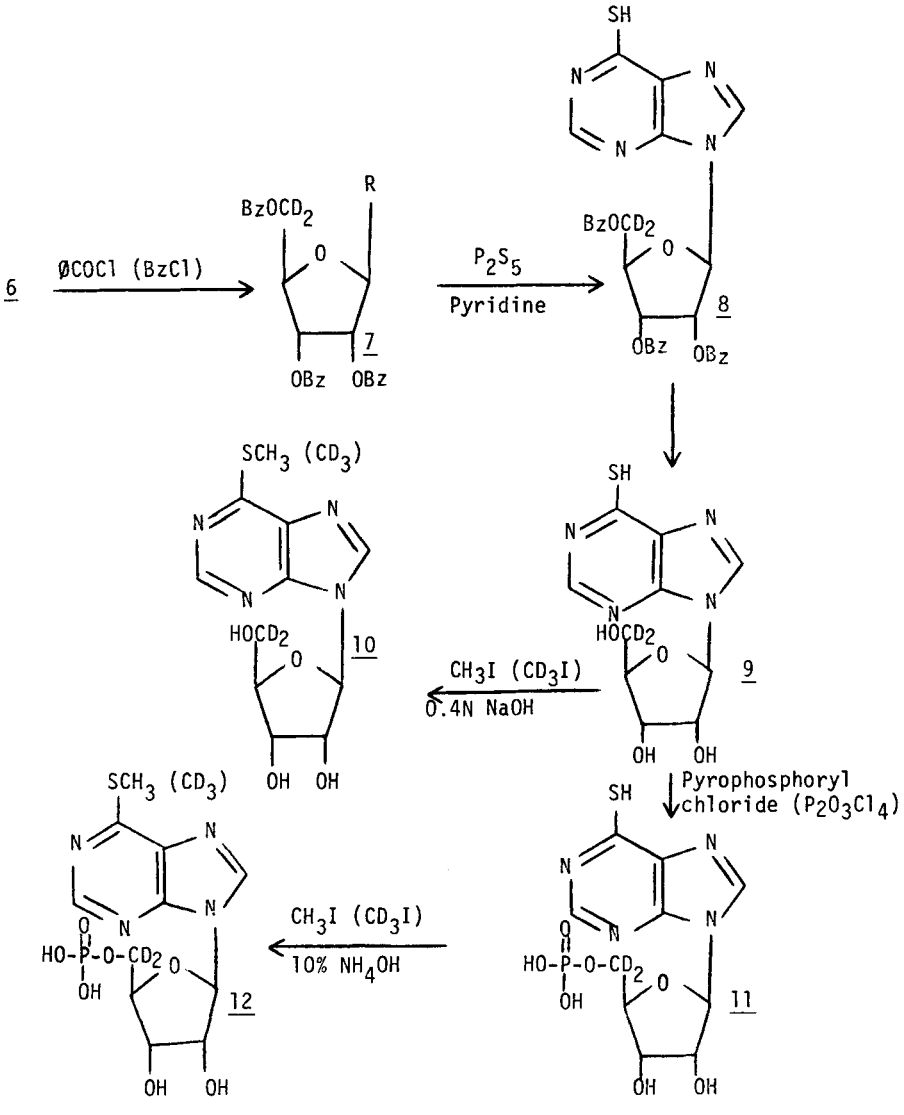
Scheme 2 depicts the conversion of 5', 5'-d_2 inosine (6) by known methods into labeled thiopurine analogs. Compound 6 is benzoylated to give compound 7 which is thiated with phosphorous pentasulfide in refluxing pyridine (18) to give compound 8. Removal of the protecting groups (18) gives MPR-d_2 (9). Conversion of MPR-d_2 to MMPR-d_2 (10) is accomplished with methyl iodide in

Scheme 1



0.4N sodium hydroxide (18). Following a procedure similar to that of Imai et al. (19), MPRP-d_2 (11) is produced by reaction of MPR-d_2 with pyrophosphoryl chloride (21). MMPRP-d_2 (12) is produced by methylation of MPRP-d_2 with methyl iodide in 10% NH_4OH. The monophosphates were collected as the barium salts after column chromatography on a Dowex 1-X8 formate column eluted with 0.3M bicarbonate buffer (19).

Scheme 2


6
ØCOCl (BzCl)
BzOCD2
R
OBz OBz
7
P2S5
Pyridine
BzOCD2
OBz OBz
8
SH
HOCD2
OH OH
9
CH3I (CD3I)
0.4N NaOH
SCH3 (CD3)
HOCD2
OH OH
10
Pyrophosphoryl chloride (P2O3Cl4)
HO-P-O-CD2
OH
11
CH3I (CD3I)
10% NH4OH
SCH3 (CD3)
HO-P-O-CD2
OH
12

6-Mercaptopurine riboside-5', 5'-d_2, 6-methylmercaptopurine riboside-5, 5'-d_2, 6-mercaptopurine riboside-5'-phosphate-5', 5'-d_2 and 6-methylmercaptopurine riboside-5'-phosphate 5', 5'-d_2 have thus been synthesized in good yield from inosine essentially by known or slight modifications of known synthetic methods. Deuterium incorporation is high with the products containing 1.8% d_0, 10.6% d_1, and 88.6% d_2 species making them suitable for GC-MS with chemical ionization and selected ion monitoring for the quantitation of these nucleosides and nucleotides from biological media.

MATERIALS AND METHODS

Inosine, 2', 3'-O-isopropylidene inosine, 6-mercaptopurine riboside, 6-methylmercaptopurine riboside and 6-mercaptopurine riboside-5'-phosphate were obtained from Sigma Chemical Co. $NaBD_4$ was obtained from Merck Isotopes. Other reagents and solvents were obtained from various chemical sources.

Melting points are uncorrected. Thin layer chromatography (TLC) was performed on Baker-flex silica gel IB2-F precoated sheets. Infra-red (IR) spectra were obtained on a Beckman IR-33 instrument. Mass spectra were obtained on a Dupont 21-492 double focusing mass spectrometer equipped with a chemical ionization source and coupled to a Varian 2740 gas chromatograph via a jet separator. Methylated nucleosides were admitted to the mass spectrometer for selected ion monitoring via a 6' glass column packed with 3% OV-17 on Supelcoport and operated at 280° C.

EXPERIMENTAL

2', 3'-O-isopropylidene inosine-5', 5'-d_2(5).

2', 3'-O-isopropylidene inosine-5'-carboxylic acid (3) is prepared in an analogous fashion to that of Schmidt and Fritz (20) by chromic acid oxidation of 2', 3'-O-isopropylidene inosine (2). 2 can be purchased from Sigma Chemical Co. or synthesized more cheaply from inosine (1) by the method of Muramatsu et al. (22).

To a suspension of 5 g (15.5 m moles) of 3 in 200 mL of acetonitrile was added 2.1 mL (15.5 m moles) of triethylamine. When the initial suspension dissolved, 1.25 mL (15.5 m moles) of ethylchloroformate was added and the mixture was stirred for three hours at room temperature (17). During this time some 3 precipitated from solution and enough triethylamine (ca. 300 µl) was added to redissolve it along with an equal amount of ethylchloroformate. At the end of three hours 1.01 g (15.5 m moles) of sodium azide in 10 mL of water was added dropwise and the solution was stirred an additional hour. The solution was filtered and the solvent was removed under vacuum. The residue was extracted once with 25 mL of ethyl ether and twice with 25 mL of cold water. The residue (4) was then resuspended in 200 mL of cold water and stirred in an ice bath. Two equivalents (1.5 g, 35.8 m moles) of sodium borodeuteride were added in portions and the solution was stirred overnight at 0-5°. The pH was then adjusted to 6 with 4N HCl and the volume was reduced to 50 mL. The solu-

tion was heated to redissolve the precipitated product and left overnight in the refrigerator. The product was collected by filtration, washed with 20 mL of cold water, and dried to yield 2.1 g (42%) of 5. The melting point and IR spectrum were identical to that of 2. Also the isobutane CI mass spectrum revealed MH^+ = 311 compared to MH^+ = 309 for 2.

Inosine-5', 5'-d_2 (6).

A stirring suspension of 2.2 g (7.2 m moles) of 5 in 40 mL of 1N HCl was heated to 60° whereupon the suspension dissolved. After 20 min. at 60° the solution was cooled and brought to pH 8 with triethylamine. The solution was evaporated in vacuo and to the residue was added 100 mL of $CHCl_3$ which dissolved the triethylamine hydrochloride leaving the product suspended in solution. After filtration and washing with $CHCl_3$ (2 x 30 mL), 1.86 g (96%) of 6 was collected (m.p. 210° decomp., CI mass spec. MH^+ = 271). This product can be recrystallized from 80% ethanol but is sufficiently pure for the next step.

6-Mercaptopurine riboside-5', 5'-d_2 (MPR-d_2) (9).

MPR-d_2 was prepared from 6 via 2', 3', 5'-tri-O-benzoyl inosine-5', 5'-d_2 (7) and 2', 3', 5'-tri-O-benzoyl-6-mercaptopurine riboside-5', 5'-d_2 (8) essentially by the methods of Fox et al. (18) in 54% yield (m.p. 201-203°; IR identical to MPR; CI mass spec. after derivatization with diazomethane (M+14) H^+ = 301 compared to m/e 299 for MPR).

6-Methylmercaptopurine riboside-5', 5'-d_2 (MMPR-d_2) (10).

MPR-d_2 was methylated with CH_3I in 0.4N NaOH again according to Fox et al. (18). Yield 63%, m.p. 164-165°. The product was indistinguishable from standard MMPR in its IR, and TLC in butanol, water, glacial acetic acid (5:3:2). Also the CI mass spectrum gave MH^+ = 301. Note that CD_3I can be used here if MMPR-d_5 is desired.

6-Mercaptopurine riboside-5'-phosphate-5', 5'-d_2 (MPRP-d_2) (11).

MPR-d_2 was phosphorylated with pyrophosphoryl chloride by the method of Imai et al. (19) and purified on a Dowex 1-X8 (formate) column (19). The phosphate was converted to the barium salt with barium acetate. (Yield 52% from MPR-d_2; IR identical to authentic MPRP).

The phosphate was permethylated using silver oxide and methyl iodide in dimethylformamide at room temperature for 18 hours (23) and the isobutane CI mass spectrum (direct probe introduction) was compared to that of permethylated MPRP. The protonated molecular ions (base peak in the spectra) revealed 1.8% d_0, 10.6% d_1 and 88.6% d_2 species.

6-Methylmercaptopurine riboside-5'-phosphate-5', 5'-d_2 (MMPRP-d_2) (12).

To a stirred suspension of 11 (100 mg, 0.199 m moles) in 50 mL of 10% ammonium hydroxide was added 5 m moles of methyl iodide. Again CD_3I can be substituted here if the d_5-analog is required. The reaction mixture was stirred 48 hours at room temperature after which time TLC (n-butanol, acetic acid, water; 5:3:2) showed the appearance of a new spot with a UV absorbance different from that of starting material. The ammonia and methyl iodide were removed under vacuum and 200 mg of barium acetate were added to the solution (ca. 20 mL), followed by the addition of 200 mL of absolute ethanol. The solution was filtered to yield 50 mg (48%) of white crystals. After permethylation (23) the CI mass spectrum was essentially identical to that of permethylated MPRP-d_2.

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