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SYNTHESIS OF LABELLED [$^{15}N_3$]-6-BROMOPURINE, A USEFUL PRECURSOR OF ^{15}N-LABELLED O^6-ALKYLGUANINES, TO BE USED AS INTERNAL STANDARDS FOR QUANTITATIVE GC-MS ANALYSES

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SUMMARY

[$^{15}N_3$]-6-Bromopurine was synthesized using readily available labelled starting materials. The ^{15}N-labelled precursor guanidine, obtained from $^{15}NH_3$ and cyanogen bromide, was condensed with ethyl cyanoacetate to give 4-hydroxy-[$^{15}N_2$]diaminopyrimidine. An additional ^{15}N isotope was incorporated by nitrosation with ^{15}N-labelled sodium nitrite. After reduction to the corresponding triaminopyrimidine and condensation with formamide, [$^{15}N_3$]guanine was obtained in 96% yield and then converted to [$^{15}N_3$]-6-thioguanine by reacting it with phosphorus pentasulfide. [$^{15}N_3$]-6-Thioguanine was readily converted to the corresponding bromopurine using bromine in the presence of methanolic hydrobromic acid. The final product was characterized by mass spectrometry. [$^{15}N_3$]-6-Bromopurine was the precursor for the subsequent synthesis of a series of O^6-alkylguanines which can be used in isotope dilution mass spectrometry.

Key Words: Nitrogen-15, 6-bromopurine, O^6-alkylguanines

INTRODUCTION

Most chemical carcinogens, in their activated form, interact with DNA and lead to the formation of adducted DNA bases, a step that appears to be relevant for the initiation of the carcinogenic process (1).

The quantitation of modified DNA bases produced by alkylating agents is very important for understanding their mechanism(s) of mutagenicity or carcinogenicity and for assessing human exposure to these compounds (2). The most reliable procedures for the unequivocal chemical characterization and quantitation of DNA adducts employ

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GC/MS methods with selected ion recording (3). Stable isotope labelled compounds are widely employed as internal standards in mass spectrometric assay procedures whenever high sensitivity and specificity are required.

This approach has been successfully applied for the quantitative determination in biological tissues of different modified DNA bases, including those resulting from DNA exposure to small alkylating agents such as N-nitroso compounds (4-7), which react with DNA to give mainly N-alkylated bases and to a lesser extent, O^6-alkylguanines and O^4-alkylthymines (8-10). O^6-alkylguanines have attracted major attention because of their mutagenic and carcinogenic potential (8).

Procedures have been reported for the introduction of stable isotope atoms into purine base and nucleoside molecules, but only a few deal with the incorporation of multiple labels (11,12). For accurate GC-MS determination at least three heavy atoms must be introduced into the molecule in order to reduce any corrections due to naturally occurring stable isotopes to negligible proportions.

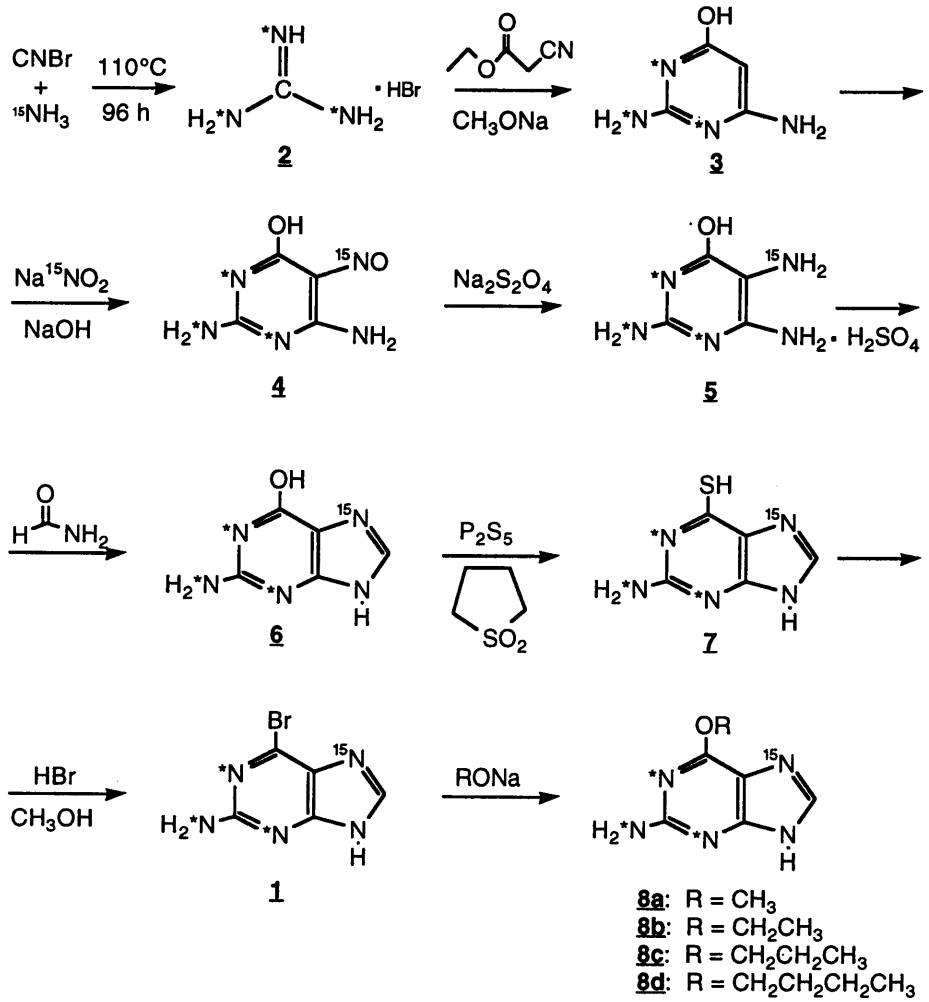
O^6-Alkylguanines labelled with stable isotopes at multiple sites are not available commercially. The present paper describes the synthesis of 6-bromoguanine labelled with three atoms of nitrogen-15 and the use of this compound as a precursor for ^{15}N-labelled O^6-alkylguanines, namely O^6-methyl-, O^6-ethyl-, O^6-propyl- and O^6-butylguanine, which can serve as internal standards in GC/MS quantitation of O^6-alkylguanines.

MATERIALS AND METHODS

Chemicals. Ammonia (98% ^{15}N-labelled), unlabelled guanine and 6-thioguanine were from Aldrich Chimica (Milan, Italy). Sodium nitrite (99% ^{15}N-labelled) was purchased from MSD Isotopes, Montreal, Canada. All other reagents were of analytical grade and used with no further purification.

Instruments. ^1H-NMR and ^{15}N-NMR spectra were recorded on a Bruker AC 200 spectrometer with tetramethylsilane (TMS) and CH_3NO_2 as internal-shift standards. Electron impact (EI) and chemical ionization (CI) mass spectra were obtained on a Finnigan MAT TSQ-700 instrument. Methane was used as CI reagent gas. HPLC analyses were done using a Beckman System Gold liquid chromatograph

programmable solvent module equipped with a Beckman System Gold diode array detector. UV spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer.



Scheme 1. Synthesis of [$^{15}N_3$]-6-bromoguanine (Two ^{15}N labels were randomly distributed between the three positions indicated by asterisks).

[$^{15}N_2$]Guanidine hydrobromide (2). This compound was synthesized according to established procedures, with minor modifications (13). Briefly, ^{15}N-ammonia (1000 mL, 45 mmol) in a break-seal flask was liquified by cooling to -70°C in an ethanol-dry ice mixture; the seal was then opened and 10 mL of cold absolute ethanol were added. The

solution was transferred to a Carius tube kept in ice, and a freshly prepared solution of cyanogen bromide (1.59 g, 15 mmol) in cold ethanol (2.5 mL) was added. The tube was sealed and heated to 110°C for 96 h in a Carius oven. The tube was then cooled in ice and opened. Unreacted ammonia was blown off by a nitrogen stream and collected in a trap containing diluted sulfuric acid. The reaction mixture was brought to dryness to give compound 2 (1.87 g, 13 mmol). Yield 88%; EI/MS, m/z 44 [M^+ - $^{15}NH_2$], m/z 45 [M^+ - NH_2], m/z 61 [M^+]. ^{15}N-NMR (D_2O): δ: -310.2 (t).

[$^{15}N_2$]-2,6-Diamino-4-hydroxypyrimidine (3). A solution of 2 (1.407 g, 9.91 mmol) and ethyl cyanoacetate (1.12 g, 9.91 mmol) in methanol (6 mL) was added dropwise to a solution of sodium (0.552 g, 24 mmol) in methanol (10 mL). The reaction mixture was refluxed for 10 h then, after cooling to room temperature, the pH was adjusted to 6 by adding glacial acetic acid. The mixture was chilled and kept in ice for 30 min, filtered and the filtrate was evaporated to dryness. The residue was washed with water, ethanol and diethyl ether and dried to give compound 3 (0.686 g, 5.36 mmol) as a white solid. Yield 54%. The purity of the compound was greater than 98% by HPLC. EI/MS, m/z 128 [M]$^+$; CI/MS, m/z 129 [M+ H]$^+$. ^{15}N-NMR (D_2O): δ -307.6 (m), -240.2 (m), -201.6 (m).

[$^{15}N_3$]-2,6-Diamino-4-hydroxy-5-nitroso-pyrimidine (4). A solution of 3 (0.27 g, 2.11 mmol) and ^{15}N-labelled sodium nitrite (0.168 g, 2.4 mmol) in 3M NaOH (3 mL) was added dropwise to stirred glacial acetic acid (3.5 mL) kept on ice (14). The pink precipitate which formed immediately was isolated by centrifugation, washed with water, ethanol and diethyl ether and dried to give compound 4 (0.247 g, 1.56 mmol). Yield 74%. CI/MS, m/z 159 [M + H]$^+$. ^{15}N-NMR (CF_3COOH/D_2O): δ -282.0 (d), -244.9 (d), -207.4 (d), 40.6, 65.9.

[$^{15}N_3$]-4-Hydroxy-2,4,5-triaminopyrimidine (5). The reaction was carried out as described, with minor modifications (15). Sodium dithionite (0.483 g, 2.77 mmol) was added in several portions to a stirred suspension of 4 (0.161 g, 1.02 mmol) in boiling water (5 mL). The reaction mixture was kept at 100°C until the pink colour had completely disappeared. The solution was then cooled and kept in an ice-bath, and after

acidification with sulfuric acid the white precipitate formed was isolated by filtration, washed with water and dried to give the sulfate of 5 (0.167 g, 0.49 mmol). Yield 48%. CI/MS (free base), m/z 145 $[M + H]^+$. ^{15}N-NMR (D_2O): δ -324.0 (s), -306.3 (m), -246.3 (s), -231.2 (br).

[$^{15}N_3$]Guanine (6). Compound 5 (0.167 g, 0.49 mmol) was added to formamide (5 mL) and the mixture was refluxed for 6 h. After cooling and addition of water (5 mL), the mixture was filtered and the precipitate was recrystalized from 1M sulfuric acid. The sulfate of 6 was suspended in water and ammonium hydroxide was added until neutral pH was reached. The suspension was filtered to give the free base 6 (0.082 g, 0.53 mmol). Yield 96%. CI/MS m/z 157 $[M + H]^+$. ^{15}N-NMR (D_2O): δ -307.6 (br), -236.1 (br), -228.4 (br), -223.3 (br).

[$^{15}N_3$]-6-Thioguanine (7). Compound 6 (0.082 g, 0.53 mmol) was added to a solution of phosphorus pentasulfide (0.613 g) in sulfolane (1 mL) (16). The mixture was heated at 170-180°C for 4 h. After cooling at room temperature, water (5 mL) was slowly added and the reaction mixture was heated at 100°C for 2h to remove the remaining phosphorus pentasulfide. The mixture was filtered, the filtrate was set aside and the solid material was suspended again in aqueous ammonia and boiled for 2 h. After filtration, the filtrate was combined with the previous one and the volume was reduced under vacuum until compound 7 started to precipitate. After adjusting the pH to 7, the mixture was kept at 4°C overnight. The precipitate was filtered, washed with water and dried to give 7 (0.06 g, 0.35 mmol). Yield 66%. CI/MS, m/z 171 $[M + H]^+$.

[$^{15}N_3$]-6-Bromoguanine (1). Compound 7 (0.059 g, 0.35 mmol) was added to a solution of methanol/aqueous hydrobromic acid (1:1, 5 mL) (17). The suspension was cooled to 8-10°C and bromine (80 µL) was added slowly under stirring. The reaction mixture was stirred at 8-10°C for 2 h. The suspension was filtered, the solid residue was washed with cold water and acetone, and dried to give compound 1 (0.02 g, 0.09 mmol). Yield 27%. EI/MS m/z 216-218 $[M]^+$. CI/MS 217-219 $[M + H]^+$.

[$^{15}N_3$]-O^6-Alkylguanines (8). [$^{15}N_3$]-O^6-Methyl- (8a), ethyl- (8b), propyl-(8c), and butylguanine (8d) were synthesized from 1 using the appropriate alkoxide solution, as previously described (6,18). The yields were over 90% for 8a, 8b, and 8c and 30% for 8d.

RESULTS AND DISCUSSION

Our synthesis scheme takes advantage of described procedures modified as necessary to obtain the labelled guanines in good yield. Scheme 1 shows the strategy for introducing three ^{15}N-labels into the molecule of 6-bromoguanine using commercially available starting materials. Two ^{15}N labels were introduced into the guanidine molecule by reacting $^{15}NH_3$ with cyanogen bromide in a Carius tube at 110°C for 96h. In agreement with the fragmentation pattern of guanidine (19), EI/MS of 2 showed a molecular ion at m/z 61 (relative abundance 41%), consistent with the introduction of two ^{15}N labels. Intense fragment ions were observed at m/z 44 (100%) and 45 (61%) due to the loss of $^{15}NH_2$• and NH_2• respectively from the molecular ion. ^{15}N-labelled guanidine hydrobromide 2 was then condensed with ethyl cyanoacetate to give [$^{15}N_2$]-2,6-diamino-4-hydroxypyrimidine 3 (14). The structure of 3 was established by ^{15}N-NMR. Since the starting guanidine bore only two ^{15}N atoms, the labels of 3 were randomly distributed between positions 1, 5 and 6 of the molecule, as suggested by ^{15}N-NMR. The third ^{15}N-label was introduced by reacting the pyrimidine 3 with ^{15}N-sodium nitrite (14). CI/MS (reagent gas methane) showed the most intense peak at m/z 159 consistent with the presence of three ^{15}N labels.

The ^{15}N-NMR spectrum of 4 confirmed the introduction of the nitroso moiety at the 5 position. The nitroso derivative 4 was reduced to the corresponding triaminopyrimidine 5 which was isolated as the sulfate salt. [$^{15}N_3$]Guanine 6 was obtained in 96% yield after condensation of 5 with formamide. Compound 6 was converted to [$^{15}N_3$]-6-thioguanine according to the procedure described by Tomsons et al. (16), by reacting it with phosphorus pentasulfide in sulfolane. [$^{15}N_3$]-6-Thioguanine was readily converted to the corresponding bromopurine 1 using bromine in the presence of methanolic hydrobromic acid. This is a versatile procedure successfully applied for the synthesis of

bromopurines from the corresponding mercaptopurines (17). The preparation of 2-amino-6-bromopurine was preferred over the classical chlorination of 6-thioguanine because it is easier to use bromine than dry chlorine gas.

Mass spectroscopic analysis of 1 was consistent with the assigned structure for [$^{15}N_3$]-6-bromoguanine. As shown in Fig. 1a, the EI mass spectrum of 1 showed two intense ions at m/z 216 and m/z 218 corresponding respectively to the $[M]^+$ and $[M+2]^+$ ions, the latter being due to the natural isotopic abundance of bromine. By analogy the CI mass spectrum showed intense ions at m/z 217 and 219 (Fig. 1b).

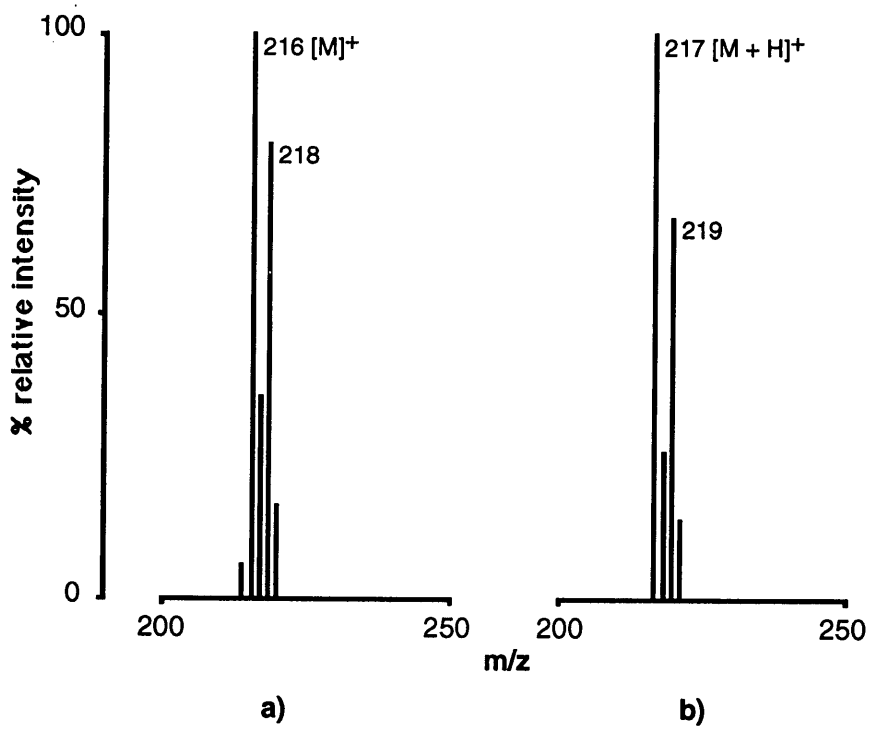


Fig. 1. Molecular ion region of the EI (a) and CI (b) mass spectra of 1.

[$^{15}N_3$]-6-Bromoguanine was readily converted to the 6-alkoxy derivative by adding it to a solution of the appropriate alcoxide (6,18). The compounds were isolated in yields ranging from 30 (O^6-butylguanine) to over 90% (O^6-methyl-, O^6-ethyl-, and O^6-propylguanine).

O^6-Alkylguanines were analyzed as their pentafluorobenzyl-trimethylsilyl derivatives by GC-MS with chemical ionization and negative ion detection and selected ion

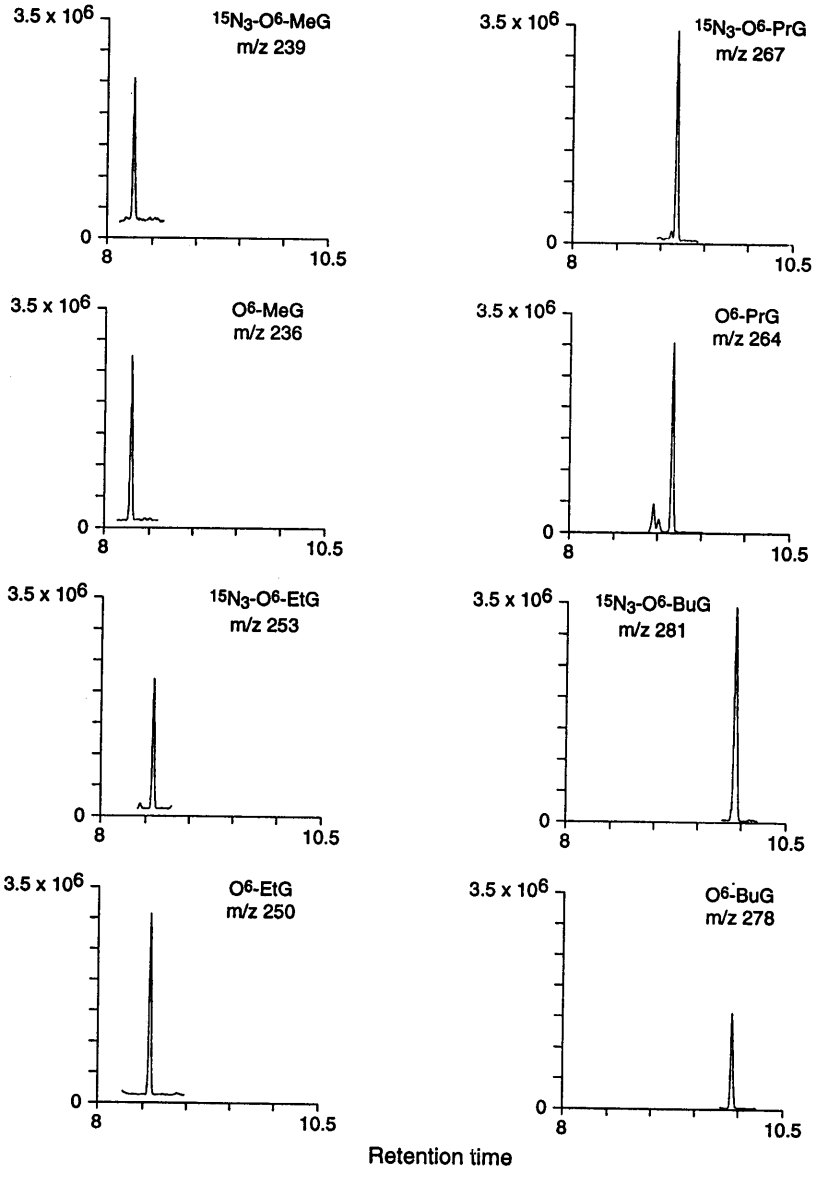


Fig. 2. Typical GC-NICI-SIR chromatogram of pentafluoribenzyl-trimethylsilyl derivatives of the $^{15}N_3$-O^6-alkylguanines indicated. Analytical conditions were as previously described (20).

recording (GC-NICI-SIR), as previously described (6). For each compound the ion at m/z [M-181]$^-$ was monitored, corresponding to the loss of the pentafluorobenzyl fragment. Figure 2 shows a typical GC-NICI-SIR chromatogram of a standard mixture of the four $^{15}N_3$-O^6-alkylguanines synthesized and the corresponding unlabelled O^6-alkylguanines. Analytical conditions were as previously described (20).

In conclusion, [$^{15}N_3$]-6-bromoguanine is a useful precursor for different ^{15}N-O^6-alkylguanines which can be used as internal standards for the quantitative determination of O^6-alkylguanines. They can also be applied in animal studies to detect biotransformation products of $^{15}N_3$-O^6-alkylguanines which can be easily distinguished from the unlabelled natural metabolites.

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