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SYNTHESIS OF ISOTOPICALLY LABELLED DNA DEGRADATION PRODUCTS FOR USE IN MASS SPECTROMETRIC STUDIES OF CELLULAR DNA DAMAGE

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SUMMARY

Thirteen substituted purines and pyrimidines bearing from three to five carbon, nitrogen and/or deuterium isotopic labels have been synthesized in yields ranging from .1 to 70%. Most of the products originate from the same small number of commercially available labelled starting materials, and in several cases one intermediate leads to two products, thus minimizing the expense and time required. The parent compounds are found in tissue as the result of DNA damage often linked with carcinogenesis and mutagenesis. The synthesized compounds serve as internal standards for the study of DNA damage using mass spectrometry.

Key Words: DNA, mass spectrometry, carcinogenesis

INTRODUCTION

Oxidative degradation of DNA to produce oxygenated derivatives of the natural purine and pyrimidine bases is often a factor in carcinogenesis, mutagenesis and ageing (1). In order to understand the role of oxidative DNA damage in biological processes, methods are needed to detect and quantify these degradation products in biological preparations. In the last few years a method has emerged for accomplishing this end by mass spectrometry (2). This has necessitated the preparation of isotopically-labelled analogs of these DNA degradation products for use as internal mass-spectral standards. To fill this need we have synthesized a series of these modified DNA bases, incorporating from three to five ^{13}C, ^{15}N and/or ^{2}H labels per molecule. Most of these compounds have already been put to use in DNA damage studies (2) and since we expect

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the mass-spectral method to gain popularity, we hereby describe the methods for their preparation.

DISCUSSION

The compounds synthesized are (Table 1): 5-hydroxy-5-methylhydantoin-[1,3-$^{15}N_2$-2-^{13}C] (1), 5-hydroxybarbituric acid-[1,3-$^{15}N_2$-2,4-$^{13}C_2$], (dialuric acid), (2), 5-formamido-2,6-diamino-4-hydroxypyrimidine-[1,3-$^{15}N_2$-(2-amino-^{15}N)-2-^{13}C] (3), 8-hydroxyguanine-[2-^{13}C-1,3-$^{15}N_2$-(2-amino-^{15}N)] (4), 4,6-diamino-5-formamidopyrimidine-[1,3-$^{15}N_2$-2-^{13}C-(5-aminoformyl-^{13}N-^{2}H)] (5), 8-hydroxyadenine-[1,3,7-$^{15}N_3$-2,8-$^{13}C_2$] (6), 5-hydroxycytosine-[1,3-$^{15}N_2$-2-^{13}C] (7), 5-hydroxyuracil-[1,3-$^{15}N_2$-2-^{13}C] (8), 6-hydroxyisobarbituric acid-[1,3-$^{15}N_2$-2-^{13}C], (isodialuric acid), (9), 5-hydroxymethyl-uracil-[4,5-$^{13}C_2$-α,α-$^{2}H_2$] (10), 5,6-dihydrouracil-[1,3-$^{15}N_2$-2-^{13}C] (11), 5,6-dihydro-thymine-[1,3-$^{15}N_2$-2-^{13}C] (12), and 5-hydroxy-5,6-dihydrothymine-[6,6,α,α,α-$^{2}H_5$] (13).

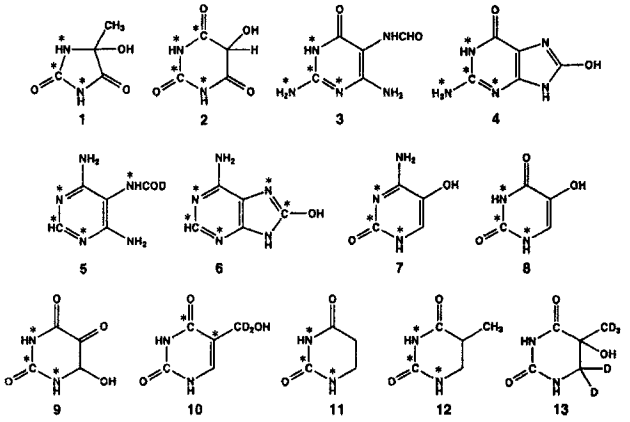
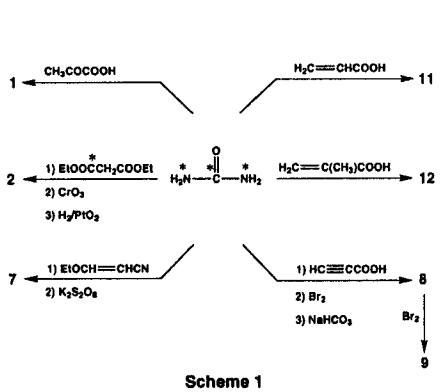


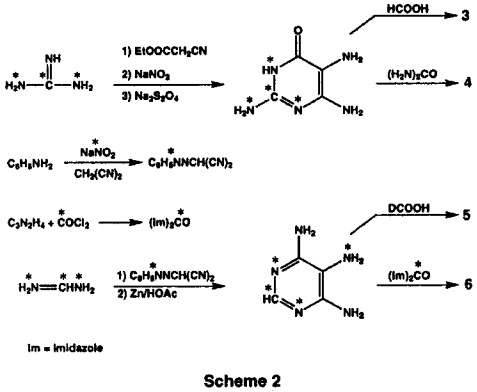
Table 1

The syntheses all employed well-known reaction sequences, long used for preparing the unlabelled compounds or intermediates leading thereto, as described in the experimental section. The only exception was for compound 6, where the published method (10) for the unlabelled material calls for a large excess of phosgene. Due to the prohibitive expense of employing a large amount of ^{13}C-labelled phosgene, it was found more expedient to first convert labelled phosgene to the diimidazole, which was then used in place of phosgene itself to give the final product in much better yield than would have resulted with phosgene directly.

The attractiveness of the method here presented results from two features. Firstly, several of the target compounds are prepared from a common intermediate, thus minimizing the number of steps needed to produce all thirteen compounds. Thus, 9 is prepared in one step from 8, while 3 and 4 are prepared in one step from the same intermediate, as is also true for 5 and 6. Secondly, eleven of the thirteen compounds can be obtained from just three fairly inexpensive labelled starting materials: urea-[^{13}C-$^{15}N_2$] (Scheme 1), guanidine-[^{13}C-$^{15}N_3$], and formamidine-[^{13}C-$^{15}N_2$] (Scheme 2). If one required only three labels per molecule for these eleven compounds, these would be the only labelled starting materials needed. Since many of our compounds had more than three labels we also used several other labelled reagents, (sodium nitrite-[^{15}N], formic acid-[^{2}H], ethyl malonate-[^{13}C], and phosgene-[^{13}C]), but since three labels per molecule are sufficient for most mass spectral applications this extra expense is not necessary.



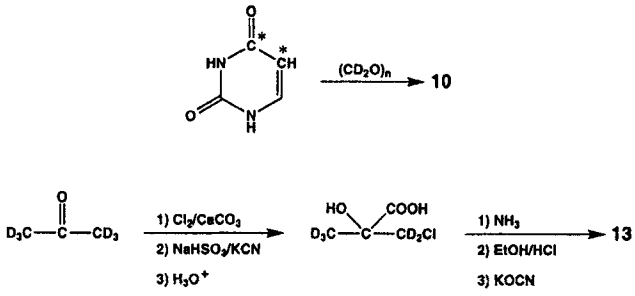
Scheme 1



Scheme 2

Two of the compounds described (10 and 13) do not originate from the above-mentioned three starting materials, but from three others which are relatively inexpensive (uracil-[4,5-$^{13}C_2$], paraformaldehyde-[$^{2}H_2$], and acetone-[$^{2}H_6$], (Scheme 3).

Since this work was completed two other syntheses of triply and quadruply labelled 8-hydroxyadenine and 8-hydroxyguanine have appeared in the literature. The first method (3) employs a somewhat lengthier route than ours to construct the pyrimidine rings. The second method (4) uses a synthetic scheme similar to ours but introduces the labels piecemeal by



Scheme 3

employing a greater variety of labelled reagents. Our method has an advantage over both of these methods in that the labels can be introduced all at once by employing a single triply labelled compound (formamidine) to construct the triaminopyrimidine ring needed for hydroxyadenine, and a single quadruply labelled compound (guanidine) to construct the triaminohydroxypyrimidine ring needed for hydroxyguanine. Neither of these starting materials proved to be significantly more expensive than versions containing a smaller number of labels.

Most of these compounds do not have reproducible melting points, but instead decompose over a fairly broad range or remain unmelted up to above 300°. However, all compounds appeared homogeneous by TLC or HPLC, except 4 which was insufficiently soluble to permit chromatographic analysis. All compounds yielded NMR spectra which matched those of the corresponding unlabelled compounds when allowance was made for the splitting of signals from atoms adjacent to ^{13}C or ^{15}N atoms. The identities of all compounds were further verified by high-resolution mass spectrometry, with the exception of 8 which proved insufficiently volatile to yield a sufficiently accurate mass measurement of its molecular ion. However, it was converted to 9 in one step, which did yield an exact mass.

Products 3, 8, 11, and 13 are obtained in low overall yields. Since all but 11 require three or more reaction steps this is not highly unusual. Compound 11 is obtained in only 7% yield even though it requires only one step, due to the high temperature and strongly acidic conditions required. This defect is offset by the simplicity of the synthesis, which has long been the method of choice for the unlabelled compound.

The lowest yield was obtained for 13. Besides the requirement of four reaction steps, this is due to the poor yield of the first step, which produces much dichloroacetone in addition to the desired monochloro compound and must be removed by distillation. However, due to the modest cost of the unlabelled starting materials, and the need for only small amounts of material as mass-spectral standards, the low yields for these compounds are not a serious drawback.

EXPERIMENTAL

General

Melting points are uncorrected. ^{1}H and ^{13}C NMR spectra were run on a Varian XL200 instrument at 200 MHZ, or a Varian Unity Plus instrument at 300 MHZ, using either D_2O or d_6 DMSO solutions with TSP as internal standard. Mass Spectra were run on a VG Micromass ZAB-2F instrument (LSIMS), or a VG 70-250 instrument (EI). TLC was run on Kodak silica gel sheets. HPLC was run on a Whatman Partisil M9 10/25 ODS column.

Although phosgene as a benzene solution is much safer to handle than pure phosgene, which is extremely toxic, it should only be opened and used under a hood.

Materials

Urea-[$^{15}N_2$-^{13}C], diethyl malonate [1-^{13}C], guanidine hydrobromide [$^{15}N_3$-^{3}C], and formamidine acetate [$^{15}N_2$-^{13}C], all of 99% isotopic purity, were obtained from ICON Services, Inc. Summit, NJ. Sodium nitrite-[^{15}N], formic acid-[1-^{2}H], and phosgene-[^{13}C] were obtained from MSD Isotopes, Montreal, Quebec, Canada. Paraformaldehyde-[$^{2}H_2$], uracil-[4,5-$^{13}C_2$], and acetone-[1,3-$^{2}H_6$] were obtained in 98+% isotopic purity from Cambridge Isotope Laboratories, Inc., Andover, MA.

5-Hydroxy-5-methylhydantoin-[1,3-$^{15}N_2$-2-^{13}C] (1) was made from pyruvic acid and urea-[$^{15}N_2$-^{13}C] following a published procedure (5) for the unlabelled material, except that the pyruvic acid was generated *in situ* from sodium pyruvate and hydrochloric acid. Yield; 35% based on urea. mp: 155° (dec.); lit. mp for unlabelled material: 166°. TLC (SiO_2; $CHCl_3$:MeOH:H_2O:

HCOOH; 85:15:1:1): one spot; R_f=.20. ^{1}H NMR (200 MHZ, d_6-DMSO) δ, 10.54 (d, 3-NH, J_{NH}=95 Hz), 8.34 (d, 1-NH, J_{NH}=95 Hz), 6.47 (s, OH), 1.35 (s, CH_3). ^{13}C NMR (200 MHZ, d_6-DMSO) δ, 176.1 (m, 4-C), 155.6 (t, 2-^{13}C, J_{CN}=19.2 Hz), 82.8 (m, 5-C), 23.4 (s, CH_3). Measured mass (LSIMS) of protonated molecular ion (134.0404) is within 2.7 mmu of calculated mass for $C_3{}^{13}CH_7O_3{}^{15}N_2$.

5-Hydroxybarbituric acid-[1,3-$^{15}N_2$-2,4-$^{13}C_2$] (2). Barbituric acid-[1,3-$^{15}N_2$-2,4-$^{13}C_2$] was prepared from diethyl malonate-[1-^{13}C] and urea-[$^{15}N_2$-^{13}C] using a standard procedure for the unlabelled compound (6). This was converted to alloxan monohydrate-[1,3-$^{15}N_2$-2,4-$^{13}C_2$] by chromium trioxide oxidation according to a published procedure for the unlabelled compound (7). Finally, the title compound was prepared by catalytic hydrogenation of the alloxan-[1,3-$^{15}N_2$-2,4-$^{13}C_2$] according to a published procedure for the unlabelled compound (8). Yield based on urea was 30%. Mp.: darkens at 197°, 229-231° (dec.). Lit. for unlabelled material: 190° (darkens), 220-230° (dec.). HPLC (H_2O:MeOH:HCOOH 95:5:0.1; 5 ml/min.) showed one peak at 1.8 min. ^{13}C NMR (300 MHZ, D_2O) δ, 79.7 (d, 5-C, J_{CC}=9.7 Hz), 153.4 (t, 2-^{13}C, J_{CN}=18.9 Hz), 172.2 (d, 4-^{13}C, J_{CN}=12.7 Hz). Measured mass (LSIMS) of protonated molecular ion (149.0256) is within 10 mmu of calculated mass for $C_2{}^{13}C_2{}^{15}N_2O_4H_5{}^+$.

5-Formamido-2,6-diamino-4-hydroxypyrimidine-[1,3-$^{15}N_2$-(2-amino-^{15}N)-2-^{13}C] dihydrogen sulfate (3). The labelled triaminohydroxy pyrimidine was made from guanidine hydrobromide-[$^{15}N_3$-^{13}C] and ethyl cyanoacetate, followed by nitrosation and reduction, according to a published procedure for the unlabelled material (9). This was converted to the formamide by refluxing with formic acid according to a published procedure for the unlabelled compound (10). The yield was 3% based on guanidine-[$^{15}N_3$-^{13}C] hydrobromide. HPLC (H_2O-MeOH 95:5; 5 ml/min.) showed one peak at 2.8 min. ^{13}C NMR (D_2O) δ, 90.0 (d, 6-C), 155.6 (m, 2- ^{13}C), 160.90 (m, 5-C), 163.8 (d, 4-C, J_{NC}=12.7 Hz), 173.3 (s, formyl C). ^{1}H NMR (D_2O) δ, 8.24 (s, Formyl H). Measured mass (EI) of molecular cation (175.0724) is within 2.3 mmu of mass for $C_4{}^{13}CH_9O_2N_2{}^{15}N_3{}^+$.

8-Hydroxyguanine-[2-^{13}C-1,3-$^{15}N_2$-(2-amino-^{15}N)] (4) was prepared from the above-described labelled triaminohydroxypyrimidine and urea according to a published procedure for the

unlabelled compound (10). The yield based on the labelled precursor was 68%. ^{13}C NMR (D_2O-NaOD, 300 MHZ) δ, 107.8 (d, 5-C, J_{CN}=7.1), 161.7 (ddd, 2-^{13}C, J_{CN}=5.6, 5.6, 19.9), 163.1-163.4 (m, 6,8-C), 167.2 (d, 4-C, J_{CN}=7.6 Hz). Measured mass (LSIMS) of molecular ion (171.0401) is within 1.4 mmu of calculated mass for $C_4{}^{13}CN_2{}^{15}N_3O_2H_6$.

4,6-diamino-5-formamidopyrimidine-[1,3-$^{15}N_2$-2-^{13}C-(5-aminoformyl-^{13}N-^{2}H)] (5). Phenylazomalononitrile-[2-^{15}N] was prepared from aniline, malononitrile and $NaNO_2$-[^{15}N] according to a published procedure for the unlabelled compound (11). This was reacted with formamidine acetate-[^{13}C-$^{15}N_2$], followed by zinc reduction to make 4,5,6-triaminopyrimidine-[2-^{13}C-1,3,5-$^{15}N_3$], isolated as the bisulfate salt, according to a published procedure for a doubly-labelled version of the same compound (12). Refluxing with DCOOH according to a published procedure for the unlabelled compound (10) produced the title product as the bisulfate salt in 70% yield based on formamidine acetate. HPLC (H_2O-MeOH 95:5; 5 ml/min.) showed one peak after 1 min. ^{13}C NMR (D_2O, 300 MHZ) δ, 93.9 (d, 5-C, J_{CN}=17.8), 151.7 (t, 2-^{13}C, J_{CN}=7.2), 158.9 (d, 4,6-C, J_{CN}=8.1 Hz). ^{1}H NMR (D_2O) δ, 8.12 (dt, Imido H, J_{NH}=209 Hz, J_{HD}=10.8 Hz). Measured mass (LSIMS) of molecular ion (158.0652) is within 0.5 mmu of calculated mass for $C_4{}^{13}CH_6{}^{2}HN_2{}^{15}N_3O$.

8-Hydroxyadenine-[1,3,7-$^{15}N_3$-2,8-$^{13}C_2$] (6). 1,1-Carbonyl diimidazole-[^{13}C] was prepared from phosgene-[^{13}C] (1.1M solution in Bz) and imidazole according to a published procedure for the unlabelled material (13). The above-described 4,5,6-triamino-pyrimidine-[2-^{13}C-1,3,5-$^{15}N_3$] (neutralized with one equiv. of aqueous KOH and evaporated to dryness) was dissolved in DMF and stirred 12 hr. with the labelled carbonyl diimidazole (2 equiv.). The mixture was evaporated under vacuum, dissolved in dilute aqueous sulfuric acid, chilled, and filtered. The solid was dissolved in dilute aqueous NaOH, charcoal was added, and the mixture filtered through Celite. Acidification of the filtrate with sulfuric acid and chilling precipitated the product in 70 % yield. HPLC (MeOH:H_2O:HCOOH-5:95:1, 5 ml/min.) showed one peak at 1 min. ^{13}C NMR (D_2O-NaOD, 300 MHZ) δ, 151.3 (s, 8-^{13}C), 171.0 (dd, 2-^{13}C, J_{CN}=9.7 Hz). ^{1}H NMR (D_2O-NaOD, 300 MHZ) δ, 7.94 (dt, 2-H, J_{CN}=199, J_{NH}=14 Hz). Measured mass (LSIMS) of molecular ion (156.0478) is within 0.5 mmu of calculated mass for $C_3{}^{13}C_2N_2{}^{15}N_3H_5O$.

5-Hydroxycytosine-[1,3-$^{15}N_2$-2-^{13}C] (7). Cytosine-[1,3-$^{15}N_2$-2-^{13}C] was prepared from ethoxyacrylonitrile and urea-[$^{15}N_2$-^{13}C] according to a published procedure for the unlabelled compound (14). The title compound was prepared from this by persulfate oxidation according to a published procedure for the unlabelled compound (15). The yield was 11% based on labelled urea. HPLC (MeOH:H_2O-2:8, 5 ml/min.) showed one peak at 2.3 min. ^{13}C NMR (D_2O-NaOD, 300 MHz) δ, 171.3 (s, 5-C), 165.6 (t, 4-C, J_{CN}=20.4, J_{CC}=20.4 Hz), 159.2 (dd, 2-^{13}C, J_{CN}=16.3, J_{CN}=9.18 Hz), 123.2 (d, 6-C, J=10.2 Hz). ^1H NMR (D_2O-NaOD, 300 MHZ) δ, 6.63 (dd, 6-H, J_{NH}=7.4, J_{CH}=3.4 Hz). Measured mass (LSIMS) of molecular ion (130.0357) is within 1.2 mmu of calculated mass for $^{13}CC_3H_5N^{15}N_2O_2$.

5-Hydroxyuracil-[1,3-$^{15}N_2$-2-^{13}C] (8). Uracil-[1,3-$^{15}N_2$-2-^{13}C] was prepared from propiolic acid and urea-[$^{15}N_2$-^{13}C] according to a published procedure for the unlabelled compound (16). 5-Bromouracil-[1,3-$^{15}N_2$-2-^{13}C] was prepared from this by stirring for 0.5 hr with bromine (1 eq) in acidic acid and filtering. The product was identical with authentic unlabelled bromouracil by TLC. Finally, the labelled bromouracil was converted to the title compound by treatment with aqueous sodium bicarbonate according to a published procedure for the unlabelled material (17). The yield based on urea was 2%. HPLC (H_2O:MeOH:HCOOH-95:5:0.1; 5 ml/min.) showed one peak at 2.1 min. ^{13}C NMR (DMSO d_6, 200 MHZ) δ, 120.5 (d, 6-C), 131.5 (d, 5-C), 149.9 (dd, 2-^{13}C, J_{CN}=16.3 Hz), 161.5 (d, 4-C). ^1H NMR (DMSO d_6) δ, 6.83 (m, CH), 8.39 (s, OH), 10.41 (d, ^{15}NH, J_{NH} = 97.4 Hz), 11.37 (d, ^{15}NH, J_{NH} = 89.8).

6-Hydroxyisobarbituric acid-[1,3-$^{15}N_2$-2-^{13}C] (9) was prepared by bromine oxidation of the above 5-hydroxyuracil-[1,3-$^{15}N_2$-2-^{13}C] according to a published procedure for the unlabelled material (18). Yield: 90%. HPLC (H_2O:HCOOH-99.9:0.1; 5 ml/min.) showed one peak at 2.0 min. ^{13}C NMR (D_2O, 300 MHZ) δ, 79.6 (d, 6-C, J_{CN} = 9.7), 91.1 (d, 5-C, J_{CN}=6.1), 156.6 (dd, 2-^{13}C, J_{CN}=20.9), 172.9 (d, 4-C, J_{CN}=10.7 Hz). Measured mass (EI) of molecular ion (147.0131) is within 1.4 mmu of calculated mass for $C_3{}^{13}CH_4{}^{15}N_2O_4$.

5-Hydroxymethyluracil-[4,5-$^{13}C_2$-α,α-$^2H_2$] (10) was prepared by reacting uracil [4,5-$^{13}C_2$] with paraformaldehyde-[$^2H_2$] according to a published procedure for the unlabelled material (19).

The yield based on uracil was 31%. HPLC (H_2O:MeOH:HCOOH-95:5:0.1; 5 ml/min.) showed one peak at 2.0 min. ^{13}C NMR (DMSO, 300 MHZ) δ, 112.5 (d, 5-^{13}C, J_{CC}=63.7 Hz), 138.7 (s, 6-C), 151.3 (s, 2-C), 163.8 (d, 2-^{13}C, J_{CC}= 63.2 Hz). ^1H NMR (d_6 DMSO, 300 MHZ) δ, 4.75 (s, OH), 7.20 (dd, 6-H, J_{CH}=9.6 Hz, J_{CH}=1.71 Hz), 10.66 (m, NH), 10.98 (m, NH). Measured mass (LSIMS) of molecular ion (147.0634) is within 9 mmu of calculated mass for $C_3{}^{13}C_2H_5D_2O_3N_2^+$.

5,6-Dihydrouracil-[1,3-$^{15}N_2$-2-^{13}C] (11) was prepared from acrylic acid and urea-[$^{15}N_2$-^{13}C] according to a published procedure for the unlabelled material (20). The yield based on urea was 7%. HPLC (H_2O:HCOOH 99.9:0.1, 5 ml/min.) showed one peak at 3.0 min. Mp. 272° (dec.). Lit. 275°. ^{13}C NMR (d_6 DMSO, 200 MHZ) δ, 30.7 (d, 5, 6-C, J_{CN}=5.4), 33.7 (d, 6,5-C, J_{CN}=8.3), 154.6 (dd, 2-^{13}C, J_{CN}=21.1), 172.1 (d, 4-C, J_{CN}=9.2 Hz). ^1H NMR δ, 2.43 (m, 6-H), 3.20 (m, 5-H), 7.46 (d, ^{15}NH, J_{NH}=93.4), 9.91 (d, ^{15}NH, J_{NH}=90.0 Hz). Measured mass (LSIMS) of molecular ion (117.0441) was within 3.7 mmu of calculated mass for $C_3{}^{13}CH_6{}^{15}N_2O_2$.

5,6-Dihydrothymine-[1,3-$^{15}N_2$-2-^{13}C] (12) was prepared from methacrylic acid and urea-[$^{15}N_2$-^{13}C] according to a published procedure for the unlabelled material (20). The yield based on urea was 12%. HPLC (H_2O:HCOOH 99.9:0.1, 5 ml/min.) showed one peak at 4.2 min. Mp. 260° (dec.). Lit. (20), unlabelled material: 264°. ^{13}C NMR (d_6 DMSO, 200 MHZ) δ, 12.2 (s, CH_3), 34.3 (d, 5,6-C, J_{CN}=5.4), 41.9 (d, 5,6-C, J_{CN}=8.7), 153.9 (dd, 2-^{13}C, J_{CN}=20.6), 173.6 (d, 4-C, J_{CN}=9.4 Hz). ^1H NMR (d_6 DMSO, 200 MHZ) δ, 1.05 (d, CH_3, J=7.0), 2.54 (m, 6-H), 2.94 (t, 6'-H, J=10.8), 3.25 (m, 5-H), 7.49 (d, NH, J_{NH}=94), 9.89 (d, NH, J_{NH}=90 Hz). Measured mass (EI) of molecular ion (131.0658) was within 9.8 mmu of calculated mass for $C_4{}^{13}CH_8{}^{15}N_2O_2$.

5-Hydroxy-5,6-dihydrothymine-[6,6,α,α,α-$^2H_5$] (13). Chloroacetone-[$^2H_5$] was prepared from hexadeuteroacetone by chlorination in the presence of calcium carbonate and D_2O according to a published procedure for the unlabelled material (21). This material was converted into β-chloro-α-hydroxy-isobutyric acid-[$^2H_5$] according to a published procedure for the unlabelled material (22), except that D_2O was used in place of H_2O as solvent in the first step. This material was then converted to the title compound by a published procedure for the unlabelled material (23). The yield based on hexadeutero-acetone was 0.1%. HPLC (H_2O:HCOOH 99.9:0.1, 5

ml/min.) showed one peak at 2.1 min. Mp: 250-253° (Lit (19) for unlabelled material: 255°). ^{13}C NMR (d_6 DMSO, 200 MHZ) δ, 66.7 (s, 5-C), 153.2(s, 2 or 4-C), 173.6 (s, 2 or 4-C). ^{1}H NMR (d_6 DMSO, 300 MHZ) δ, 5.71 (s, OH), 7.50 (s, 1-H), 9.93 (s, 3-H).

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