SYNTHESIS OF HEXALABELED THYMINE AND THYMIDINE

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SUMMARY

The synthesis of hexalabeled thymine and thymidine are described and field ionization mass spectra are recorded for these materials. Field ionization mass spectrometry is preferred in the utilization of multilabeled compounds as tracers in biomedical applications.

Organic molecules substituted by a sufficient number of nonradioactive isotopic atoms, so-called molecular tracers,[1] can be used in biological tracer studies or in isotope dilution analysis with the same degree of sensitivity as radioactively labeled molecules.[2] Such multilabeled materials may be assayed by mass spectrometry and their abundance compared to that of the unlabeled species. The method of ionization which produces the highest yield of isotopically unscrambled parent ions is field ionization.[2,3] Field ionization mass spectrometry has another advantage over other methods of ionization; by virtue of its nonfragmenting nature, interference by fragment ions of impurities with the same molecular weight as the material of interest or its multilabeled analog is less likely.[4]

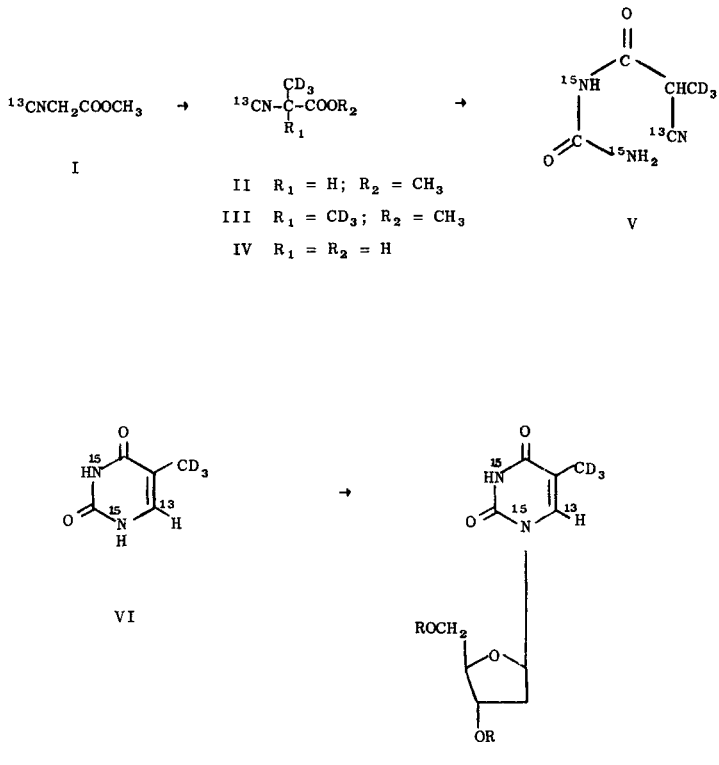
In order to keep the natural isotopic background below 10^{-6} and thus allow a reasonably high sensitivity of detection of molecular tracers, these have to be multilabeled to produce M+5 to M+7 species in the molecular weight range 100 to 200, and correspondingly M+7 to M+12 for compounds with molecular weights between 200 and 300.

Following this reasoning, hexalabeled thymidine, labeled on the pyrimidine entity, has been synthesized from multilabeled precursors to be used in a number of different biological[5] and clinical studies including the measurement of thymidine uptake by leukemic cells. The tracer may be measured mass spectrometrically either as the parent ion of the thymidine or following hydrolysis as thymine. In the latter case, the natural background of M+6 thymine (132 amu) is lower than 2×10^{-6} of the abundance of the unlabeled material.[4] The synthesis of these materials and their mass spectra are presented in this paper.

Route of Synthesis of Hexalabeled Thymidine

The synthesis of multilabeled thymidine is outlined in Scheme 1. Since only four nonexchangeable protons are available in thymine for substitution by deuterium, it was necessary to consider replacement of C, N or O atoms by their stable isotopes. ^{15}N or ^{18}O are more desirable than ^{13}C for this purpose because they are currently available in > 98% isotopic purity as compared to 85-90% for ^{13}C. However, the route utilized for construction of the thymine ring was especially convenient for incorporation of ^{15}N and ^{13}C labels.[6] In addition, since some of the intermediates were used in the synthesis of other labeled pyrimidine nucleosides, it was more economical to use ^{15}N and ^{13}C as labels in addition to ^{2}H.

Methyl cyano-^{13}C-acetate (I) was prepared[7] from sodium chloroacetate and sodium cyanide-^{13}C with esterification of the resultant cyanoacetic acid by methanolic hydrogen chloride. The cyanoester was alkylated by trideuteromethyl iodide-sodium hydride in tetrahydrofuran to afford the trideutero cyano-^{13}C-propionate (II) in 65% yield. The yield of mono alkylated product[8] was dependent upon use of less than a stoichiometric amount of CD_3I to minimize the bis-methylation product (III). The ester (II) could be separated by careful fractional distillation from unreacted



Scheme 1

I and the small amounts of (III) so obtained. Alkaline hydrolysis of III afforded the acid (IV).

Condensation[9] of the cyano acid (IV) with bis-^{15}N-urea in the presence of acetic anhydride gave the multilabeled ureido compound (V). Hydrogenation of V with D_2 in aqueous (H_2O) media resulted in reduction of the cyano group with concomitant cyclization and elimination of ammonia to yield (49%) thymine-1,3-^{15}N-5-d_3-6-^{13}C (VI). The expected insertion of deuterium at C-6 did not occur because of the exchange reaction between solvent (H_2O) and D_2, resulting in thymine (m + 6). However, in later experiments, to avoid the possibility of isotopic exchange of D_2 with H_2O,

the reduction of V was carried out with D_2 in D_2O, and successfully labeled the thymine at the 6-position with deuterium. Thus, for future mass spectrometric work with thymine (m + 7) will be prepared according to this scheme.

Incorporation of the sugar moiety to prepare multilabeled thymidine was carried out according to the general method of Gupta and Bubbar,[10] previously utilized in the synthesis of 5-hydroxymethyl-2-deoxyuridine. Thymine was treated with trimethylsilyl chloride and the resulting bis-silyl compound was immediately coupled with 1-chloro-2-deoxy-3,5-di-O-p-toluylribofuranose[11] in acetonitrile at 25°. From the anomeric mixture a 33% yield of the desired α-anomer (VII) was obtained after crystallization from methanol. Removal of the toluoyl blocking groups by transesterification ($NaOCH_3$-CH_3OH) afforded thymidine-1,3-^{15}N-5-CD_3-6-^{13}C (VIII).

Mass Spectra of Thymine and Thymidine

Compared with the highly fragmented mass spectrum of thymine obtained by classical electron impact (Figure 1a) the field ionization spectrum of the same compound contains essentially only the parent ion (Figure 1b). The field ionization mass spectra of thymidine and of M+6 thymidine are presented in Figures 2a and 2b. Note here some thermal decomposition of the nucleoside which produces the pyrimidine at masses 126 and 132 respectively as well as desoxyribose (116 amu).

The field ionization mass spectrum over a short mass range of a 1:1 mixture of thymine and M+6 thymine (VI) is presented in Figure 3. The peaks at masses 127 and 133 respectively are the result of the expected M+1 species due to the natural abundances of ^{13}C, ^{15}N, and ^{2}H. The peaks at masses 128 and 134 respectively are predmoinantly those of the ^{18}O carrying species due to the natural abundance of this isotope. The peak at mass 131 produced by the pentalabeled thymine ions, because each of the isotopic reactants used in the synthesis, containing ^{13}C, ^{15}N, and ^{2}H, carry a small fraction of unlabeled material. Figure 4 demonstrates that the amount of unlabeled thymidine in the hexalabeled material is

less than one part in 10^4. This is close to the theoretical level of isotopic background considering the isotopic purity of the labeled precursors.

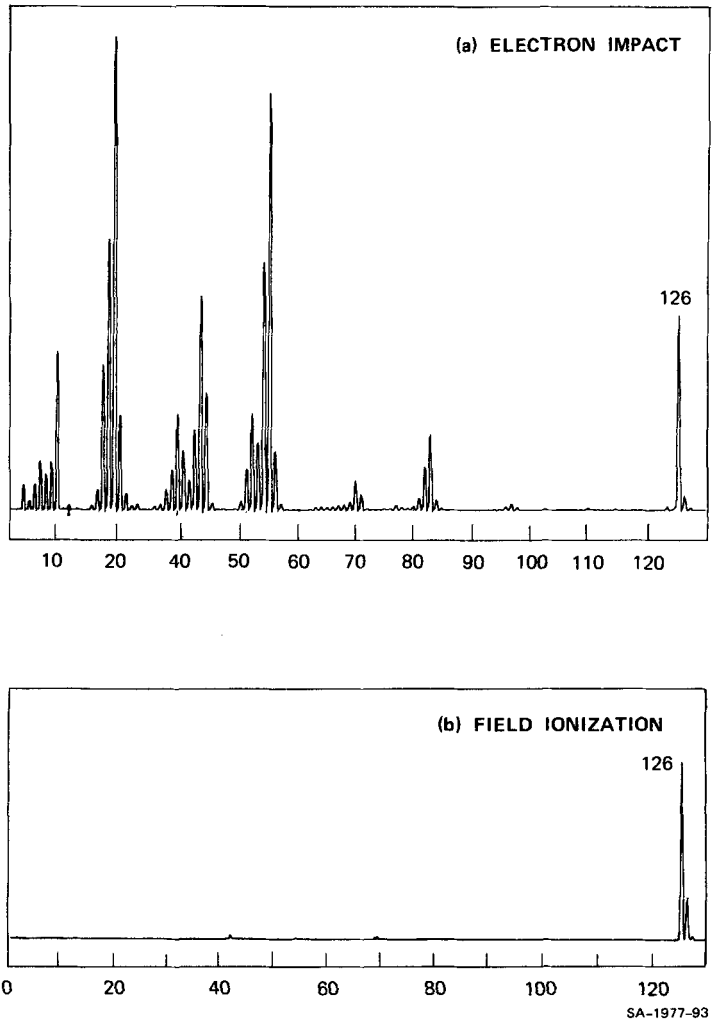


FIGURE 1 MASS SPECTRA OF THYMINE (MW 126)

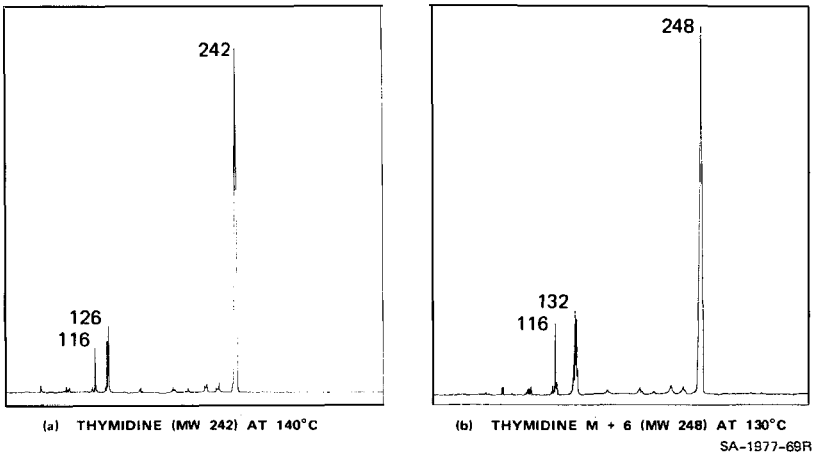


FIGURE 2 FIELD IONIZATION SPECTRA OF THYMIDINE

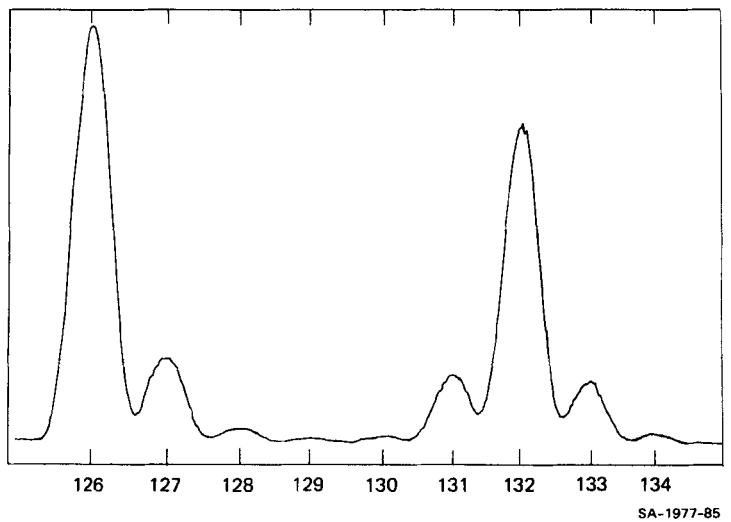


FIGURE 3 MASS SPECTRUM OF 1:1 MIXTURE OF THYMINE AND HEXALABELED THYMINE

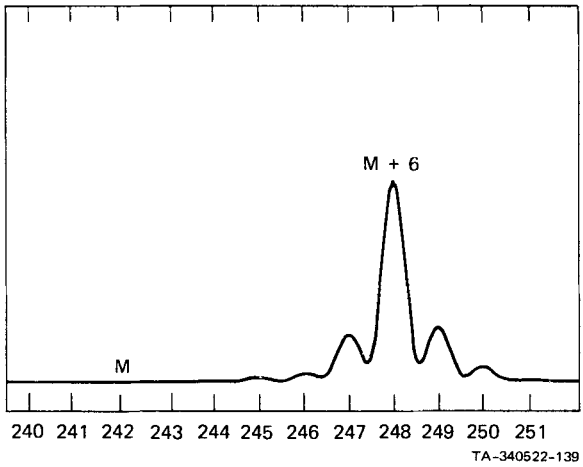


FIGURE 4 MASS SPECTRUM OF THYMIDINE M + 6

EXPERIMENTAL

Methyl 2-Cyano-^{13}C-propionate-3,3,3-d_3 (II)

Sodium hydride (5.80 g, 0.13 mole, 55% oil suspension) was washed with tetrahydrofuran to remove oil and suspended in 200 ml of tetrahydrofuran. The slurry was added over 20 minutes to an ice-cold, stirred mixture of methyl cyano-^{13}C-acetate[7] (I, 20.0 g, 0.20 mole), methyl iodide-d_3 (18.4 g, 0.13 mole) and 200 ml of tetrahydrofuran. The mixture was stirred another 10 minutes and the solvent removed in vacuo. The residue was treated with 200 ml of dichloro methane, filtered to remove sodium iodide and the filtrate was evaporated to leave 21.7 g of yellow oil. The mixture was carefully distilled through a spinning band column at 20 mm, collecting several small fractions from 63-78°. GC analysis showed the cuts boiling at 73-75° (7.55 g) were nearly pure mono CD_3 ester (II), while 1.10 g of the methyl cyano-^{13}C-isobutyrate-d_6 (III) was obtained from the 63-69° fractions. Unreacted I (8.31 g) remained in the pot residue. Another 0.97 g of II was obtained by preparative gas chromatography of intermediate fractions rich in compound II; total yield was 8.47 g (65% based on recovered I); NMR ($CDCl_3$) 3.90 δ (3H, singlet $COOCH_3$), 3.64 (1H, doublet, $J_{H-^{13}C}$ = 12 Hz).

2-Cyano-^{13}C-propionic Acid-3,3,3-d_3 (IV)

A solution of 8.4 g of the ester (II) in 100 ml of 6% sodium hydroxide in methanol was kept at room temperature for 2 hours and evaporated to dryness in vacuo. The residue was dissolved in 90 ml of 20% hydrochloric acid and the solution evaporated. The residue was stirred with 200 ml of an ether-acetone (1:1) mixture. This mixture was dried with magnesium sulfate, filtered and the salts washed with ether. The filtrate was evaporated to leave a pale yellow oil that was vacuum dried to leave 7.16 g (97%); NMR ($CDCl_3$) 3.72 δ (1H, doublet, $J_{H-^{13}C}$ = 12 Hz).

2-Cyano-^{13}C-propionyl-3,3,3-d_3-urea-1,3-^{15}N (V)

A mixture of 3.70 g (36 mmole) of the cyano acid (IV), 2.50 g (40 mmole) of bis-^{15}N-urea and 4.5 ml (45 mmole) of acetic anhydride was heated on a steam bath for 2 hours. Water (25 ml) was added and the mixture heated for another 30 minutes. The resulting pale yellow solution was chilled for one hour and the crystalline precipitate collected. The cake was washed twice with 10 ml portions of water and dried in vacuo to afford 3.05 g (58%, mp 190-191° (lit.[9] 192°); NMR (d_6-DMSO-$CDCl_3$; 1:1) 3.91 δ (1H, doublet, $J_{H-^{13}C}$ = 12 Hz), 10.30 (1H, COO<u>H</u>), 7.35 (2H, doublet, $J_{H-^{15}N}$= 88 Hz, $^{15}N\underline{H}_2$), 9.60 (1H, doublet, $J_{H-^{15}N}$ = 92 Hz, -CO-$^{15}N\underline{H}$-CO-).

Thymine-1,3-^{15}N-6-^{13}C-α,α,α-d_3 (VI)

A mixture of 2.41 g (17 mmole) of the ureide (V), 1.0 ml (17 mmole) of acetic acid, 0.20 g of platinum oxide and 120 ml of water (H_2O) was stirred under one atmosphere of deuterium gas at 70° for 24 hours. A total of 1.38 equiv. of gas was absorbed. The catalyst was removed by filtration and the filtrate concentrated in vacuo to a volume of 30 ml. The crystalline precipitate was collected and dried to leave 0.87 g. Further concentration to 15 ml gave a second crop of 0.18 g for a total yield of 1.05 g (49%); TLC (chloroform-methanol, 9:1) and UV spectra

were identical to natural thymine. Field ionization mass spectrometry showed a molecular ion of 132 indicating the absence of deuterium label at the 6-position. The product from unlabeled runs was identical with natural thymine in all respects.

Ureide, prepared from the labeled cyano-acid (IV) and ordinary urea, was hydrogenated with deuterium gas in H_2O to afford thymine-6-^{13}C-α,α,α-d_3; FIMS molecular weight 130; NMR (d_6-DMSO) 4.84 δ (1H, doublet, $J_{H-^{13}C}$ = 59 Hz, C_6-H). However, reduction of the same ureide with D_2 in D_2O gave thymine-6-^{13}C-6,α,α,α-d_4; FIMS MW 131; NMR (d_6-DMSO) no proton at C_6.

3',5'-Di-p-toluoyl Thymidine-1,3-^{15}N-6-^{13}C-α,α,α-d_3 (VII)

A mixture of 0.91 g (6.8 mmole) of labeled thymine (VI), 2.57 g (23.6 mmole) of chlorotrimethylsilane, 2.6 ml (18.8 mmole) of triethylamine and 20 ml of benzene was stirred at ambient temperature for 17 hours. Precipitated triethylamine hydrochloride was removed by filtration and the filtrate evaporated *in vacuo*. The residual bis-silylated thymine was dissolved in 15 ml of acetonitrile and treated with 2.65 g (6.8 mmole) of 1-chloro-3,5-di-p-toluoyl-2-deoxyribofuranose.[11] The mixture was stirred at ambient temperature for 21 hours and evaporated *in vacuo*. The residue was partitioned between 25 ml of dichloromethane and 20 ml of water. The aqueous portion was extracted with another 10 ml of dichloromethane and the combined extracts washed with 10 ml of water. The extract was dried over magnesium sulfate and evaporated to leave a foamy residue. The material was twice recrystallized from 12 ml portions of methanol to afford 1.28 g (39%), mp 197-198° (lit.[12] mp 197°C).

A second crop of 0.80 g appeared to be a mixture of anomers inseparable by conventional column chromatography.

Thymidine-1,3-^{15}N-6-^{13}C-α,α,α-d_3 (VIII)

A solution of 1.28 g of the blocked nucleoside (VII) in 5 ml of methanol containing 30 mg of sodium methoxide was refluxed under nitrogen for 19 hours. The solvent was evaporated *in vacuo* and the residue par-

titioned between 20 ml of water and 10 ml of ether. Evaporation of the aqueous solution afforded 0.54 g of a colorless gum. Two crystallizations from ethanol yielded 0.42 g (65%), mp 183-184°C (lit.[12] mp 187°); TLC (methanol-chloroform, 1:4; silica gel) showed a single spot at R_f 0.30, identical with natural thymidine; NMR (d_6-acetone) multiplets due to deoxyriboside at 2.1 δ, 3.5-4.5 δ, 6.1 δ; 7.65 δ (1H, triplet of doublets, $J_{H-^{13}C}$ = 178 Hz, $J_{H-^{15}N}$ = 2 Hz), 9.66 (1H, doublet, $J_{H-^{15}N}$ = 88 Hz).

Mass Spectrometric Measurements

The mass spectra were measured on an SRI constructed field ionization mass spectrometer which has been described elsewhere.[2,4]

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