Spirodihydantoin Is a Minor Product of 5-Hydroxyisourate in Urate Oxidation

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Experimental Procedures:

***General Information**:* All reagents were of commercial quality and used as received. A Thermosil-Keystone hypercarb column (150 × 3 mm, 5 μm) was used for most HPLC and LC-MS analyses on Agilent 1100 Series HPLC and LC/MSD systems, respectively. In a typical LC-MS analysis, the mobile phase consisted of 0.4% formic acid in water (A) and 0.4% formic acid in acetonitrile (B) with 0-3% B over 10 min at a flow of 0.2 mL/min. Selective ion monitoring (SIM) at 185.1/188.1 (positive ion mode) or 183.1/186.1 (negative ion mode) was used to detect the labeled and unlabeled spirodihydantoin. In a typical HPLC analysis, the mobile phase consisted of ammonium bicarbonate (50 mM, pH 7.4) (A) and acetonitrile (B) with 0-20% B over 30 min at a flow rate of 0.3 mL/min. Uric acid and allantoin were quantitated by HPLC-UV using external standards. Spirodihydantoin was quantitated by isotope-dilution mass spectrometry in positive or negative ion mode using labeled spirodihydantoin as the internal standard. High-resolution FT-MS analyses were conducted on a Bruker FTMS. ESI-MS analyses were conducted on an Agilent ESI-TOF MS. Allantoin, spirodihydantoin, guanidinohydantoin and spiroiminodihydantoin bases and their corresponding ^{18}O-labeled compounds, 1-methylallantoin, 3-methylallantoin, and 1-methylspirodihydantoin were all dissolved in water. The ESI-TOF MS analyses were conducted on the hypercarb column. The mobile phase consisted of 0.4% formic acid in water (A) and 0.4% formic acid in acetonitrile (B) with a gradient of 0-9% B over 30 min at a flow rate of 0.2 mL/min. The NMR experiments were conducted on a Varian 500 MHz instrument. Both spirodihydantoin and 1-methylspirodihydantoin were dissolved in D_6-DMSO and the ^{13}C-NMR data were collected overnight.

Synthesis of Spirodihydantoin and 2-^{13}C-1,3-$^{15}N_2$-Spirodihydantoin. The synthesis of spirodihydantoin (Spd) was a modification of Poje's procedure(*1*). Acetonitrile was added to the mixture of anhydrous 4-hydroxy-2,5-dioxo-4-imidazolidinecarboxyureide and trifluoroacetic anhydride. The resulting mixture was heated at 75 °C for 10 min. Solvents were evaporated *in vacuo* to afford a viscous yellow residue, to which methanol (20 mL) was added to give a precipitate. The precipitate was recrystallized from water as a colorless solid (ESI-MS: 185.1 $[M+H]^+$; 183.1 $[M-H]^-$, 140.1 $[M-H-NHCO]^-$; FT-MS (negative ion mode): theoretical mass of $[M-H]^-$: 183.0149; actual mass: 183.0152; ^{13}C NMR (D_6-DMSO): δ

169.6, 155.9, 83.0 and 76.2 ppm). The synthesis of 2-^{13}C-1,3-$^{15}N_2$-spirodihydantoin was similar to that of spirodihydantoin except that ^{13}C, $^{15}N_2$-labeled urea was used (FT-MS: theoretical mass: 186.0149; actual mass: 186.0123 $[M-H]^-$; ESI-MS: 188.1 $[M+H]^+$; 186.1 $[M-H]^-$, 143.1 $[M-H-NHCO]^-$, 142.1$[M-H-^{15}NHCO]^-$, and 141.1 $[M-H-^{15}NH^{13}CO]^-$). Both spiro compounds were further purified by HPLC on a 4.6 $\times$ 250 mm Zorbax Eclipse XDB C8 column with 100% water as mobile phase at a flow rate of 1.0 mL/min. Retention times for both the labeled and unlabeled spiro compounds were 2.8 min.

Oxidation of Uric Acid by 2 equivalents of Na_2IrCl_6 at Various pH. Uric acid (901 μM) and labeled Spd (0.9 μM) were dissolved in potassium phosphate (KPi) buffers (0.1 M, pH at 6.7, 7.4, 8.0, 9.5, 10.2, 11.2 and 12.2). Na_2IrCl_6 (1.8 mM) was added (*2*) and the resulting mixture stirred at room temperature for 30 min. Each oxidation was carried out in duplicates with control experiments where no Na_2IrCl_6 was added.

Dose Response of Oxidation of Uric Acid by Na_2IrCl_6 at pH 8.0. Uric acid (919 μM) and labeled Spd (0.9 μM) were dissolved in KPi buffer (0.1 M, pH 8.0). Na_2IrCl_6 (0.2, 0.5, 1, 2, 5, 10 or 20 equivalents relative to uric acid) was added and the resulting mixture stirred at room temperature for 30 min. Each oxidation was carried out in duplicates with control experiments where no Na_2IrCl_6 was added.

Oxidation of Uric Acid by 2 equivalents of Na_2IrCl_6 at Various Temperatures. Uric acid (930 μM), labeled Spd (1.4 μM), and Na_2IrCl_6 (1.9 mM) were held in KPi buffer (0.1 M, pH 7.4) at 0 °C, 24, 50, 62 and 84 °C for 30 min with stirring. Each oxidation was carried out in duplicates with control experiments where no Na_2IrCl_6 was added.

Dose Response of Oxidation of Uric Acid by Peroxynitrite. Uric acid (925 μM) and labeled Spd (0.7 μM) were dissolved in a buffer containing KPi (150 mM) and sodium bicarbonate (25 mM) (pH 7.2) and varying equivalents of peroxynitrite relative to uric acid (0.2, 0.5, 1 and 2) were added to separate reactions in bolus fashion. Uric acid concentration was reduced to 171 μM when 10 and 20 equivalents of peroxynitrite were applied. Each oxidation was carried out in duplicates with control experiments where no peroxynitrite was added.

Oxidation of Uric Acid by Uricase/O_2. Urate oxidase (1 unit) was added to an oxygenated solution of uric acid (918 μM) and labeled Spd (0.7 μM) (in pH 7.4 KPi buffer)(*3*). O_2 was gently bubbled through the mixture for 15 min. The reaction mixture was applied to Amicon YM-10 centrifugal spin columns and the filtrate was concentrated by freeze-drying for the analysis of Spd.

Oxidation of Uric Acid by Horseradish Peroxidase/H_2O_2 with or without Presence of NO_2^-. Horseradish peroxidase (3.9 μM), uric acid (753 μM), labeled Spd (0.9 μM), DTPA (99 μM), and $NaNO_2$ (10.8 mM) were gently mixed in KPi buffer (0.1 M, pH 7.4). H_2O_2 (12.7 mM) was added to the mixture to initiate the oxidation(*4*). The reaction mixture was stirred at room temperature for 8 h and applied to Amicon YM-10 centrifugal spin columns. The filtrate was concentrated for the analysis of uric acid, allantoin and Spd. For the oxidation without the presence of NO_2^-, an equivalent volume of water was added instead of the nitrite solution.

Oxidation of Uric Acid by Myeloperoxidase/H_2O_2 with or without Presence of NO_2^-. Myeloperoxidase (0.02 μM), uric acid (733 μM), labeled Spd (0.8 μM), DTPA (94.3 μM), and $NaNO_2$ (10.3 mM) were gently mixed. H_2O_2 (0.06 mM) was added to the mixture to initiate the oxidation(*4*). The reaction mixture stirred at room temperature for 8 h and was applied to Amicon YM-10 centrifugal spin columns. The filtrate was concentrated for the analysis of uric acid, allantoin and Spd. For the oxidation without NO_2^-, an equivalent volume of water was added instead of the nitrite solution.

Synthesis of 4-Hydroxy-1-methyl-2,5-dioxo-4-imidazolidinecarboxyureide and 1-Methylspirodihydantoin. The synthesis of 4-hydroxy-1-methyl-2,5-dioxo-4-imidazolidinecarboxyureide was a modification of Poje's procedure (*1*). To a warm aqueous alloxan solution (1.7 M), methylurea (2.4 M) was added. The resulting solution was kept at 70 °C for 20 min to give a precipitate, which was filtered and recrystallized from ethanol to afford light pink crystals of 4-hydroxy-1-methyl-2,5-dioxo-4-imidazolidinecarboxyureide (52%) (ESI-MS: 217.2 $[M+H]^+$, 215.2 $[M-H]^-$). The synthesis of 1-methylspirodihydantoin was similar to that of spirodihydantoin except that a 2-hour heating time at 80 °C was employed. 1-Methylspirodihydantoin was purified by HPLC with a Zorbax Eclipse XDB C8 column (mobile phase consisting of water and acetonitrile with acetonitrile from 0 -3.5% over 7 min at a flow rate

of 1.0 mL/min). HPLC fractions between 5 and 7 min were collected and freeze-dried to afford pure 1-methylspirodihydantoin (ESI-MS: 197.1 $[M-H]^-$ and 154.1 $[M-H-NHCO]^-$, 199.1 $[M+H]^+$ and 216.1 $[M+NH_4]^+$); FT-MS: theoretical mass: 197.0305 $[M-H]^-$; actual mass: 197.0309; ^{13}C-NMR(D_6-DMSO): δ 169.4, 168.2, 155.9, 155.5, 75.2 and 24.7 ppm).

Oxidation of N9-Methyluric Acid by 2 equivalents of Na_2IrCl_6. To an N9-methyluric acid solution (954 μM) (in 0.1 M, pH 7.4 KPi buffer), Na_2IrCl_6 (1.9 mM) was added. The resulting solution was stirred at room temperature for 48 h. To an aliquot of the reaction mixture, 1-methylspirodihydantoin (82 μM) was added. In another aliquot of the reaction mixture an equivalent volume of KPi buffer (pH 7.4, 0.1 M) was added as a control. Both the spiked solution and the control solution were analyzed by HPLC on the hypercarb column. HPLC conditions were as above except that the acetonitrile gradient was from 0-60% over 20 min. 1-Methylspirodihydantoin and 1-methylallantoin were eluted after 8.5 and 9.8 min, respectively.

Synthesis and Purification of Spiroiminodihydantoin and Guanidinohydantoin Bases. To a Na_2IrCl_6 solution (7.4 mM) (in 0.1 M, in pH 11.2, 9.2, 7.3 and 5.3 KPi buffer, respectively), 8-oxo-7,8-dihydroguanine was added to saturation. The resulting suspension was stirred at room temperature for 48 h then filtered. The pH of the filtrate was adjusted to 7.5 using either phosphoric acid or sodium hydroxide, and the products in these solutions were analyzed by HPLC (on a 4.6 × 250 mm LC-18-DB column, Supelco). The mobile phase consisted of 1% acetonitrile and 99% water at a flow rate of 1.0 mL/min. Spiroiminodihydantoin and guanidinohydantoin bases eluted after 3.5 min and 4.7 min, respectively. The remaining filtrate was freeze-dried and purified by silica gel flash chromatography. Products were eluted with 100 mL 1:1 methanol/ethyl acetate. Guanidinohydantoin (the major product at pH 5.3) and spiroiminodihydantoin (the major product at pH 11.2) were further purified by HPLC using the LC-18-DB column as before.

Oxidation of 8-oxo-7,8-dihydroguanine with 2 equivalents of Na_2IrCl_6 at Different pH. To an 8-oxo-7,8-dihydroguanine solution (23.4 μM) (in 0.1 M KPi buffer with pH 9.5, 10.2 or 11.2), Na_2IrCl_6 (46.8 μM) was added. The resulting solution was stirred at room temperature for 6 h, then the pH of the solution

was adjusted to 6.9 using 85% phosphoric acid. An aliquot of the acidified solution was concentrated by freeze-drying for the quantitation of spiroiminodihydantoin and guanidinohydantoin bases.

Oxidation of Allantoin by 10 equivalents of Na_2IrCl_6 or 10 equivalents of Peroxynitrite. To an allantoin solution (0.81 mM) (0.1 M, pH 7.4 KPi buffer), Na_2IrCl_6 (8.1 mM) was added. For reactions with peroxynitrite (7 mM), allantoin (0.7 mM) was dissolved in a buffer containing KPi (150 mM) and sodium bicarbonate (25 mM) (pH 7.2) and peroxynitrite was added in bolus fashion. The reactions were stirred at room temperature for 4 h. Remaining allantoin was quantitated by HPLC according to the typical procedure in the general information.

Decomposition of Allantoin in KPi Buffer (pH 11.2 or 12.2). Allantoin (0.1 mM) was dissolved in KPi buffer (0.1 M, pH 11.2 or 12.2). The decomposition of allantoin with time was monitored by HPLC according to the procedure descried in the general information.

Oxidation of N7-Methyluric Acid by 2 equivalents of Na_2IrCl_6. To N7-methyluric acid (454 μM) (in 0.1 M, pH 7.4 KPi buffer), Na_2IrCl_6 (1.9 mM) was added. 1-Methylallantoin and 1-methylspirodihydantoin were isolated by HPLC on a Hypercarb column using acetonitrile (B) and 10 mM ammonium acetate (A) as mobile phases. A gradient from 0-30% B over 20 min and a flow rate of 0.3 mL/min were used. 1-Methylspirodihydantoin and 1-methylallantoin containing fractions were collected between 7.5 -11.5 min and 11.5-15 min, respectively.

Synthesis and Decomposition of 5-Chloro-N7-methylisouric Acid. 5-Chloro-N7-methylisouric acid was prepared according to literature(*5*). Briefly, N7-methyluric acid (23 mg) was suspended in 15 mL of dry chloroform. The suspension was cooled in an ice-bath while Cl_2 gas was bubbled through for 30 min. The green colored mixture was stirred at 0 °C for 3 h. Chloroform was evaporated to afford a solid residue. The solid (2 mg) was dissolved in 10 mL of KPi buffer (0.1 M, pH 7.4) and stirred at room temperature for 1 h. 3-Methylallantoin, 2-methylspirodihydantoin and remaining N7-methyluric acid were isolated by HPLC on the hypercarb column. In the control experiments where N7-methyluric acid was dissolved in the same buffer, no 2-methylspirodihydantoin or 3-methylallantoin was detected.

Oxidation of 8-oxo-7,8-dihydroguanine by 2 equivalents **Na_2IrCl_6** ***in $H_2^{16}O$ and $H_2^{18}O$.*** 8-oxo-7,8-dihydroguanine (233 uM) was dissolved in KPi buffer (0.1 M, pH 9.5). An aliquot (1.5 mL) of the solution was freeze-dried and reconstituted with 1.5 mL of $H_2^{18}O$ (isotopic purity > 95%). Na_2IrCl_6 (167 uL of 4.2 mM in $H_2^{18}O$ water, 2 equivalents) was then added. The oxidation in $H_2^{16}O$ was carried out similarly. The resulting mixture was stirred at room temperature for 6 h. Guanidinohydantoin and spiroiminodihydantoin bases were purified using the Hypercarb column. The mobile phases were acetonitrile (B) and ammonium acetate (10 mM, pH 7) (A) and a gradient from 0-20 B% over 30 min at a flow rate of 0.3 mL/min were used. The retention times of guanidinohydantoin and spiroiminodihydantoin bases were 7.6 and 13.5 min, respectively. The HPLC fractions containing both compounds were collected and analyzed by LC-ESI-TOF MS.

Oxidation of Uric Acid by 2 equiv**alents of** **Na_2IrCl_6** ***in $H_2^{16}O$ and $H_2^{18}O$.*** Uric acid (1 mM) was dissolved in KPi buffer (0.1 M, pH 9.5). An aliquot of the uric acid solution (0.6 mL) was freeze-dried and reconstituted with 0.6 mL of $H_2^{18}O$. The solution was mixed with 2-^{13}C-1,3-$^{15}N_2$-labeled Spd (3.3 nmol) and Spd (5.4 nmol). To the solution, Na_2IrCl_6 (28.2 uL of 4.2 mM in $H_2^{18}O$, 2 equivalents) was added. The mixture was stirred at room temperature for 1 h. The oxidation in $H_2^{16}O$ was carried out similarly, but with the addition of 3.3 nmol of labeled Spd only. Spd and allantoin were purified by the Hypercarb column as described immediately above. The retention times of Spd and allantoin were at 9.0 and 13.3 min, respectively. The HPLC fractions containing allantoin and Spd were collected, freeze-dried and analyzed by LC-ESI-TOF MS.

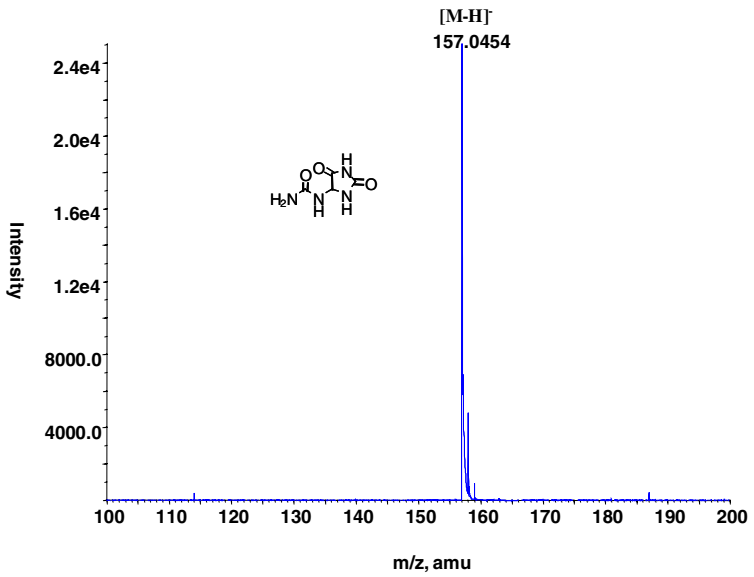
Stability of Spirodihydantoin under Alkaline pH. Spirodihydantoin (0.27 mM) was dissolved in KPi buffer (50 mM, pH 12.2). Aliquots of the solution were analyzed using HPLC on the hypercarb column at different time points (at 0 min, 30 min, and 4 h). The mobile phase consisted of 10 mM NH_4OAc (A) and acetonitrile (B) with a gradient of 0-28% B over 40 min at a flow rate of 0.2 mL/min. Spirodihydantoin was eluted after 11 min. No significant degradation of spirodihydantoin was observed in 4 hours.

Oxidation of 8-OxodG by Na_2IrCl_6 at pH 9.5. 8-OxodG (1.8 mM) was dissolved in KPi buffer (50 mM, pH 9.5). To the solution Na_2IrCl_6 (3.6 mM) was added to completely oxidize 8-oxodG. The reaction mixture was analyzed using HPLC on the hypercarb column. The mobile phase consisted of 10

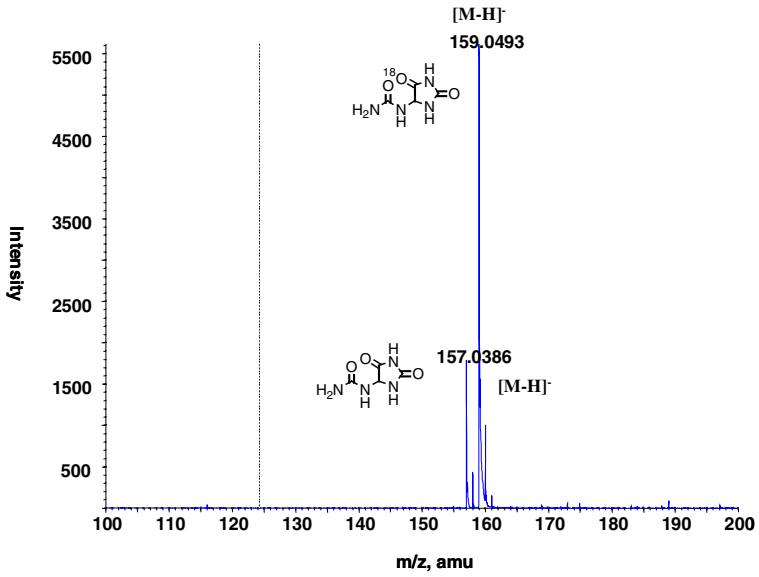
mM NH_4OAc (A) and acetonitrile (B) with a gradient of 0-28% B over 40 min at a flow rate of 0.2 mL/min. Spiroiminodihydantoin nucleoside was eluted after 12.0 and 13.6 min. No guanidinohydantoin nucleoside was observed.

Mass Spectra of Allantoin, Guanidinohydantoin, Spiroiminodihydantoin, 2-Methylspirodihydantoin, and 3-Methylallantoin

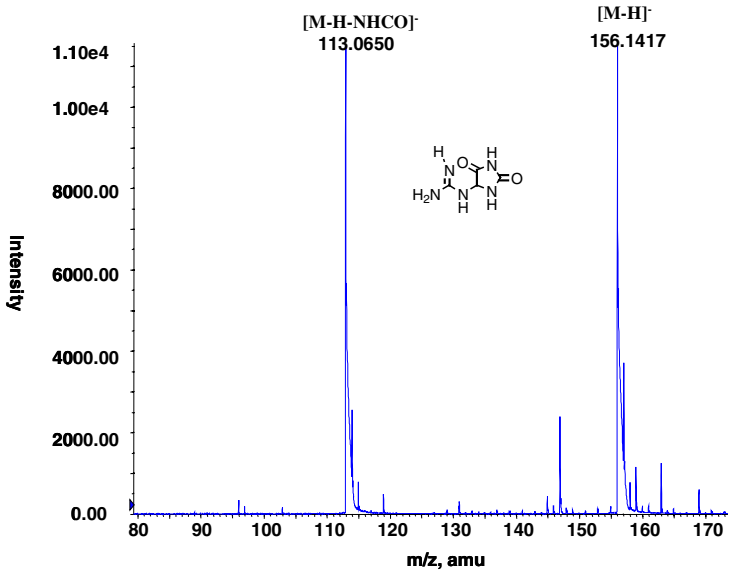
Allantoin from oxidation of uric acid in H_2O



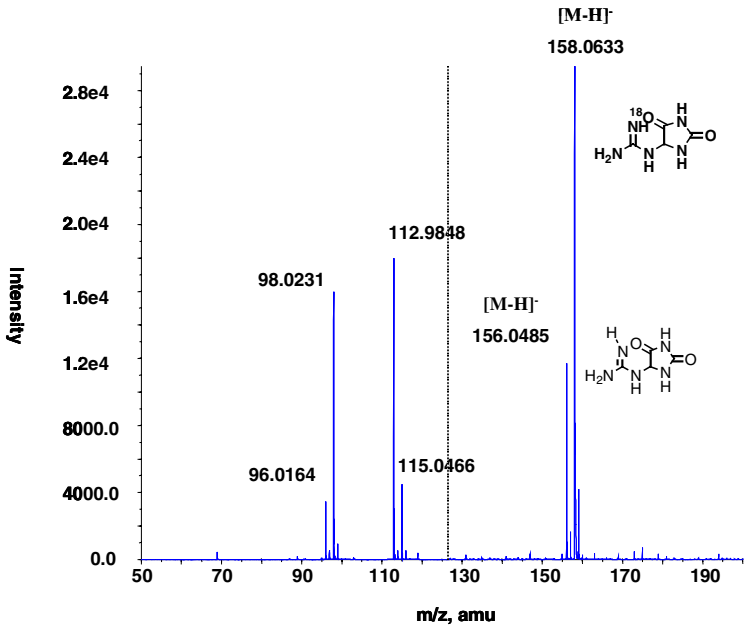
Allantoin from oxidation of uric acid in $H_2{}^{18}O$



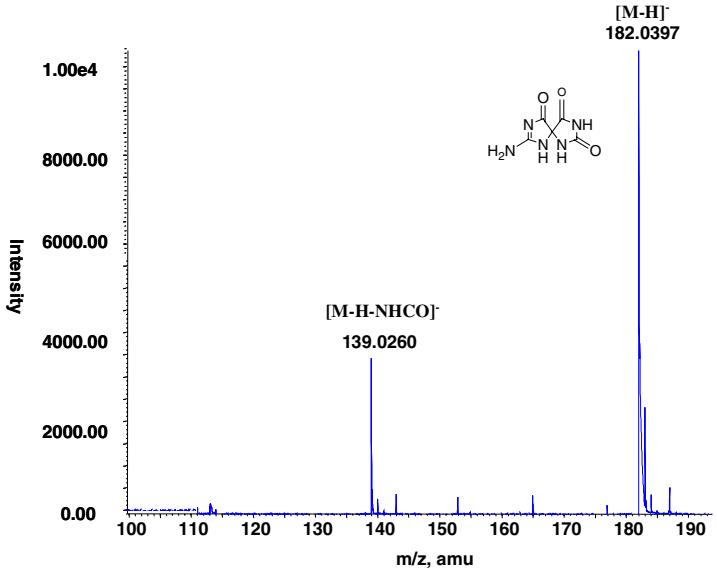
Guanidinohydantoin base from the oxidation of 8-oxo-7,8-dihydroguanine in H_2O



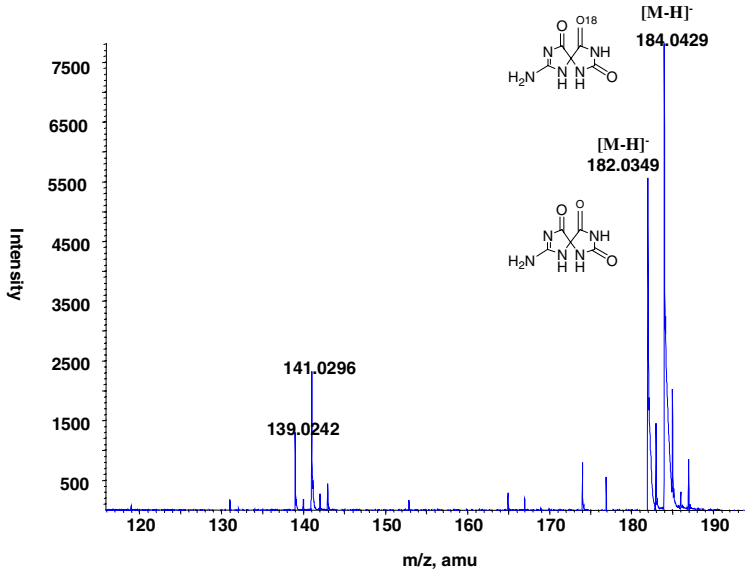
Guanidinohydantoin base from the oxidation of 8-oxo-7,8-dihydroguanine in $H_2{}^{18}O$



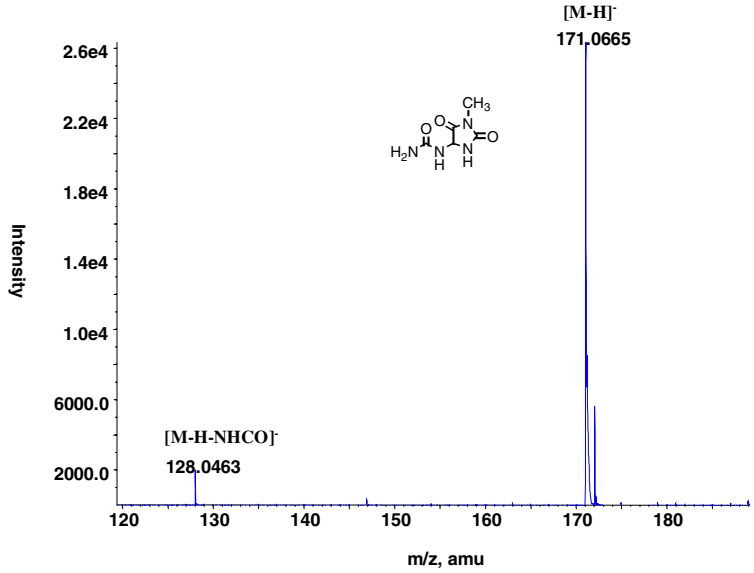
Spiroiminodihydantoin base from oxidation of 8-oxo-7,8-dihydroguanine in H_2O



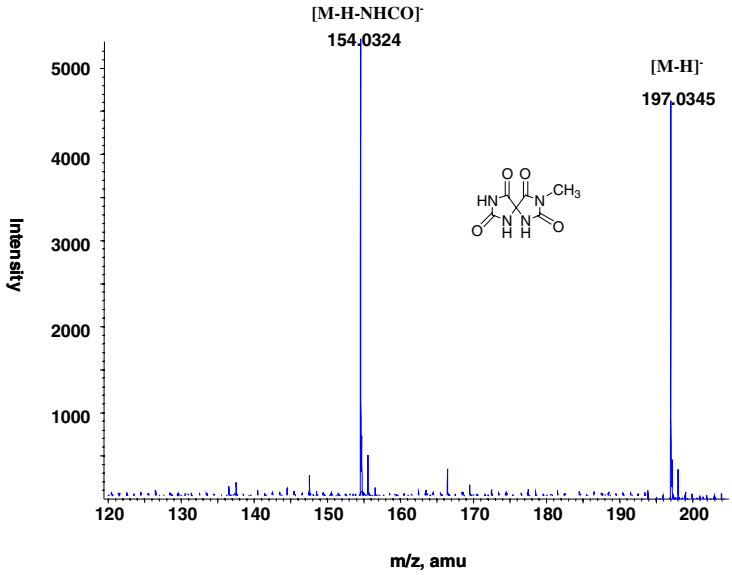
Spiroiminodihydantoin base from oxidation of 8-oxo-7,8-dihydroguanine in $H_2{}^{18}O$



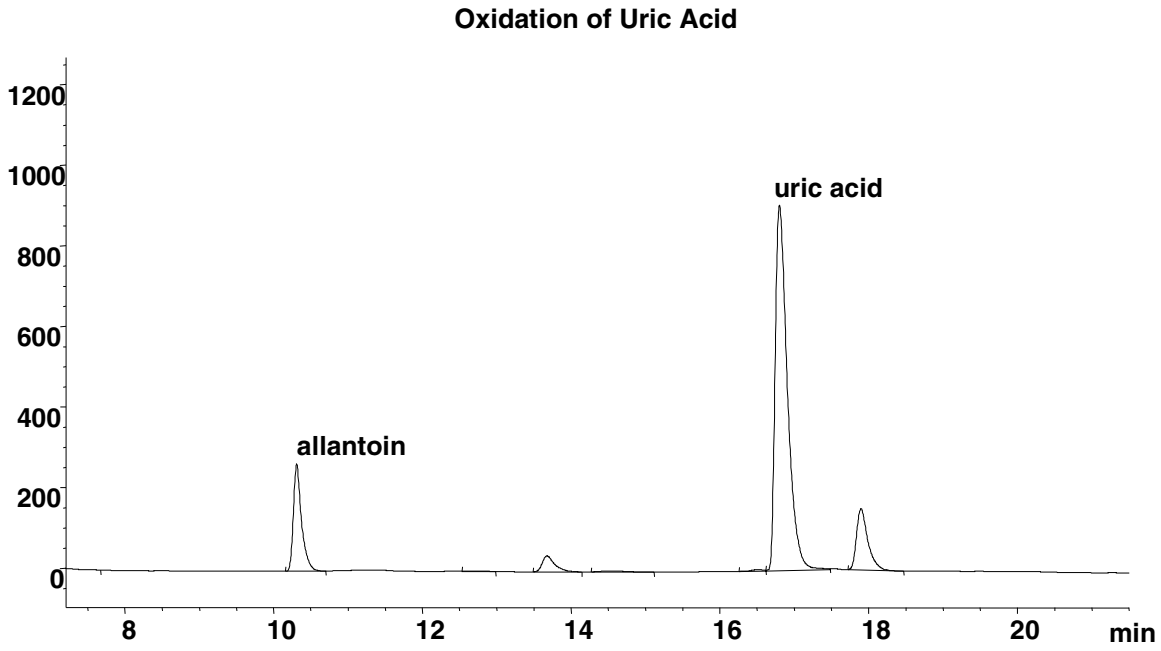
3-Methylallantoin from oxidation of N7-methyluric acid or decomposition of 5-Cl-N7-methylisouric acid

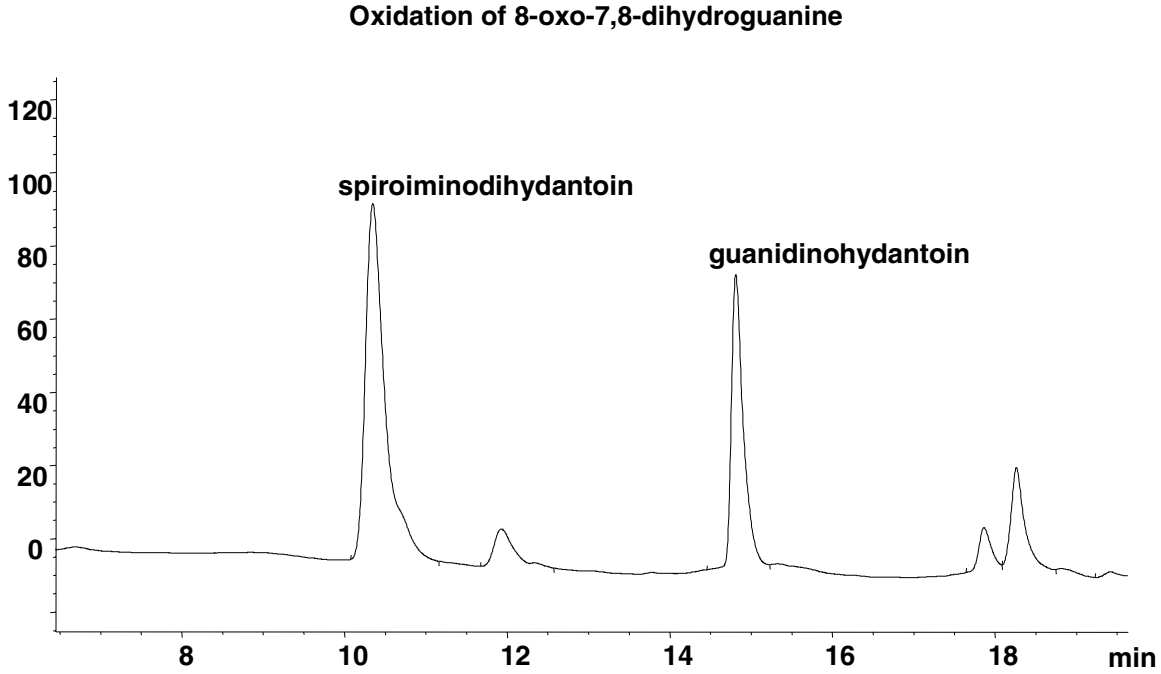


2-Methylspirodihydantoin from oxidation of N7-methyluric acid or decomposition of 5-Cl-N7-methylisouric acid

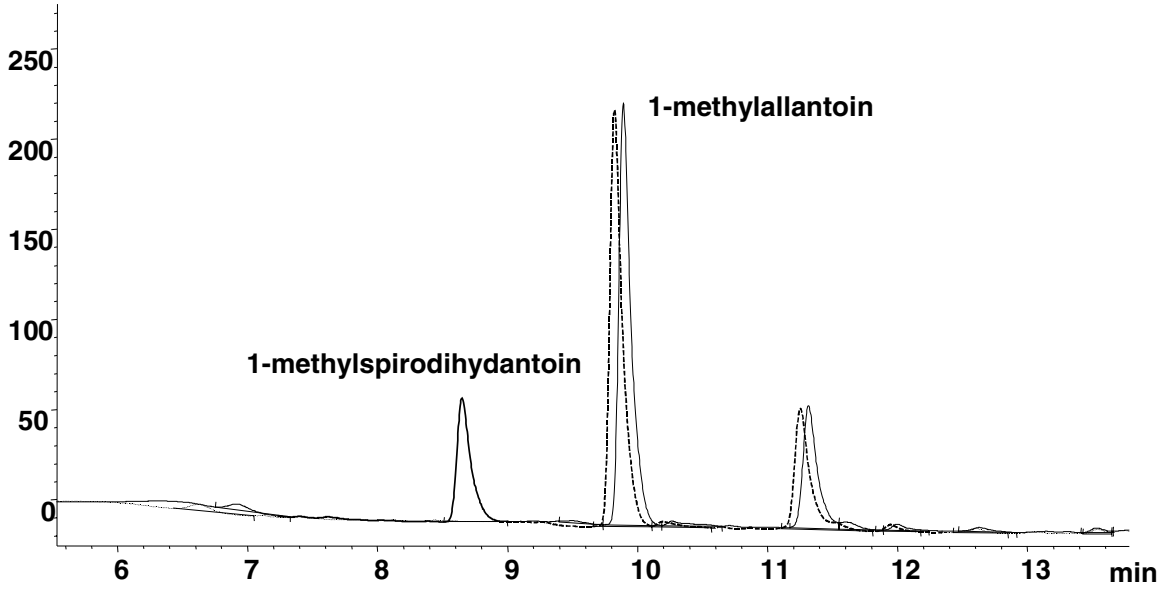


HPLC Chromatograms of Oxidation of Uric Acid, 8-oxo-7,8-dihydroguanine, N9-methyluric acid, N7-methyluric acid and Decomposition of 5-Chloro-N7-methylisouric Acid

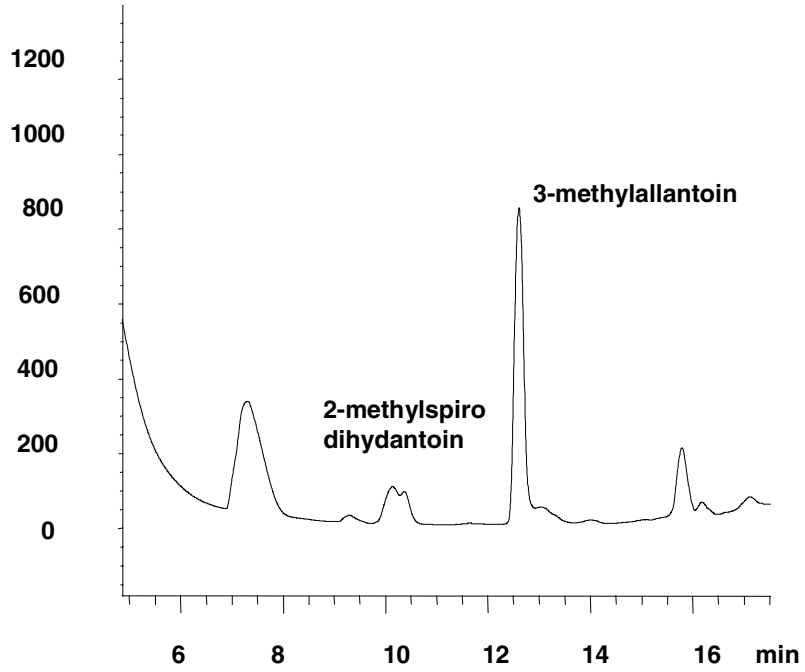




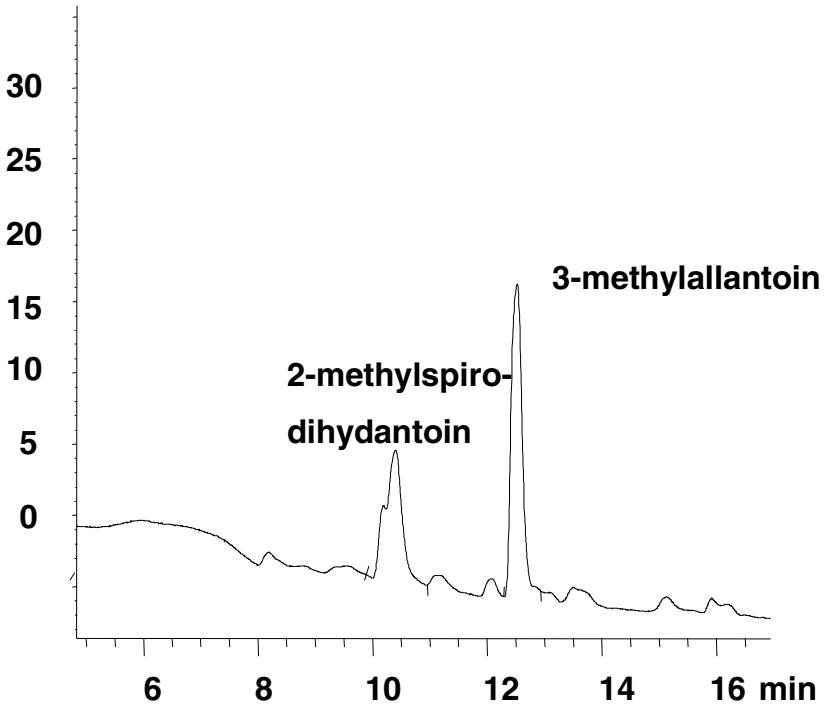
Oxidation of N9-Methyluric Acid (dotted line: without spiking of 1-methylSpd; solid line: with spiking of 1-methylSpd)



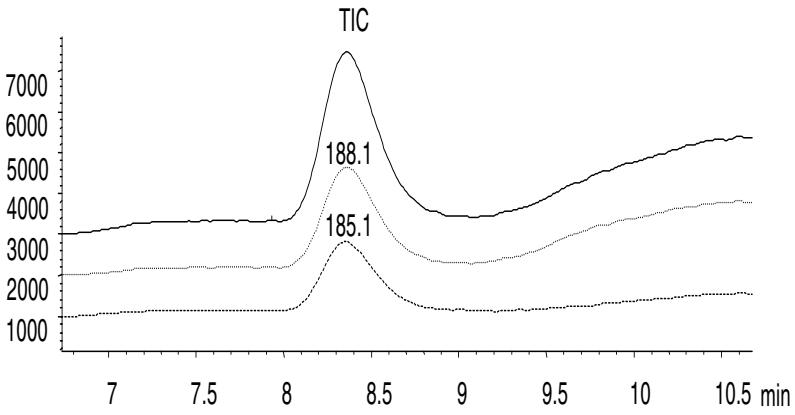
Oxidation of N7-Methyluric Acid



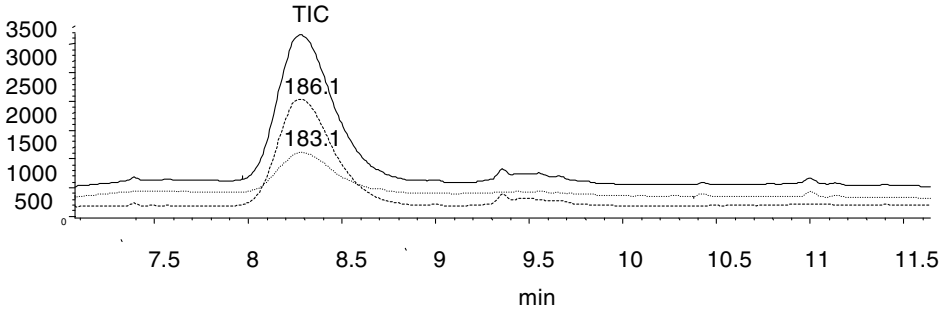
Decomposition of 5-Chloro-N7-methylisouric acid



Selective Ion Monitoring Chromatograms of Detection of Spirodihydantoin with Labeled Spirodihydantoin as Internal Standard (A: positive ion mode; B: negative ion mode).



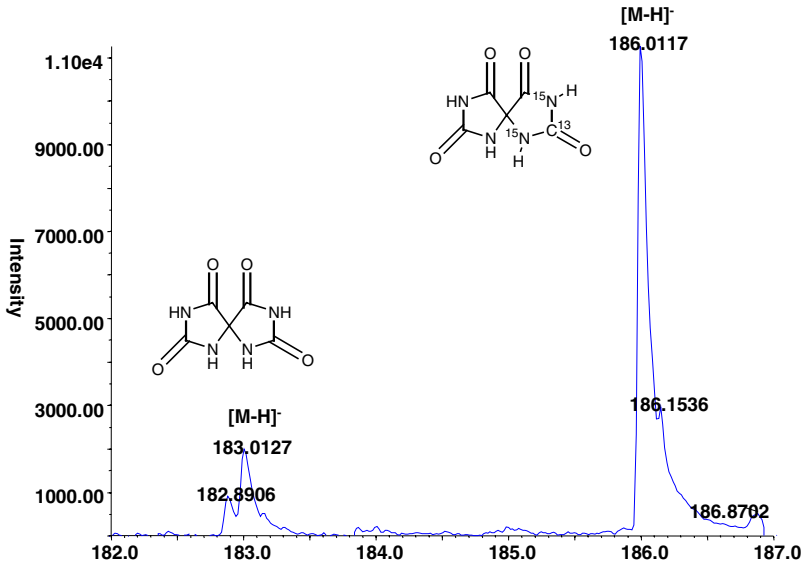
A



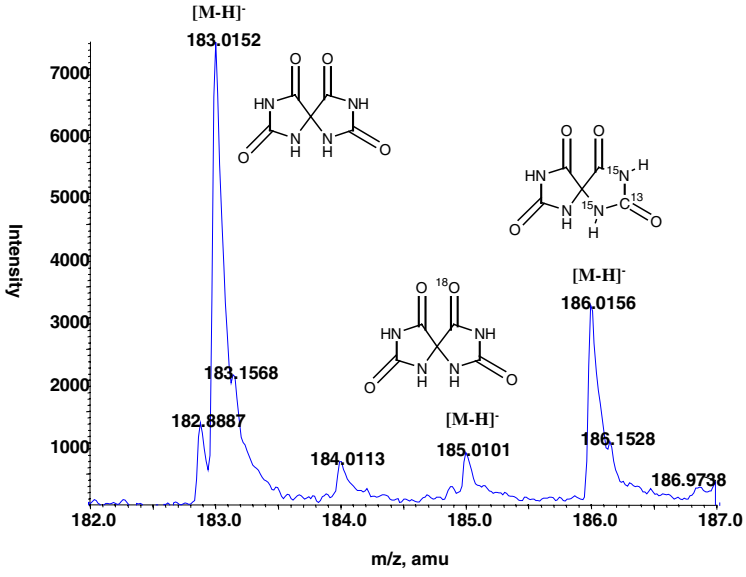
B

Mass Spectra of Detection of ^{18}O-Labeled Spirodihydantoin with Spirodihydantoin and Labeled Spirodihydantoin as Internal Standards

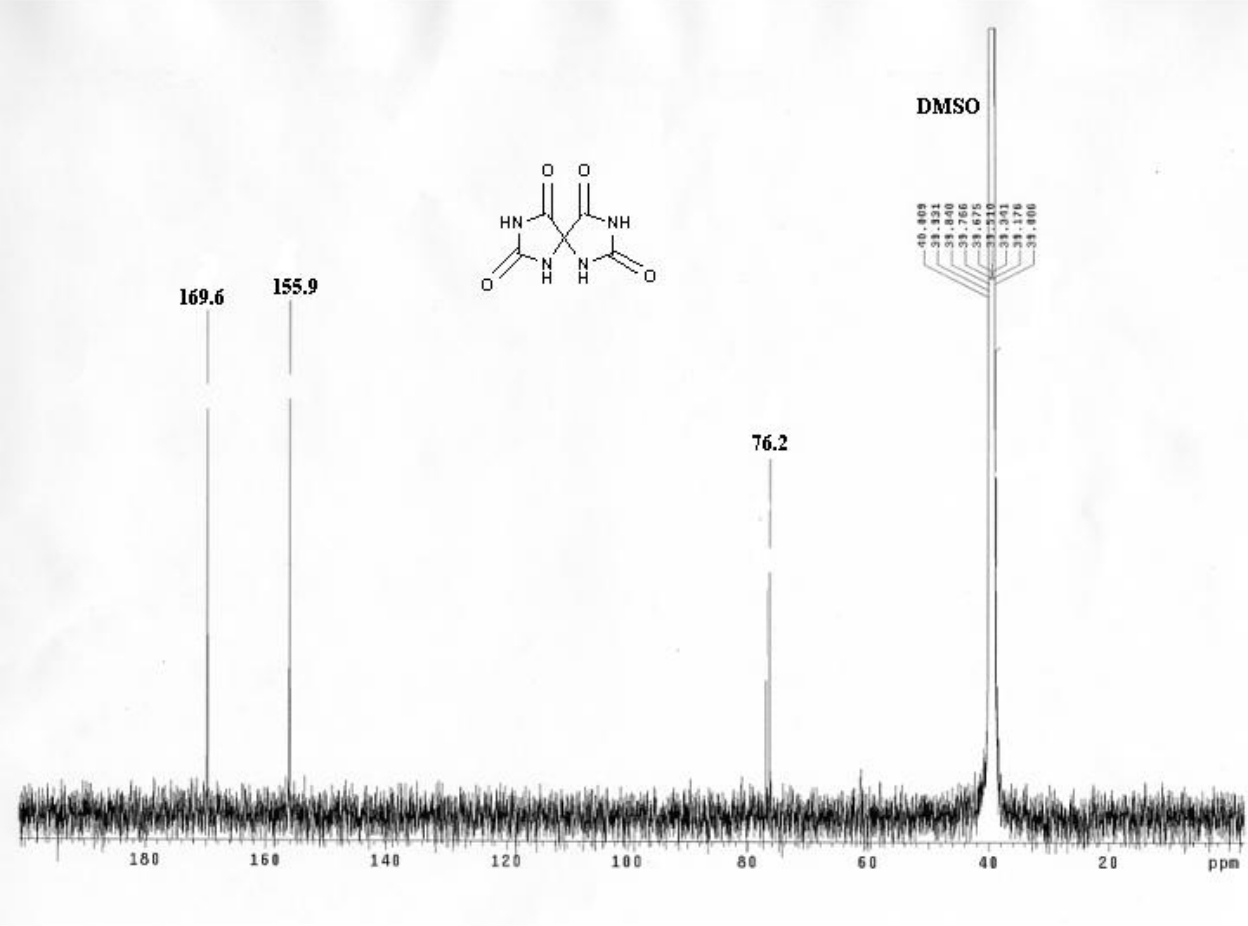
Formation of Spirodihydantoin in H_2O with Labeled Spirodihydantoin as the Internal Standard

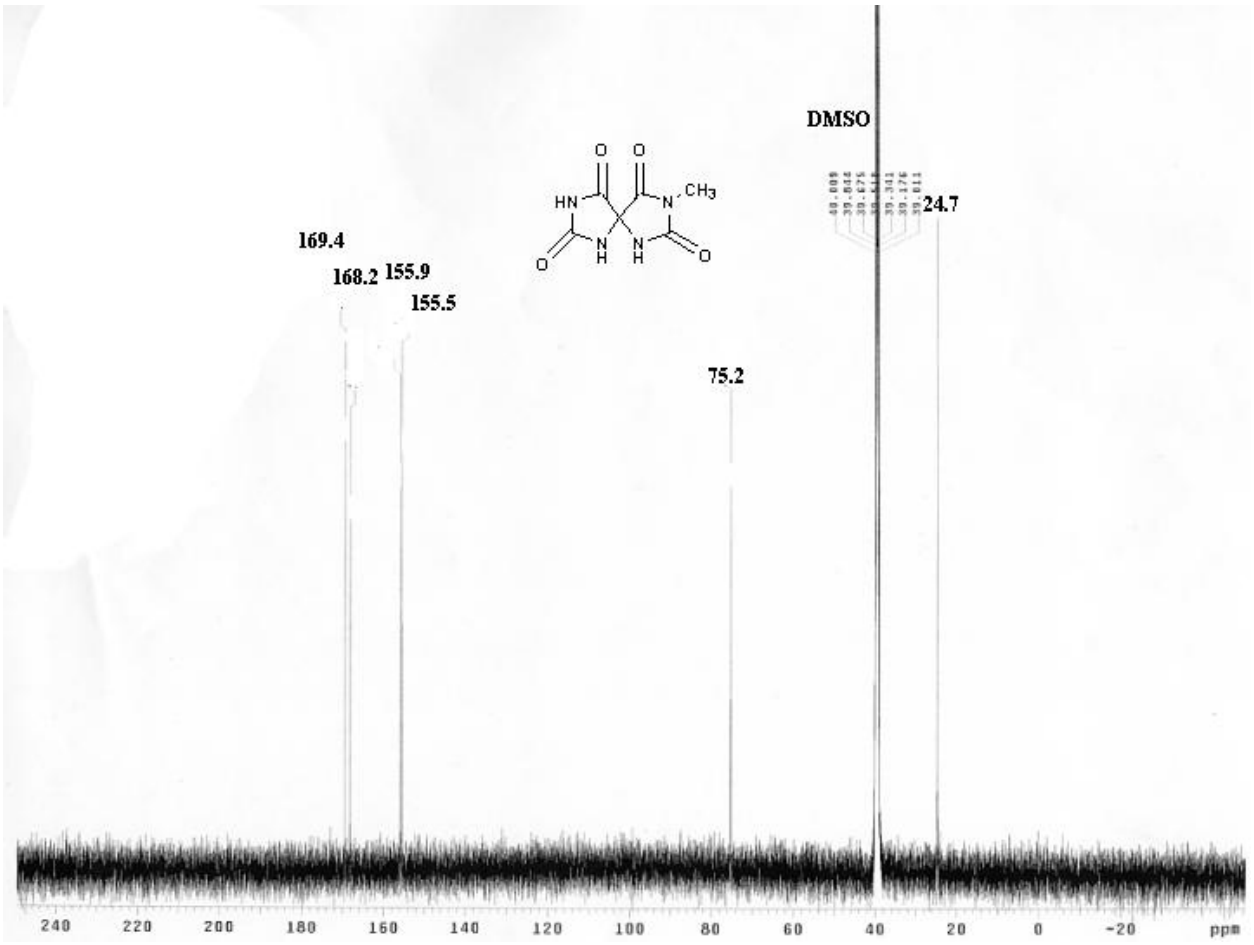


Formation of ^{18}O-Labeled Spirodihydantoin in $H_2{}^{18}O$ with both Unlabeled and Labeled Spirodihydantoin as Internal Standards

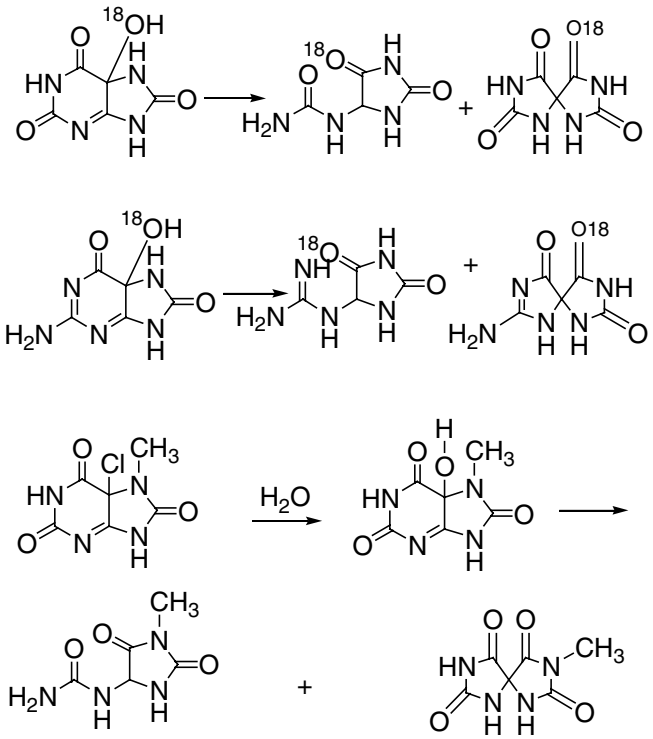


^{13}C-NMR of Spirodihydantoin and 1-Methylspirodihydantoin

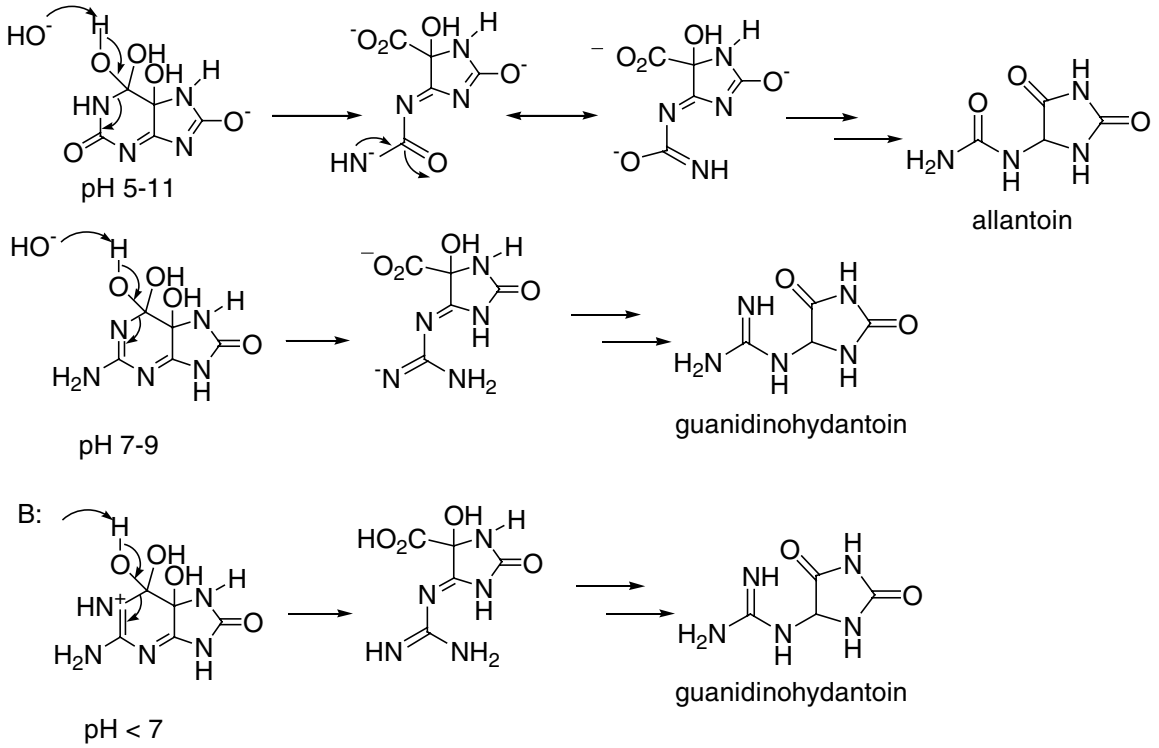



DMSO
24.7
169.4
168.2
155.9
155.5
75.2
240
220
200
180
160
140
120
100
80
60
40
20
0
-20
ppm

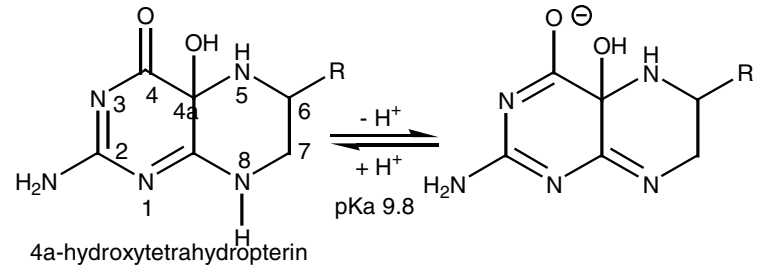
Scheme 1: ^{18}O-Labeling Experiments and Decomposition of 5-Chloro-N7-methylisouric acid



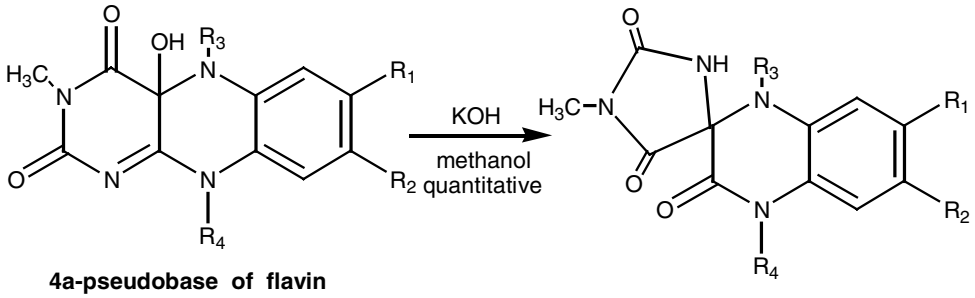
Scheme 2: Decomposition of 5-OH-isourate and 5-OH-8-oxo-7,8-dihydroguanine



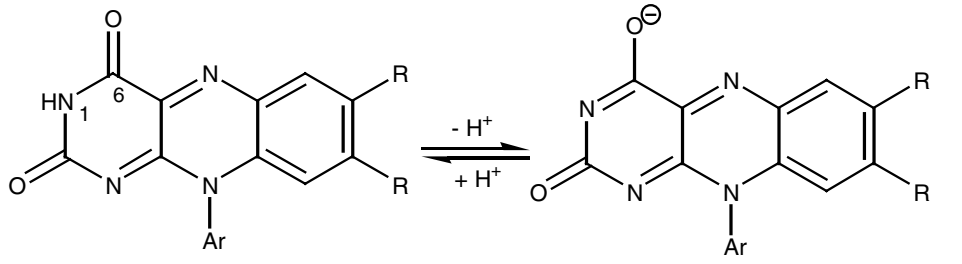
Physico-chemical Properties of Stable Analogs of 5-OH-isourate and 5-OH-8-oxo-7,8-dihydroguanine



4a-hydroxytetrahydropterin



4a-pseudobase of flavin



flavin

enolized C6 carbonyl slow to hydrolysis

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