ORGANIC LETTERS

2004
Vol. 6, No. 19
3417−3420

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

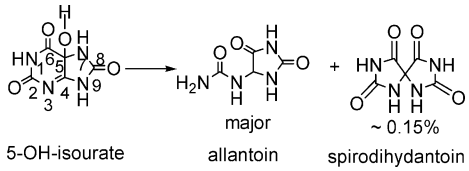
Hongbin Yu,[†] Jacquin C. Niles,[†] John S. Wishnok,[†] and Steven R. Tannenbaum*[,†,‡]

Biological Engineering Division and Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Room 56-731A, Cambridge, Massachusetts 02139

srt@mit.edu

Received July 26, 2004

ABSTRACT



Spirodihydantoin is a minor product from oxidation of uric acid (∼0.15% yield), while spiroiminodihydantoin is a major product from oxidation of 8-oxo-7,8-dihydroguanine (37% yield, pH 10.2). High pH and temperature favor the formation of both spiro compounds. ^{18}O labeling experiments and in situ generation and decomposition of 5-hydroxy-N7-methylisouric acid indicate that spirodihydantoin and allantoin and spiroiminodihydantoin and guanidinohydantoin are products of 5-hydroxyisourate and 5-hydroxy-8-oxo-7,8-dihydroguanine intermediates, respectively.

8-OxodG,[1] an important biomarker of DNA oxidative damage, is prone to further oxidation. Nucleosides **dGh** and **dSp** are two major products[2,3] from decomposition of **5-OH-8-oxodG**, by analogy to **5-OH-UA** from oxidation of **UA**.[4,5] Recently the long postulated **5-OH-8-oxoguanosine** was detected by NMR at low temperature[6] and rearranged to **Sp** nucleoside at room temperature. Hydration/decarboxylation of C6-carbonyl of **5-OH-8-oxodG** leads to **dGh**, similar to the formation of **Alla** from **5-OH-UA**, while the rearrangement of **5-OH-8-oxodG** leads to **dSp**. Interestingly, a similar rearrangement of **5-OH-UA** to **Spd** has not been reported. In fact, during extensive study of oxidation of **UA** in the past few decades, only one communication has provided indirect evidence for the formation of **Spd** from oxidation of **UA**.[7] We report here detection and quantitation of this compound from oxidation of **UA** by isotope dilution mass spectrometry. Mechanistic studies to confirm **Spd** as a product of **5-OH-UA** and the comparison between the product distribution of oxidation of **8-oxoG** and **UA** are also reported.

UA was oxidized by Na_2IrCl_6,[3] peroxynitrite,[8] NO_2,[9] and uricase/O_2,[5] and **8-oxoG** was oxidized by Na_2IrCl_6. The first three oxidants are known to oxidize **8-oxodG** to **dSp** via **5-OH-8-oxodG**, and uricase/O_2 generates **5-OH-UA** intermediate from oxidation of **UA**.[5] Our preliminary investigation indicated that the yield of **Spd** was very low. To unambiguously detect and quantitate this compound, authentic **Spd**

[†] Biological Engineering Division.
[‡] Department of Chemistry.

(1) Abbreviations used: 8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxodG; 8-oxo-7,8-dihydroguanine, 8-oxoG; uric acid, UA; spirodihydantoin, Spd; spiroiminodihydantoin, Sp; guanidinohydantoin, Gh; 5-hydroxyisourate, 5-OH-UA; 5-hydroxy-N7-methylisouric acid, 5-OH-N7-methylUA; 5-hydroxy-8-oxoguanine, 5-OH-8-oxoG; 5-hydroxy-8-oxodG, 5-OH-8-oxodG; allantoin, Alla; horseradish peroxidase, HRP; myeloperoxidase, MPO.

(2) Luo, W.; Muller, J. G.; Rachlin, E. M.; Burrows, C. J. *Chem. Res. Toxicol.* **2001**, *14*, 927−938.
(3) Luo, W.; Muller, J. G.; Rachlin, E. M.; Burrows, C. J. *Org. Lett.* **2000**, *2*, 613−616.
(4) Modric, N.; Derome, A. E.; Ashcroft, S. J. H.; Poje, M. *Tetrahedron Lett.* **1992**, *33*, 6691−6694.
(5) Kahn, K.; Serfozo, P.; Tipton, P. A. *J. Am. Chem. Soc.* **1997**, *119*, 5435−5442.
(6) McCallum, J.; Foote, C. S. *Abstracts of Papers*, 227th National Meeting of the American Chemical Society, Anaheim, CA, March 28−April 1, 2004; American Chemical Society: Washington, DC, 2004.
(7) Modric, N.; Poje, M.; Gojmerac-Ivsic, A. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1685−1686.
(8) Niles, J. C.; Wishnok, J. S.; Tannenbaum, S. R. *Org. Lett.* **2001**, *3*, 963−966.
(9) Shafirovich, V.; Cadet, J.; Gasparutto, D.; Dourandin, A.; Geacintov, N. E. *Chem. Res. Toxicol.* **2001**, *14*, 233−241.

10.1021/ol048547w CCC: $27.50
Published on Web 08/25/2004

and 2-^{13}C-1,3-$^{15}N_2$-**Spd** were synthesized on the basis of a modification of Poje's procedure.[10] Addition of acetonitrile as a cosolvent shortened the reaction time and increased the yield of **Spd**. The identities of both **Spd** compounds were confirmed by high-resolution MS (theoretical mass of $[M - H]^-$ of **Spd** 183.0149, actual mass 183.0152; theoretical mass of $[M - H]^-$ of labeled **Spd** 186.0149, actual mass 186.0123). In negative ion mode of ESI-TOF MS, unlabeled and labeled **Spd** also gave fragment ions of 140.0 (from **Spd**), 141.0, 142.0, and 143.0 (from labeled **Spd**) (Figure 1), corresponding to the loss of neutral fragments NHCO,

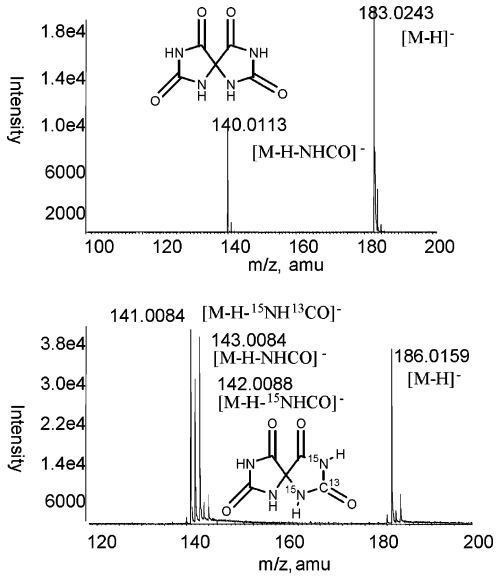


Figure 1. Mass spectra of **Spd** and 2-^{13}C-1,3-$^{15}N_2$-**Spd**

^{15}NHCO, or ^{15}NH^{13}CO from $[M - H]^-$, which are typical for compounds containing a hydantoin structure.[3,11] The isotopic purity of labeled **Spd** was 99.4% based on ESI-MS measurements. Additionally, ^{13}C NMR of **Spd** indicated a resonance at 76.2 ppm, assigned as the spiro carbon because of its similar position to quaternary carbon resonances in other spiro compounds.[3,7,12]

Na_2IrCl_6 was used extensively in oxidation of **UA** and **8-oxoG** because of its ability to generate the 5-OH intermediates. **Alla** and **Spd** and **Gh** and **Sp** bases were isolated from oxidation of **UA** and **8-oxoG**, respectively. The identities of these compounds were confirmed by comparing their HPLC retention times, UV spectra, and mass spectrometric properties with those of the authentic standards or those reported in the literature.[2,3] **Alla**, **Gh**, and **Sp** bases were quantitated with the external standards by HPLC. **Spd** was quantitated by isotope dilution mass spectrometry with labeled **Spd** as the internal standard. The dose−response, temperature, and pH dependence of the formation of **Spd** and **Alla** and the pH dependence of the formation of **Sp** and **Gh** bases are summarized in Table 1. **Alla** represented the bulk of the products from oxidation of **UA**, and its yield increased in a dose-dependent fashion up to 2 equiv of Na_2IrCl_6, which completely oxidized **UA**. Interestingly, **Alla** was not detectable when >5 equiv of Na_2IrCl_6 was used due to its further oxidation (products not determined). Similar oxidation of **dGh** was observed by Burrows and co-workers.[2] In the temperature and pH dependence studies, 2 equiv of Na_2IrCl_6 was used to maximize the yield of **Spd** without further oxidation of **Alla**. The yield of **Alla** was in the range of 67−94% except at high pH and temperature, where it dropped significantly, partly due to its decomposition under these conditions.[13] The yield of **Spd** was in the range of 0.08−0.92% and demonstrated a modest dose response. However, the temperature and pH dependence were more significant, with high pH and temperature favoring its formation (no decomposition of **Spd** observed at pH 12.2 in 4 h). Control experiments with no addition of Na_2IrCl_6 were conducted to accompany the above studies. No oxidation of **UA** was observed, and **Spd** was not detectable. **Gh** and **Sp** bases represented the major products from oxidation of **8-oxoG**. Their formation showed significant pH dependence, with high pH favoring the formation of **Sp** base and disfavoring that of **Gh** base. No **Sp** base was detectable at pH < 8, and its yield increased to 58% at pH 11.2. It is important to note that similar pH and temperature dependence were observed for the formation of **dSp** from oxidation of **8-oxodG**.[3,14]

Results of oxidation of **UA** by peroxynitrite ($ONOO^-$) and enzyme-mediated oxidants are summarized in Table 2 (HRP/H_2O_2, HRP/H_2O_2/NO_2^-,[15] MPO/H_2O_2, MPO/H_2O_2/NO_2^-,[15] and uricase/O_2 designated as 1−5, respectively). The dose response of the formation of **Spd** and **Alla** from $ONOO^-$

Table 1. Summary of Oxidation of **UA** and **8-oxoG** by Na_2IrCl_6[a]

dose response (pH 8, **UA**)			temp dependence (pH 8, **UA**)			pH dependence (**UA** and **8-oxoG**)				
Ir (equiv)	**Spd** (%)	**Alla** (%)	*T* (°C)	**Spd** (%)	**Alla** (%)	pH	**Spd** (%)	**Alla** (%)	**Sp** (%)	**Gh** (%)
0.2	0.15	47	0	0.15	67	6.7	0.09	94	nd	major
0.5	0.08	55	24	0.11	78	7.4	0.09	81	nd	major
1	0.10	76	50	0.34	80	8.0	0.10	74	minor	major
2	0.10	75	62	0.55	82	9.5	0.15	84	12	79
5	0.11	nd	84	0.92	40	10.2	0.17	84	37	57
10	0.12	nd				11.2	0.19	28	58	33
20	0.16	nd				12.2	0.16	15		

[a] Nd = not detectable; all yields based on consumed **UA** and **8-oxoG**.

Table 2. Summary of Oxidation of **UA** by $ONOO^-$ and Enzyme-mediated Oxidants[a]

$ONOO^-$ (equiv)	**Spd** (%)	**Alla** (%)	enzyme	**Spd** (%)	**Alla** (%)
0.2	nd	3	1	0.04	8
0.5	nd	8	2	0.04	10
1.0	0.09	12	3	0.06	21
2.0	0.14	16	4	0.07	19
10	0.06	nd	5	0.006	56
20	0.14	nd			

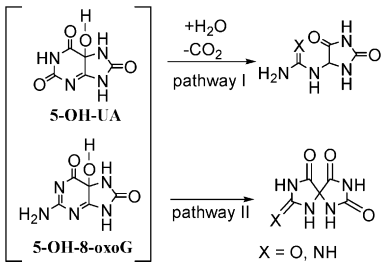
[a] Nd = not detectable.

oxidation was carried out in pH 7.2 potassium phosphate and sodium bicarbonate buffer via bolus addition of $ONOO^-$. The yield of **Alla** was <16% when < 2 equiv of $ONOO^-$ were used and not detectable at >10 equiv of $ONOO^-$ treatment. The formation of **Spd** (~0.1% yield) showed no significant dose response. In the uricase/O_2 oxidation, the yield of **Alla** was 56%, while the yield of **Spd** (0.006%) was significantly lower than that in oxidation of **UA** by Na_2IrCl_6. In the **HRP**- or **MPO**-mediated oxidation, the yields of **Alla** and **Spd** were in the range of 8−21% and 0.04−0.07%, respectively, and did not differ significantly if NO_2^- was present, suggesting no significant oxidation by NO_2. Control experiments with no addition of $ONOO^-$ or enzymes were carried out to ensure no false positive formation of **Spd**.

To confirm that **Spd** and **Alla** and **Sp** and **Gh** bases are products of **5-OH-UA** and **5-OH-8-oxoG** intermediates, respectively, we carried out oxidation of **UA** and **8-oxoG** by Na_2IrCl_6 both in $H_2{}^{16}O$ and $H_2{}^{18}O$ (95% enriched) in pH 9.5 phosphate buffer. The isolated **Spd**, **Alla**, **Sp**, and **Gh** bases were analyzed by ESI-TOF MS in negative ion mode, and those reactions done in $H_2{}^{16}O$ gave $[M - H]^-$ at 183.0, 157.0, 182.0, and 156.1, respectively. From oxidation in $H_2{}^{18}O$, $[M - H]^-$ ions at 185.0, 159.0, 184.0, and 158.1 were observed (see Supporting Information), suggesting that the OH group in **5-OH-UA** and **5-OH-8-oxoG** comes from water and that the oxygen atom is retained in the products. This supports the intermediacy of **5-OH-UA** and **5-OH-8-oxoG**. The definitive proof that **Spd** is a product of **5-OH-UA** would require independent synthesis of **5-OH-UA** and isolation of **Spd** from its decomposition. Because **5-OH-UA** is very unstable, we used **5-OH-N7-methylUA** as its surrogate because oxidations of **UA** and **N7-methylUA** proceed via similar intermediates, namely, **5-OH-UA** and **5-OH-N7-methylUA**.[16] **5-OH-N7-MethylUA** was generated in situ from hydration of **5-chloro-N7-methylUA** in potassium phosphate buffer.[17] **2-Methyl-Spd** (minor product) and **3-methyl-Alla** (major product) were isolated from the decomposition of **5-chloro-N7-methylUA** as well as from the oxidation of **N7-methylUA** by Na_2IrCl_6. This unambiguously demonstrates that these two compounds are products of **5-OH-N7-methylUA** in the oxidation of **N7-methylUA** and strongly suggests that **Spd** and **Alla** are products of **5-OH-UA** in the oxidation of **UA**.

Formation of analogous intermediates and similar pH dependence of formation of the spiro compounds suggest that the oxidation of **UA** and **8-oxoG** share similar pathways (Scheme 1). The significant difference between the yields

Scheme 1. Decomposition of **5-OH-UA** and **5-OH-8-oxoG**

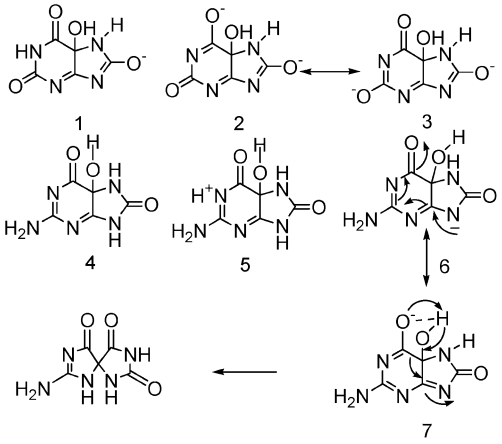


of **Spd** and **Sp** base is very intriguing. The absence of significant formation of **1-methyl-Spd** (<9% yield) from oxidation of **N9-methylUA** by Na_2IrCl_6 excluded the role of N9-substituents in causing the low yield of Sp. The transient existence of **5-OH-UA** and **5-OH-8-oxoG** has limited efforts to probe their decomposition mechanism. However, the physicochemical properties of stable analogues suggest that deprotonation of 5-OH facilitates the formation of spiro compounds via pathway **II**,[18,19] and enolization of the C6 carbonyl slows the hydrolysis of the N1−C6 amide bond and pathway **I**.[20]

On the basis of observations from Burrows[21,22] and our group,[23] we propose pathways **I** and **II** (Scheme 1) to be competitive. The predominant form of **5-OH-UA** in pH range 7−11 is the N9-deprotonated species (**1**)[5,24] (Scheme 2), while the predominant form of **5-OH-8-oxoG** is the neutral species (**4**)[25] at pH 7. At pH ~ 10, N9 of **5-OH-8-oxoG** is deprotonated (**6**) (N9 pK_a ~ 9 using structural similarity

(10) Poje, M.; Paulus, E. F.; Rocic, B. *J. Org. Chem.* **1980**, *45*, 65−68.
(11) Volk, K. J.; Yost, R. A.; Brajter-Toth, A. *Anal. Chem.* **1989**, *61*, 1709−1717.
(12) Johnson, F.; Huang, C.-Y.; Yu, P.-L. *Environ. Health Perspect. (Suppl.)* **1994**, *6*, 143−149.
(13) Vogels, G. D.; De Windt, F. E.; Bassie, W. *Recl. Trav. Chim. Pays-Bas* **1969**, *88*, 940−950.
(14) Leipold, M. D.; Muller, J. G.; Burrows, C. J.; David, S. S. *Biochemistry* **2000**, *39*, 14984−14992.
(15) Van der Vliet, A.; Eiserich, J. P.; Halliwell, B.; Cross, C. E. *J. Biol. Chem.* **1997**, *272*, 7617−7625.
(16) Wrona, M. Z.; Owens, J. L.; Dryhurst, G. *J. Electroanal. Chem.* **1979**, *570*, 371−387.
(17) Subramanian, P.; Nguyen, N. T.; Dryhurst, G.; Hutzenlaub, W.; Pfleiderer, W. *J. Electroanal. Chem. Interfacial Electrochem.* **1989**, *263*, 353−371.
(18) Iwata, M.; Bruice, T. C.; Carrell, H. L.; Glusker, J. P. *J. Am. Chem. Soc.* **1980**, *102*, 5036−5044.
(19) Bruice, T. C.; Miller, A. *J. Chem. Soc., Chem. Commun.* **1980**, 693−694.
(20) Smith, S. B.; Bruice, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 2875−2881.
(21) Luo, W.; Muller, J. G.; Burrows, C. J. *Org. Lett.* **2001**, 2801−2804.
(22) Ye, Y.; Muller, J. G.; Luo, W.; Mayne, C. L.; Shallop, A. J.; Jones, R. A.; Burrows, C. J. *J. Am. Chem. Soc.* **2003**, 13926−13927.
(23) Niles, J. C.; Wishnok, J. S.; Tannenbaum, S. R. *Chem. Res. Toxicol.* In press.
(24) Telo, J. P. *Org. Biomol. Chem.* **2003**, *1*, 588−592.
(25) Lazarus, R. A.; DeBrosse, C. W.; Benkovic, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 6871−6872.

Scheme 2. Ionization States of **5-OH-UA** and **5-OH-8-oxoG**



between 4*a*-OH-tetrahydropterins and **5-OH-8-oxoG**[26]). Since hydration of the C6 carbonyl is merely a hydrolysis of the N1−C6 amide bond,[5] the rate-limiting step is most likely the collapse of the tetrahedral intermediates. For **5-OH-UA** at pH < 11, the collapse is facilitated by delocalization of the negative charge onto the C2 oxygen (see Scheme 2 in Supporting Information). For **5-OH-8-oxoG**, at pH 7−9, such facilitation is not available, although hydration of C6 carbonyl still takes place. At pH > 9, the anionic species of **5-OH-8-oxoG** (**6** and **7**) predominates and enolization of C6 carbonyl slows the hydrolysis of N1−C6 amide bond due to electrostatic repulsion between C6−O^- and −OH.[20] However, the N1-protonated species of **5-OH-8-oxoG** (**5**) predominates at pH < 7 and is susceptible to hydration since the positively charged guanidine moiety is a good leaving group. Thus, pathway **I** is favored at pH < 11 for **5-OH-UA**, while for **5-OH-8-oxoG** it is favored at pH < 7 and disfavored at pH > 9. This may partly explain why the yield of **Alla** was above 70% at pH below 10.2 from the oxidation of **UA** and the pH dependence of formation of **Gh** base in pH range 5−11. High pH favored the formation of **Spd** and **Sp** base, suggesting involvement of deprotonation of 5-OH. As the pH rises to >10, a significant portion of **5-OH-8-oxoG** is the anionic species (**7**). Enolization of the C6 carbonyl may generate intramolecular hydrogen bonding between C6−O^- and 5-OH, which may facilitate deprotonation of 5-OH since C6−O^- can act as a proton acceptor in proximity (Scheme 2). The pH dependence of formation of the **Sp** and **Gh** bases supports this hypothesis. As the pH rises, the anionic species of **5-OH-8-oxoG** (**7**) becomes more and more dominant along with a gradual increase in the yield of **Sp** base and a decrease in the yield of **Gh** base. At pH > 11, N1 of **5-OH-UA** may also be deprotonated to enolize the C6 or C2 carbonyl (species **2** and **3** in Scheme 2). As in the case of **5-OH-8-oxoG**, enolization of C6 carbonyl would slow pathway **I** and facilitate pathway **II**. Our data showed that the yields of **Alla** at pH > 11 were significantly lower than those at pH < 10.2 due to its decomposition. However, a significant increase in the yield of **Spd** was not observed. One possibility is that reaction pathways besides **I** and **II** are in effect at such high pH and **II** is always a minor pathway. Another possibility is that the enolization takes place at the C2 carbonyl, thus facilitated deprotonation of 5-OH via intramolecular hydrogen bonding is not likely as in the case of **5-OH-8-oxoG**. It is important to note that although our data are consistent with the above hypotheses, more experimental or theoretical data are needed to fully understand this intriguing chemistry.

Acknowledgment. This work was supported by NIH Grant No. CA26731 and by the MIT Center for Environmental Health Sciences with a grant from the NIEHS (ES002109-26). We thank Agilent Technologies for access to the 1100 MSD and ESI-TOF MS.

Supporting Information Available: Experimental procedures, mass spectra of products, LC-MS and HPLC chromatograms, ^{13}C NMR of **1-methyl-Spd** and **Spd**, and relevant schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

OL048547W

(26) Bailey, S. W.; Rebrin, I.; Boerth, S. R.; Ayling, J. E. *J. Am. Chem. Soc.* **1995**, *117*, 10203−10211.