Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 2159–2162

2′,3′-Didehydro-2′,3′-dideoxynucleosides are degraded to furfuryl alcohol under acidic conditions

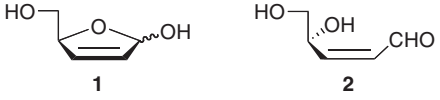
Junxing Shi,[a,b,†] Adrian S. Ray,[c,‡] Judy S. Mathew,[a,b] Karen S. Anderson,[c] Chung K. Chu[d] and Raymond F. Schinazi[a,b,*]

[a]*Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30323, USA*
[b]*Emory University School of Pediatrics and Veterans Affairs Medical Center, Medical Research 151H, 1670 Clairmont Road, Decatur, GA 30033, USA*
[c]*Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520-8066, USA*
[d]*Department of Pharmacology and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, USA*

Received 30 October 2003; accepted 6 February 2004

Abstract—2′,3′-Didehydro-2′,3′-dideoxynucleosides are clinically relevant antiviral agents. These nucleosides could be degraded under acidic conditions. Acidic stability studies showed the D4N had the following increasing stability order: D4G < cyclo-D4G ⩽ RVT < D4T with half-lives ranging from less than 2 min to 35 days. A concerted A-1 mechanism has been proposed for the acidic cleavage of D4-nucleosides. The cleavage products were characterized as furfuryl alcohol and the corresponding nucleobase. Furfuryl alcohol is an agent found in many everyday food products. The biological results demonstrated that furfuryl alcohol had neither anti-HIV activity nor cytotoxicity in vitro, suggesting the acid instability of D4-nucleosides is unlikely to have an impact on the toxicity of these nucleoside analogs in humans.

2′,3′-Didehydro-2′,3′-dideoxynucleosides belong to an important class of antiviral agents, especially for the treatment of human immunodeficiency virus (HIV) infection. Among this class of nucleosides, D-D4T (Stavudine)[1] is an approved anti-HIV agent. Two other nucleosides, RVT (Reverset, D-D4FC)[2] and L-D4FC (Elvucitabine)[3] are in advanced Phase II clinical trials. In addition, several analogs of this class, for example, D-2′,3′-didehydro-2′,3′-dideoxycytidine[4] (D-D4C) and L-2′,3′-didehydro-2′,3′-dideoxyadenosine[5] (L-D4A) are also potent antiviral agents. All these nucleosides are metabolized to their triphosphates in vivo, which act as viral DNA chain terminators, due to the lack of the 3′-hydroxyl groups. Although the acidic instability of D4-nucleosides is well known, the study on their glycosidic bond cleavage has not drawn much attention.[6] This is in contrast to the nucleosides bearing natural sugars, which have been investigated intensively.[6–30] In these studies, the cleavage products were assigned as corresponding nucleobase and sugar without full characterization. This is true especially for ribo- and 2′-deoxyribonucleosides. However, for D4-nucleosides, the cleavage product formed from the sugar portion is still ambiguous. Only recently, Ray et al.[31] proposed 2-hydroxy-5-hydroxymethyl-2,5-dihydrofuran (**1**, Fig. 1) in two isomeric forms as a major cleavage product and an α/β unsaturated aldehyde (**2**, Fig. 1) as a minor product of D-2′,3′-didehydro-2′,3′-dideoxyguanosine (D-D4G) degradation under neutral aqueous conditions based on proton NMR. These sugar products could be a possible reason for degraded D4Gs relatively high cytotoxicity. Therefore, further investigation of the cleavage of the D4-nucleosides is warranted. Having an unambiguous



1 2

Figure 1. Proposed cleavage products of D-D4G by Ray et al.[31]

Keywords: HIV; Nucleoside; Furfuryl alcohol; Reverset; Degradation; Cleavage.
* Corresponding author. Tel.: +1-404-728-7711; fax: +1-404-728-7726; e-mail: rschina@emory.edu
† Present address: Pharmasset Inc., 1860 Montreal Road, Tucker, GA 30084, USA.
‡ Present address: Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, USA.

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.02.031

structure of the cleavage products of D4-nucleosides would be helpful in understanding the biological and pharmacokinetic profiles of these nucleosides, and should prove useful in designing improved antiviral agents. We have investigated the acid cleavage of some D4-nucleosides, and herein report our results on the susceptibility of these nucleosides to acid catalyzed degradation, as well as the biological activity of the cleavage products.

Two series of experiments were designed for four D4-nucleosides, namely, RVT, D-D4T, D-D4G, and D-cyclo-D4G (Fig. 2). First, the acidic cleavage products (cleavage experiment) were determined, and the second study focused on the acidic stability of these nucleosides (stability experiment). For the cleavage experiment, harsher conditions were adopted in order to facilitate the process. When subjected to an acidic aqueous solution (HOAc) under increased temperature (35–50 °C), these nucleosides were cleaved to produce two products as evidenced by thin-layer chromatography (TLC). One of the products was the corresponding nucleobase, as confirmed by TLC and HPLC in comparison with an authentic sample. The other product was scarcely visible on a TLC plate under UV light, but showed a strong purple color after spraying with 5% H_2SO_4/EtOH and heating. A cleavage reaction using one of our large stocked D4-nucleosides, RVT, resulted in the isolation of a colorless oily product upon extraction of the reaction mixture. This product was determined to be furfuryl alcohol based on TLC, ^1H, and ^{13}C NMR data of this compound as compared to those of an authentic sample obtained from a commercial source. The other three D4-nucleosides, D-D4T, D-D4G, and D-cyclo-D4G, also resulted in furfuryl alcohol as the cleavage product, which was confirmed by TLC in two development systems ($CHCl_3$/MeOH 95:5, and EtOAc/hexanes 4:1).

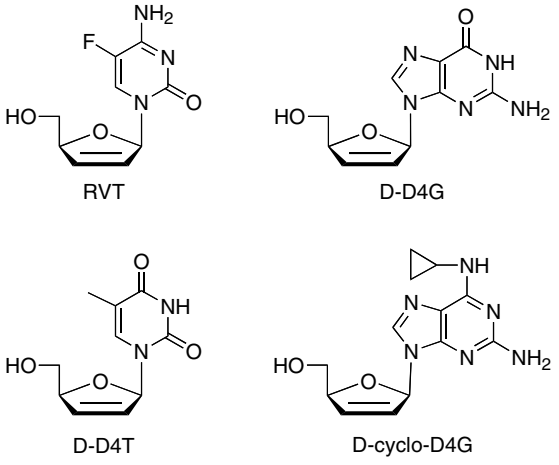
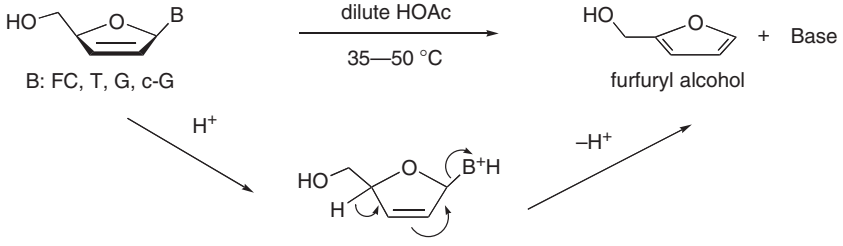


Figure 2. Structure of D4-nucleosides studied.

For the stability experiment, an aqueous pH 2 phosphate buffer under ambient temperature was utilized for these nucleosides, and the products were assayed by reverse phase HPLC. The reaction solutions were also checked by TLC using the above-mentioned systems at the last time point (24 h). The TLC results confirmed the presence of furfuryl alcohol for all the four tested nucleosides under milder acidic conditions.

Cleavage of the D4-nucleosides. RVT (500 mg) was dissolved in water (10 mL) and HOAc (0.5 mL) was added. The solution (pH ~ 3–4) was stirred at 35–40 °C for 1 h, then extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were washed with water, dried (Na_2SO_4), filtered, and evaporated to give 125 mg (58%) of product as colorless oil. As this product was water soluble and highly volatile, no attempt was made to optimize the yield. This product was compared with an authentic sample of furfuryl alcohol purchased from Sigma-Aldrich Co. (catalog no 18,593-0) using ^1H and ^{13}C NMR and TLC (visualized after spraying with 5% H_2SO_4 in EtOH and heating), and found to have identical properties in all respects. TLC: 0.49 ($CHCl_3$/MeOH, 95:5), 0.68 (EtOAc/hexanes, 4:1); ^1H NMR (DMSO-d_6) δ 7.58 (d, $J = 1.2$ Hz, 1H, H-5), 6.38 (dd, $J = 1.2$ and 3.2 Hz, 1H, H-4), 6.27 (d, $J = 3.2$ Hz, 1H, H-3), 5.19 (br, 1H, D_2O exchangeable, OH), 4.37 (s, 2H, CH_2OH); ^{13}C NMR (DMSO-d_6) δ 155.4 (s, C-2), 142.1 (d, C-5), 110.3 (d, C-4), 106.9 (d, C-3), 55.6 (t, CH_2OH).

The other three D4-nucleosides, D-D4T, D-D4G, and D-cyclo-D4G, were also processed in a similar way (0.3 mL HOAc in 3 mL of water, pH ~ 3), but on a one-tenth scale (each with 50 mg) and at a higher temperature (50 °C) (Scheme 1). Each reaction was monitored by TLC using two developing systems ($CHCl_3$/MeOH 95:5, and EtOAc/Hexanes 4:1) and compared with an authentic sample of furfuryl alcohol. All the reactions resulted in the formation of the base and a single product that has the same R_f value as furfuryl alcohol by TLC.



Scheme 1. Acidic cleavage of the D4-nucleosides and the possible mechanism.

Stability of the D4-nucleosides. As D4-nucleosides were relatively stable under basic and neutral conditions,[31] only the acidic stability of the nucleosides (RVT, D-D4T, D-D4G, and D-cyclo-D4G) was investigated. A small amount of D4-nucleoside (about 1 mg) was dissolved in NaH_2PO_4 buffer (0.05 M, pH 2.0, 200 μL) and the solution was stirred at ambient temperature for 24 h. A sample was taken at 1 and 24 h, and checked by reverse phase HPLC (Varian Prostar 210; Alltech Zorbax SB-C18 4.6×150 mm (5 μM) column; solvent A: 0.05 M triethyl ammonium bicarbonate; solvent B: 70% acetonitrile in water; starting from 100% A, by increasing B to 50% at 15 min, then to 100% B at 20 min, and maintaining for 25 min). From the HPLC data, the half-life ($t_{1/2}$) of the D4N was estimated and is shown in Table 1.

The biological activity of furfuryl alcohol was evaluated in comparison with RVT. The anti-HIV-1 activity was assayed in activated human peripheral blood mononuclear (PBM) cells, and the cytotoxicity was determined in PBM, CEM, and Vero cells, as described previously.[32] The mitochondrial toxicity was also examined utilizing the previously described method.[32] In addition, the three nucleobases, that is, 5-fluorocytosine, thymine, and guanine, were also tested for cytotoxicity. It is clear that furfuryl alcohol demonstrated neither anti-HIV activity nor cytotoxicity in the tested cells (Table 2). The nucleobases 5-fluorocytosine and thymine were also nontoxic in the three cell lines up to 100 μM, whereas guanine showed minimal toxicity in CEM and Vero cells. The mitochondrial toxicity result in liver cells revealed that furfuryl alcohol has no significant effect on mitochondrial DNA levels up to 100 μM (data not shown). Furthermore, the compound did not increase lactic acid production in these cells.

For acidic cleavage of ribo- and 2′-deoxyribonucleosides, two mechanisms were postulated. One was a Schiff base mechanism, which involves protonation of the sugar ring oxygen, opening of the sugar ring to form a Schiff base, water attack on the Schiff base followed by cleavage of the glycosidic bond.[7–14] The other, an A-1 mechanism, involves the protonation of the nucleobase, followed by the direct cleavage of the glycosidic bond.[6,15–21] Our data fit the A-1 mechanism better and does not support the Schiff base mechanism, because the formation of furfuryl alcohol is better explained without the sugar ring opening. Under our experimental conditions, no sugar ring opening product was detected. The proposed mechanism is depicted in Scheme 1. Aromatization of the furan ring with a concerted process of C1′–N1 glycosidic bond cleavage and the release of a proton from C4′ is the driving force of the reaction that only occurs during cleavage of D4-nucleosides.

Although the acidic cleavage products of the D4-nucleosides proved to be nontoxic, the reason for the cytotoxicity caused by D-D4G in aqueous medium is still unknown. Since the toxicity was due to the cleavage of D-D4G in neutral aqueous solution, the mechanism might be different from cleavage under acidic conditions (mimicking stomach conditions). Thus, the intermediates proposed by Ray et al.[31] could not be excluded. However, it has been shown that certain purine nucleosides can be further hydrolyzed at the imidazole ring of the nucleobase, giving rise to C8–N9 bond cleavage product even under neutral aqueous conditions.[21,26] If this is true, D-D4G would produce the cleavage product 2,4-diamino-5-formamido-6-hydroxypyrimidine. As this compound is not commercially available, the preparation and evaluation of this compound would be necessary to prove this hypothesis, and may provide a clue as to the cause of D-D4Gs cytotoxicity.

In conclusion, certain important antiviral D4N are unstable under acidic conditions. A concerted A-1 mechanism is proposed for the acidic cleavage of D4N. The cleavage product, furfuryl alcohol, has neither anti-HIV activity nor cytotoxicity in culture. These results are consistent with the presence of furfuryl alcohol in many common food items including popcorn, coffee, and peanuts,[33] and its general acceptance as being nontoxic with limited exposure. These studies are most relevant to degradation, which occurs in the stomach under acidic conditions and suggests a limited risk of toxicity associated with D4-nucleoside acid degradation

Table 1. Estimated half-life of the D4-nucleosides at 20 °C in 0.05 M pH 2 NaH_2PO_4 buffer

Nucleoside	Half-life ($t_{1/2}$, min)
RVT	45
D-D4T	50,000
D-D4G	<2
D-cyclo-D4G	41

Table 2. Anti-HIV-1 activity and cytotoxicity of furfuryl alcohol and nucleobases relative to Reverset (RVT)

Compound	Anti-HIV-1 activity in PBM cells (μM)		Cytotoxicity (IC_{50}, μM) in		
	EC_{50}	EC_{90}	PBM	CEM	Vero
RVT	0.067	1.9	>100	>100	>100
Furfuryl alcohol	>100	>100	>100	>100	>100
5-Fluorocytosine	ND[a]	ND[a]	⩾100	>100	>100
Thymine	ND[a]	ND[a]	>100	>100	>100
Guanine	ND[a]	ND[a]	>100	64.3	54.9
AZT[b]	0.002	0.02	>100	30.9	29.0
Cycloheximide[b]	ND[a]	ND[a]	0.46	0.08	0.53

[a] ND: not determined.

[b] Positive controls for anti-HIV activity (AZT) and toxicity (cycloheximide).

and the need for enterically coated drugs. Further studies are needed to characterize and understand the activities of sugar moieties released during systemic metabolism of D4-nucleosides, such as those formed from D-D4T.[34]

Acknowledgements

The authors acknowledge Ann Hobbs, Jason P. Grier, and Phil M. Tharnish for their excellent technical work, and Drs. Kyoichi A. Watanabe, Jinfa Du, and Lieven Stuyver for their helpful discussions. R.F.S. is supported in part by NIH grant 1R37-AI-41980 and the Department of Veterans Affairs. C.K.C. and R.F.S. are supported by NIH grant 1-R01-AI-25899. K.S.A. is supported by NIH grant GM49551. A.S.R. was supported by NIH, National Research Service Award 5 T32 GM07223 from the National Institute of General Medical Sciences. R.F.S. and C.K.C. are the founders of Pharmasset Inc. and did not receive any funding from the company for these studies. Reverset is currently in Phase II clinical trials led by Pharmasset Inc.

References and notes

1. Lin, T.-S.; Schinazi, R. F.; Prusoff, W. H. *Biochem. Pharmacol.* **1987**, *36*, 2713.
2. Schinazi, R. F.; Mellors, J.; Bazmi, H.; Diamond, S.; Garber, S.; Gallagher, K.; Geleziunas, R.; Klabe, R.; Pierce, M.; Rayner, M.; Wu, J.-T.; Zhang, H.; Hammond, J.; Bacheler, L.; Manion, D. J.; Otto, M. J.; Stuyver, L.; Trainor, G.; Liotta, D. C.; Erickson-Viitanen, S. *Antimicrob. Agents Chemother.* **2002**, *46*, 1394.
3. Lin, T.-S.; Luo, M.-Z.; Liu, M.-C.; Zhu, Y.-L.; Gullen, E.; Dutschman, G. E.; Cheng, Y.-C. *J. Med. Chem.* **1996**, *39*, 1757.
4. Lin, T.-S.; Schinazi, R. F.; Chen, M. S.; Kinney-Thomas, E.; Prusoff, W. H. *Biochem. Pharmacol.* **1987**, *36*, 311.
5. Bolon, P. J.; Wang, P.; Chu, C. K.; Gosselin, G.; Boudou, V.; Pierra, C.; Mathé, C.; Imbach, J.-L.; Faraj, A.; Alaoui, M. A.; Sommadossi, J.-P.; Pai, S. B.; Zhu, Y.-L.; Lin, J.-S.; Cheng, Y.-C.; Schinazi, R. F. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1657.
6. Garrett, E. R.; Mehta, P. J. *J. Am. Chem. Soc.* **1972**, *94*, 8532.
7. Kenner, G. W. In *The Chemistry and Biology of Purines, Ciba Symposium*; Wolstenholme, G. E. W., O'Connor, C. M., Eds.; Little, Brown and Co.: Boston, Mass, 1957; pp 312–313.
8. Dekker, C. A. *Ann. Rev. Biochem.* **1960**, *29*, 453.
9. Micheel, F.; Heesing, A. *Chem. Ber.* **1961**, *94*, 1814.
10. Michelson, A. M. *The Chemistry of Nucleosides and Nucleotides*; Academic: New York, NY, 1963; p 26.
11. Ulbricht, T. L. V. *Comp. Biochem.* **1963**, *8*, 196.
12. Pfitzner, K. E.; Moffatt, J. G. *J. Org. Chem.* **1964**, *29*, 1508.
13. Garrett, E. R.; Seydel, J. K.; Sharpen, A. J. *J. Org. Chem.* **1966**, *31*, 2219.
14. Gin, J. B.; Dekker, C. A. *Biochem.* **1968**, *7*, 1413.
15. Shapiro, R.; Kang, S. *Biochem.* **1969**, *8*, 1806.
16. Zoltewicz, J. A.; Clark, D. F.; Sharpless, T. W.; Grahe, G. *J. Am. Chem. Soc.* **1970**, *92*, 1741.
17. Zoltewicz, J. A.; Clark, D. F. *J. Org. Chem.* **1972**, *37*, 1193.
18. Panzica, R. P.; Rousseau, R. J.; Robins, R. K.; Townsend, L. B. *J. Am. Chem. Soc.* **1972**, *94*, 4708.
19. Hevesi, L.; Wolfson-Davidson, E.; Nagy, J. B.; Bruylants, A. *J. Am. Chem. Soc.* **1972**, *94*, 4715.
20. Romero, R.; Stein, R.; Bull, H. G.; Cordes, E. H. *J. Am. Chem. Soc.* **1978**, *100*, 7620.
21. Lonnberg, H.; Lehikoinen, P. *Nucleic Acids Res.* **1982**, *10*, 4339.
22. Notari, R. E.; Witiak, D. T.; DeYoung, J. L.; Lin, A. J. *J. Med. Chem.* **1972**, *15*, 1207.
23. Lindahl, T.; Karlström, O. *Biochem.* **1973**, *12*, 5151.
24. Singer, B.; Kröger, M.; Carrano, M. *Biochem.* **1978**, *17*, 1246.
25. Lin, L.-G.; Bakthavachalam, V.; Cherian, X. M.; Czarnik, A. W. *J. Org. Chem.* **1987**, *52*, 3113.
26. Ratsep, P. C.; Pless, R. C. *J. Org. Chem.* **1988**, *53*, 3241.
27. Nair, V.; Buenger, G. S. *J. Org. Chem.* **1990**, *55*, 3695.
28. Marquez, V. E.; Tseng, C. K.-H.; Mitsuya, H.; Aoki, S.; Kelley, J. A.; Ford, H., Jr.; Roth, J. S.; Broder, S.; Johns, D. G.; Driscoll, J. S. *J. Med. Chem.* **1990**, *33*, 978.
29. Wagner, J. R.; Decarroz, C.; Berger, M.; Cadet, J. *J. Am. Chem. Soc.* **1999**, *121*, 4101.
30. Novak, M.; Ruenz, M.; Kazerani, S.; Toth, K.; Nguyen, T.-M.; Heinrich, B. *J. Org. Chem.* **2002**, *67*, 2303.
31. Ray, A. S.; Yang, Z.; Chu, C. K.; Anderson, K. S. *Antimicrob. Agents Chemother.* **2002**, *46*, 887.
32. Stuyver, L. J.; Lostia, S.; Adams, M.; Mathew, J. S.; Pai, B. S.; Grier, J.; Tharnish, P. M.; Choi, Y.; Chong, Y.; Choo, H.; Chu, C. K.; Otto, M. J.; Schinazi, R. F. *Antimicrob. Agents Chemother.* **2002**, *46*, 3854.
33. Shibamato, T. *J. Agric. Food Chem.* **1977**, *25*, 206.
34. Cretton, E. M.; Zhou, Z.; Kidd, L. B.; McClure, H. M.; Kaul, S.; Hitchcock, M. J.; Sommadossi, J. P. *Antimicrob. Agents Chemother.* **1993**, *37*, 1816.