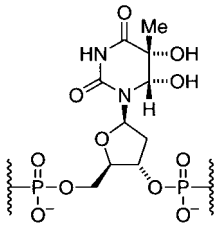
Synthesis of Thymine Glycol Containing Oligonucleotides from a Building Block with the Oxidized Base**

Shigenori Iwai*

Thymine glycol (5,6-dihydro-5,6-dihydroxythymine) is a major form of DNA base damage generated by oxidation with a hydroxyl radical[1] or by ionizing radiation.[2] The *cis*-5*R*,6*S* form (shown as part of an oligonucleotide in **1**) is produced preferentially by the oxidation of thymidine or oligonucleotides,[3] while it was reported that γ irradiation of DNA resulted in the formation of the two *cis* isomers (5*R*,6*S* and 5*S*,6*R*) in equal amounts.[4] The *cis* forms are in equilibrium with the *trans* isomers in solution.[5] Although mutation frequency is low,[6] this damage can block DNA replication.[7] Thymine glycol is excised by enzymes such as endonuclease III[8] and endonuclease VIII[9] in cells, and in vitro experiments showed that this damage is also repaired by the human nucleotide excision repair system.[10]

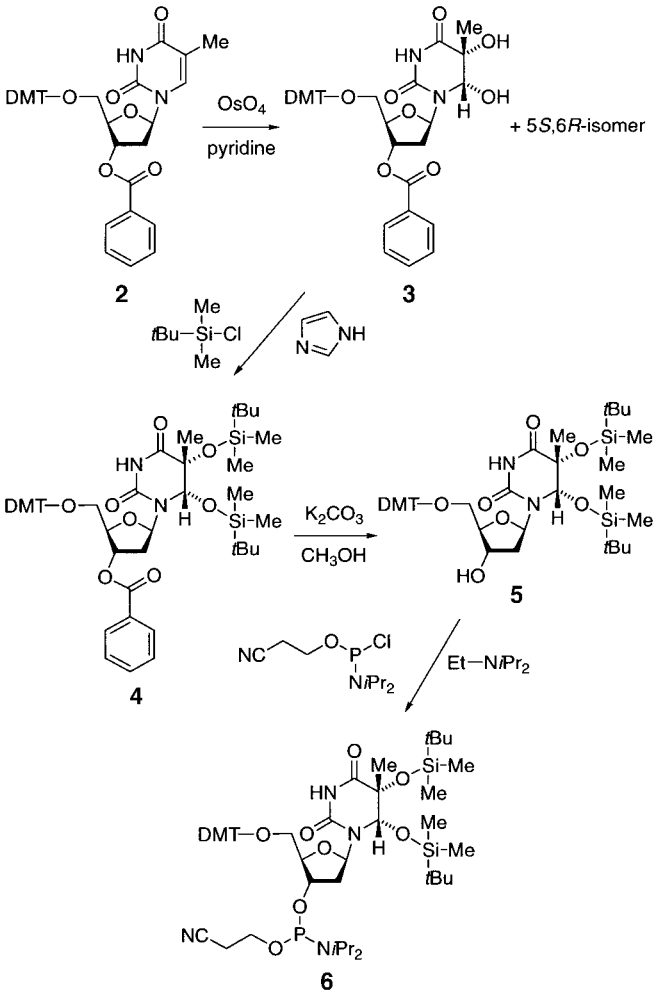


1

In spite of the biological significance of thymine glycol, chemical synthesis of oligonucleotides containing this damage has not been reported hitherto. Such oligonucleotides have been prepared by the oxidation of short oligomers, which contain thymine at a single site, with $KMnO_4$ or OsO_4. However, oxidation with $KMnO_4$ gives various products, which make identification and isolation of the desired product extremely difficult.[3b, 6b, 11] When OsO_4 is used as an oxidizing agent, thymine glycol is produced almost exclusively, but its yield is very low.[6b, 12] Furthermore, the chain length and sequence are limited in these postsynthetic oxidation methods.

In this study, I tried to develop a method for the direct incorporation of a thymine glycol building block into oligonucleotides. The problem was that this oxidized base is labile under the alkaline conditions normally used for the deprotection of the other nucleobases after coupling.[5] This problem might be solved, to some extent, by the use of a (4-*tert*-butylphenoxy)acetyl (tBPA) protecting group on the nucleobases; this group can be removed by a short ammonia treatment at room temperature.[13] It was also expected that protection of the hydroxyl functions of thymine glycol might improve the stability of this base moiety.

The design and the synthesis of the building block are shown in Scheme 1. The *tert*-butyldimethylsilyl (TBDMS) group, which is commonly used for RNA synthesis,[14] was chosen for the protection of the hydroxyl functions at the C5 and C6 positions, because the groups at these positions should be removed at the final step, after the deprotection with ammonium hydroxide.



Scheme 1. Synthesis of the thymine glycol building block **6**.

First, thymidine protected with the 4,4′-dimethoxytriphenylmethyl (DMT) and benzoyl groups at the 5′ and 3′ positions, respectively, was oxidized with OsO_4.[15] Barvian and Greenberg stated that they used a catalytic amount of OsO_4 to prepare thymidine glycol,[16] but an equimolar amount was required in my case. Two products were obtained, and ^{1}H NMR analysis revealed that they were the two isomers of *cis*-thymine glycol. The ratio of the major and minor products was 6:1, as determined by ^{1}H NMR spectroscopy. The configuration of each isomer was assigned by comparison of the NOESY spectra of the products. For both isomers, a crosspeak was found between H6 and the methyl proton, but no NOE effect was detected between H6 and H1′. These observations indicated that the two hydroxyl groups were in the *cis* orientation and that the base moiety was in the *anti* conformation around the glycosyl bond. For the minor isomer, a strong NOE was detected between H6 and H2′, whereas the corresponding crosspeak was too weak to be discriminated from the noise for the major product. As suggested by Vaishnav et al.,[3a] it can be concluded from this difference that the configuration of the thymine glycol used in this study was 5*R*,6*S*.

[*] Dr. S. Iwai
Biomolecular Engineering Research Institute
6-2-3 Furuedai, Suita, Osaka 565-0874 (Japan)
Fax: (+81) 6-6872-8219
E-mail: iwai@bioorg.beri.co.jp

[**] I am grateful to Ms. R. Yuuji and Dr. K. Hirayama, Ajinomoto Company Inc., and Dr. H. Urata, Osaka University of Pharmaceutical Sciences, for obtaining the high-resolution mass data.

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/home/angewandte/ or from the author.

  0044-8249/00/11221-4032 $ 17.50+.50/0

The next step was the protection of the hydroxyl functions at C5 and C6. Although the C5 hydroxyl is tertiary, it was silylated without difficulty. Detection of the imino proton in the NMR analysis demonstrated that the second silylation did not occur at the oxygen atom at the C2 or C4 positions. The benzoyl group of **4** was removed with potassium carbonate in methanol,[17] and the 3′-hydroxyl function of **5** was phosphitylated with (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite.[18]

Using the building block **6** and the tBPA-protected phosphoramidites of the other nucleosides, a 6-mer, d(GCTgAGC), a 13-mer, d(ACGCGATgACGCCA), and a 30-mer, d(CTCGTCAGCATCTTgCATCATACAGTCAGTG), were synthesized on a solid support (Tg represents thymine glycol). Since the coupling yield of **6** was low (74–78%) when the standard cycle was used, the reaction time was prolonged to 5 min when coupling **6**, to obtain a nearly 100% yield. After the chain assembly, the oligonucleotides were cleaved from the support, simultaneously deprotected with 28% aqueous ammonium hydroxide at room temperature for 2 h, and then treated with 1.0 M tetrabutylammonium fluoride in tetrahydrofuran for 16 h to remove the TBDMS group. Following the method described previously,[6b] d(GCTgAGC) was prepared by oxidation of d(GCTAGC) with OsO_4 and was used as the authentic 6-mer for comparison. The authentic 13-mer was also prepared in the same way.

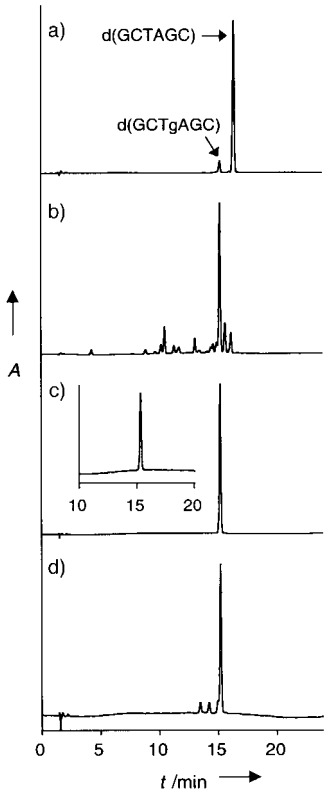


Figure 1. HPLC analysis of the 6-mer with an acetonitrile gradient of 2.5–10% during 20 min. Elution profiles monitored at 254 nm are shown. a) A mixture after the OsO_4 oxidation of d(GCTAGC). b) A crude mixture after deprotection of d(GCTgAGC) synthesized using the building block **6**. c) The 6-mer purified from the mixture shown in (b). The inset is the trace obtained from coinjection with the authentic 6-mer. d) Treatment of the purified 6-mer with ammonium hydroxide at room temperature for 2 h.

Figure 1 shows the results of the HPLC analysis of the 6-mer. As shown in trace (b), a large peak was detected at the same retention time as that of the OsO_4-oxidized product in trace (a). The product giving the main peak was purified by HPLC, and its identity with the authentic 6-mer was demonstrated by coinjection, as shown by trace (c). Treatment of the purified 6-mer with ammonium hydroxide at room temperature for 2 h resulted in degradation of approximately 18% of this oligonucleotide (trace (d)). Since the peaks corresponding to the degraded products were not found in the crude mixture, it is concluded that the protection of the hydroxyl groups of thymine glycol increased the stability of this oxidized base under alkaline conditions. However, after cleavage from the support, treatment with ammonia at 55 °C for 12 h resulted in complete degradation of the thymine glycol containing oligonucleotide, even if ethanol was added to prevent the removal of the TBDMS group (data not shown).[19] The HPLC elution profiles of the crude 13- and 30-mers are shown in Figure 2, and the identity of the 13-mer with the authentic one was demonstrated by coinjection.

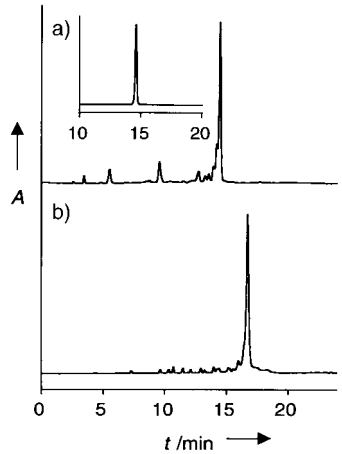


Figure 2. HPLC analysis of the 13-mer (a) and the 30-mer (b). The inset is the trace obtained from a coinjection of the purified 13-mer with the authentic one. The acetonitrile gradients were 5–13% and 5–15% for the 13- and 30-mers, respectively.

After purification with HPLC, the oligonucleotides were analyzed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Although characterization of the 30-mer was not successful because of broad peaks, the difference between the undamaged and thymine glycol containing oligonucleotides was equal to the mass of two hydroxyl groups, as shown in Figure 3.

As described above, a method for the synthesis of oligonucleotides containing thymine glycol at any site within defined sequences has been developed. In my previous studies, oligonucleotides containing the [6–4] photoproduct, which is one of the major forms of DNA damage caused by ultraviolet light, were synthesized,[20] and these oligonucleotides,[21] as well as those containing other DNA lesions,[22] have been used in various studies. Similarly, thymine glycol containing oligonucleotides synthesized by this method will contribute significantly to studies of the molecular biology of DNA repair.

Received: April 20, 2000 [Z15022]

[1] R. Adelman, R. L. Saul, B. N. Ames, *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 2706–2708.
[2] a) R. Teoule, C. Bert, A. Bonicel, *Radiat. Res.* **1977**, *72*, 190–200; b) K. Frenkel, M. S. Goldstein, G. W. Teebor, *Biochemistry* **1981**, *20*, 7566–7571.
[3] a) Y. Vaishnav, E. Holwitt, C. Swenberg, H.-C. Lee, L.-S. Kan, *J. Biomol. Struct. Dyn.* **1991**, *8*, 935–951; b) H. C. Kung, P. H. Bolton, *J. Biol. Chem.* **1997**, *272*, 9227–9236.
[4] G. Teebor, A. Cummings, K. Frenkel, A. Shaw, L. Voituriez, J. Cadet, *Free Radical Res. Commun.* **1987**, *2*, 303–309.

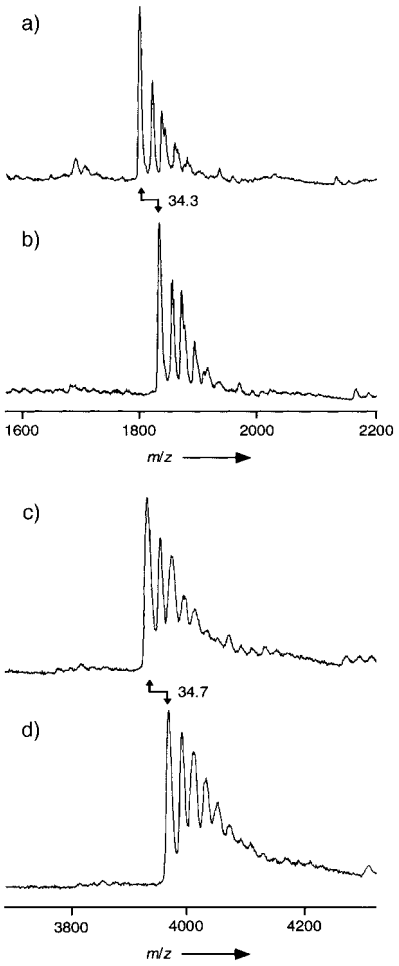


Figure 3. MALDI-TOF MS analysis of: a) d(GCTAGC); b) d(GCTgAGC); c) d(ACGCGATACGCCA); and d) d(ACGCGATgACGCCA).

[5] M. J. Lustig, J. Cadet, R. J. Boorstein, G. W. Teebor, *Nucleic Acids Res.* **1992**, *20*, 4839 – 4845.

[6] a) R. C. Hayes, L. A. Petrullo, H. Huang, S. S. Wallace, J. E. LeClerc, *J. Mol. Biol.* **1988**, *201*, 239 – 246; b) A. K. Basu, E. L. Loechler, S. A. Leadon, J. M. Essigmann, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 7677 – 7681.

[7] a) H. Ide, Y. W. Kow, S. S. Wallace, *Nucleic Acids Res.* **1985**, *13*, 8035 – 8052; b) J. M. Clark, G. P. Beardsley, *Nucleic Acids Res.* **1986**, *14*, 737 – 749; c) R. C. Hayes, J. E. LeClerc, *Nucleic Acids Res.* **1986**, *14*, 1045 – 1061; d) J. M. McNulty, B. Jerkovic, P. H. Bolton, A. K. Basu, *Chem. Res. Toxicol.* **1998**, *11*, 666 – 673.

[8] a) B. Demple, S. Linn, *Nature* **1980**, *287*, 203 – 208; b) M. Dizdaroglu, J. Laval, S. Boiteux, *Biochemistry* **1993**, *32*, 12105 – 12111; c) S. Ikeda, T. Biswas, R. Roy, T. Izumi, I. Boldogh, A. Kurosky, A. H. Sarker, S. Seki, S. Mitra, *J. Biol. Chem.* **1998**, *273*, 21585 – 21593.

[9] R. J. Melamede, Z. Hatahet, Y. W. Kow, H. Ide, S. S. Wallace, *Biochemistry* **1994**, *33*, 1255 – 1264.

[10] J. T. Reardon, T. Bessho, H. C. Kung, P. H. Bolton, A. Sancar, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 9463 – 9468.

[11] C. D'Ham, A. Romieu, M. Jaquinod, D. Gasparutto, J. Cadet, *Biochemistry* **1999**, *38*, 3335 – 3344.

[12] J. Y. Kao, I. Goljer, T. A. Phan, P. H. Bolton, *J. Biol. Chem.* **1993**, *268*, 17787 – 17793.

[13] N. D. Sinha, P. Davis, N. Usman, J. Pérez, R. Hodge, J. Kremsky, R. Casale, *Biochimie* **1993**, *75*, 13 – 23.

[14] N. Usman, K. K. Ogilvie, M.-Y. Jiang, R. J. Cedergren, *J. Am. Chem. Soc.* **1987**, *109*, 7845 – 7854.

[15] J. S. Baran, *J. Org. Chem.* **1960**, *25*, 257.

[16] M. R. Barvian, M. M. Greenberg, *J. Org. Chem.* **1993**, *58*, 6151 – 6154.

[17] W. H. A. Kuijpers, J. Huskens, L. H. Koole, C. A. A. van Boeckel, *Nucleic Acids Res.* **1990**, *18*, 5197 – 5205.

[18] N. D. Sinha, J. Biernat, J. McManus, H. Köster, *Nucleic Acids Res.* **1984**, *12*, 4539 – 4557.

[19] J. Stawinski, R. Strömberg, M. Thelin, E. Westman, *Nucleic Acids Res.* **1988**, *16*, 9285 – 9298.

[20] a) S. Iwai, M. Shimizu, H. Kamiya, E. Ohtsuka, *J. Am. Chem. Soc.* **1996**, *118*, 7642 – 7643; b) T. Mizukoshi, K. Hitomi, T. Todo, S. Iwai, *J. Am. Chem. Soc.* **1998**, *120*, 10634 – 10642.

[21] a) Y. Fujiwara, S. Iwai, *Biochemistry* **1997**, *36*, 1544 – 1550; b) K. Hitomi, S.-T. Kim, S. Iwai, N. Harima, E. Otoshi, M. Ikenaga, T. Todo, *J. Biol. Chem.* **1997**, *272*, 32591 – 32598; c) H. Kamiya, S. Iwai, H. Kasai, *Nucleic Acids Res.* **1998**, *26*, 2611 – 2617; d) K. Sugasawa, J. M. Y. Ng, C. Masutani, S. Iwai, P. J. van der Spek, A. P. M. Eker, F. Hanaoka, D. Bootsma, J. H. J. Hoeijmakers, *Mol. Cell* **1998**, *2*, 223 – 232; e) Y. Fujiwara, C. Masutani, T. Mizukoshi, J. Kondo, F. Hanaoka, S. Iwai, *J. Biol. Chem.* **1999**, *274*, 20027 – 20033.

[22] J. Butenandt, L. T. Burgdorf, T. Carell, *Synthesis* **1999**, 1085 – 1105.

A Concise Stereoselective Route to the Pentacyclic Frameworks of Arisugacin A and Territrem B**

Luke R. Zehnder, Richard P. Hsung,* Jiashi Wang, and Geoffrey M. Golding

Arisugacin A (**1a**, Scheme 1) was first isolated from *Penicillium* sp. Fo-4259 as a potent and selective inhibitor of acetylcholinesterase (AChE) with an IC_{50} value of 1 nM.[1] Because of the potential therapeutic value of **1a** in the treatment of Alzheimer's disease,[2] and the structural similarity of **1a** to important natural products such as territrems (for example, territrem B (**2**))[3] and pyripyropenes,[4] we have been exploring a number of different synthetic routes that may be suitable for an efficient and stereoselective synthesis of arisugacins or territrems.[5, 6] Our recent work involving a formal [3+3] cycloaddition reaction using α,β-unsaturated iminiums and diketo systems[7–9] allowed us to envision a highly concise entry to these natural products. We report here our success in using a stereoselective variant of this formal cycloaddition reaction to construct the pentacyclic frameworks of arisugacin A (**1a**) and territrem B (**2**).

An advanced pentacyclic intermediate such as **3** that is suitable for the syntheses of arisugacins and territrems can be obtained readily by a stereoselective formal [3+3] cycloaddition reaction using the 4-hydroxy-2-pyrone **4**[10] and α,β-unsaturated iminium **5** (Scheme 1). The critical stereoinduction to be achieved in this key reaction is the stereochemistry

[*] Prof. R. P. Hsung, L. R. Zehnder, J. Wang, G. M. Golding
Department of Chemistry
University of Minnesota
Minneapolis, MN 55455 (USA)
Fax: (+1) 612-626-7541
E-mail: hsung@chem.umn.edu

[**] This work was supported by the University of Minnesota.

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/home/angewandte/ or from the author.

  0044-8249/00/11221-4034 $ 17.50+.50/0