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Synthesis and Mutagenesis of the Butadiene-derived N3 2'-deoxyuridine adducts

Priscilla H. Fernandes[#], Linda C. Hackfeld[¶], Ivan D. Kozekov[§], Richard P. Hodge[¶], and R. Stephen Lloyd^{#,*}

[#]Center for Research on Occupational and Environmental Toxicology, and Department of Molecular and Medical Genetics, Oregon Health and Science University, Portland, Oregon 97239-3098

[¶]National Institute of Environmental Health Sciences Center, and Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555

[§]Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37235.

Abstract

1,3-Butadiene is a known carcinogen and mutagen that acts through a variety of metabolic intermediates that react with DNA, forming stable and unstable lesions on dG, dA, dC and dT. The N3 2'-deoxyuridine adducts are a highly stable, stereoisomeric mixture of adducts derived from the reaction of cytosine with the monoepoxide metabolite of butadiene, followed by spontaneous deamination. In this study, the phosphoramidites and subsequent oligodeoxynucleotides containing the N3 2'-deoxyuridine adducts have been constructed and characterized. Using a single-stranded shuttle vector DNA, the mutagenic potential of these adducts has been tested following replication in mammalian cells. Replication past the N3 2'-deoxyuridine adducts was found to be highly mutagenic with an overall mutation yield of ~ 97%. The major mutations that were observed were C to T transitions and C to A transversions. *In vitro*, these adducts posed a complete block to both the Klenow fragment of *Escherichia coli* polymerase I and polymerase ϵ , while these lesions were significantly blocking to polymerase δ . These data suggested a possible involvement of bypass polymerases in the *in vivo* replication of these lesions. Overall these findings indicate that the N3 2'-deoxyuridine adducts are highly mutagenic lesions that may contribute to butadiene-mediated carcinogenesis.

Introduction

1,3-Butadiene (BD)¹ is a high volume, industrial chemical used in the manufacture of synthetic rubber, plastics and resins. BD is also a constituent of cigarette smoke, automobile exhaust and gasoline vapor, thus making it a relevant environmental hazard (1, 2). It is an established rodent carcinogen with its effect being more pronounced in mice than rats (3, 4). BD-induced specific genetic alterations lead to the development of lymphomas, brain tumors and mammary adenocarcinomas in mice (5–7). Occupational exposure to BD is correlated with an increased risk of developing leukemia (8–11). BD has been classified by the International Agency for Research on Cancer as a probable human carcinogen (12). More recently, it has been classified by the National Toxicology Program as a known human carcinogen (13) and by the US Environmental Protection Agency as carcinogenic to humans by inhalation (14).

*To whom correspondence should be addressed: 3181 SW Sam Jackson Park Rd, Portland, Oregon 97239, Tel: 503-494-9957; Fax: 503-494-6831, Email: lloydst@ohsu.edu

BD toxicity is mediated by its metabolic activation to reactive epoxides (*reviewed in* ¹⁵). BD is primarily oxidized to 1,2-epoxy-3-butene (EB) by cytochrome P450 2E1 and 2A6. EB can be further oxidized to 1,2,3,4-diepoxbutane (DEB). EB can also be hydrolyzed by epoxide hydrolase to 1,2-dihydroxy-3-butene that can be metabolized by cytochrome P450 to hydroxymethylvinylketone (HMK) (16). 3,4-epoxy-1,2-butanediol (EBD) can be produced

¹Abbreviations:

BD	1,3-Butadiene
EB	1,2-epoxy-3-butene
DEB	1,2,3,4-diepoxbutane
EBD	3,4-epoxy-1,2-butanediol
HMK	hydroxymethylvinylketone
BD N3-dU	N3 2'-deoxyuridine
Kf	Klenow fragment of <i>E. coli</i> polymerase I
pol δ	polymerase delta
PCNA	proliferating cell nuclear antigen
pol ε	polymerase epsilon
ss	single-stranded
DMT-dU	5'-O-Dimethoxytrityl-2'-deoxyuridine
DMT-Cl	Dimethoxytrityl chloride
TLC	thin layer chromatography
Rf	relative front
DMT-dU-Tol	5'-O-DMT-3'-O-toluoyl-2'-deoxyuridine
DMT-dU-(Bu-OSi)-Tol	5'-O-DMT-3'-O-toluoyl -N3-(2-O-tBDMSi-3-butene) -2'-deoxyuridine
DMT-dU-Bu-OSi	5'-O-DMT-2'-deoxyuridine-N3-(2-O-tBDMSi-3-butene)
DMT-dU-BuOSi-CE	5'-O-DMT-2'-deoxyuridine-N3-(2-O-tBDMSi-3-butene)-3'-O-(O-cyanoethyl-N,N-diisopropyl)-phosphoramidite
tBDMSi	tert-butyldimethylsilyl
CGE	capillary gel electrophoresis.

through partial hydrolysis of DEB and/or epoxidation of hydrolyzed EB. EB, DEB, EBD and HMVK can react with nucleophilic sites in DNA to form their corresponding adducts (17-19). DEB is more genotoxic than EB, which in turn is more genotoxic than BDE (17, 20, 21). The high genotoxicity of DEB has been attributed to its ability to form DNA-DNA cross-links (22-24). The ability of EB, DEB and BDE to induce toxicity is also affected by polymorphisms in the enzymes that metabolize them. For instance, an increase in *hprt* mutant frequency was observed in humans with polymorphic variants that confer reduced microsomal epoxide hydrolase activity that is responsible for the detoxification of EB and DEB (25).

The mechanisms underlying BD-induced carcinogenesis are not clearly understood. Since covalent modification of DNA represents the initial step to carcinogenesis, one may gain a better understanding of this process through the study of DNA adducts of BD. To date, many BD-derived adducts have been detected following treatment of DNA *in vitro*, as well as in DNA isolated from exposed organisms. These include N1, N², and N7 adducts of deoxyguanosine (26-29), N1, N3, N⁶ adducts of adenine (30-33) and the N3 adducts of cytidine and thymidine (34, 35). Some of these adducts (e.g. N7 guanine and N3 adenine) are unstable, giving rise to apurinic sites. Site-specific mutagenesis studies have made it possible to examine the role of some of the other adducts. These include previous studies in our lab that have demonstrated that N⁶ deoxyadenosine and N² deoxyguanosine adducts are weakly mutagenic in *E. coli* (36-37), while the adducts of N1 deoxyinosine and the N⁶,N⁶-deoxyadenosine intrastrand cross-link were considerably mutagenic in *E. coli* and mammalian cells (38).

The role of BD-derived pyrimidine adducts has not yet been examined, particularly the N3 cytosine adducts formed from the reaction of EB with deoxycytidine or calf thymus DNA (31, 35). These adducts were unstable due to epoxidation and they subsequently deaminated to form the corresponding 2'-deoxyuridine (BD N3-dU) adducts. These BD N3-dU adducts were stable towards hydrolysis in DNA and as such, represent a potential source of mutagenesis, since this type of alkylation not only changes the base pairing of the parent nucleoside, but also has the potential to block DNA replication enzymes. Mutations at GC base pairs are critical events in BD mutagenicity as indicated by studies of mutations in *lacI* and *lacZ* mice and *hprt* mutational analyses in rodents and humans (reviewed in³⁹), thus suggesting modification of cytosine by BD metabolites and formation of the N3-dU adducts *in vivo*.

Therefore, the current study focuses on the synthesis of these BD N3-dU adducts, their incorporation into oligodeoxynucleotides and their subsequent use in cellular and *in vitro* studies to examine their effects on replication.

Materials and Methods

Materials

All solvents and reagents for the synthesis of the BD N3-dU were obtained from Fisher-Acros or Aldrich Chemicals except for bis-N,N,N',N'-tetraisopropyl-cyanoethyl phosphine which was obtained from Chemgenes (Cambridge, MA). MALDI-TOF mass spectra of modified oligodeoxynucleotides were performed on an ABI Voyager 4211 system using α -cyano-4-hydroxycinnamic acid as the matrix and calibrated against 10 kDa and 4 kDa oligodeoxynucleotide standards from Genosys. T4 DNA ligase, T4 polynucleotide kinase, *EcoRV*, and Klenow fragment of *E. coli* polymerase I (Kf) were obtained from New England BioLabs (Ipswich, MA). S1 nuclease and proteinase K were purchased from Invitrogen (Rockville, MD). Calf thymus DNA polymerase delta (pol δ) and proliferating cell nuclear antigen (PCNA) were generous gifts from Dr. K. M. Downey (University of Miami, Miami, FL). DNA polymerase epsilon (pol ϵ) from HeLa cells was kindly provided by Dr. S. Linn (University of California, Berkeley, CA) and yeast DNA pol δ was a generous gift from Dr. P.M. Burgers (Washington University School of Medicine, St. Louis, MO). Single stranded

(ss) pMS2 DNA was a generous gift from Dr. M. Moriya (State University of New York, Stony Brook, NY). [γ ³²P] Ribo-ATP was purchased from PerkinElmer Life Science (Boston, MA). Bio-Spin columns were purchased from Bio-Rad (Hercules, CA). Centricon 100 concentrators were obtained from Amicon Inc. (Beverly, MA). Dulbecco's modified Eagle's medium, fetal bovine serum, Opti-MEM (reduced serum medium), L-glutamine, antibiotic-antimycotic, and Lipofectin reagent for tissue culture were obtained from Invitrogen (Rockville, MD). Trypsin-EDTA and HEPES buffer were purchased from Cellgro Mediatech (Herndon, VA). COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). Nondamaged 12-mer and 32-mer with a deoxycytosine (dC) in place of BD N3-dU, were purchased from Midland Reagent Co. (Midland, TX).

Synthesis of the BD N3-dU adducts and their incorporation into oligodeoxynucleotides

a) Synthesis of 5'-O-Dimethoxytrityl-2'-deoxyuridine (DMT-dU)—Synthesis of DMT-dU was performed based on methods described by Beaucage et al. (40). 2'-dU (5 g, 21.9 mmol) was co-evaporated twice with anhydrous pyridine in a 250 ml flask, and re-dissolved with 100 ml fresh anhydrous pyridine under argon. Dimethoxytrityl chloride (DMT-Cl) (4.45 g, 13.14 mmol) was added at 0°C (ice bath) and stirred under argon for 30 min. A second addition of DMT-Cl (4.45 g, 13.14 mmol) was then introduced at 0°C and after 10 min the ice bath was removed and the reaction was stirred for 2 h. The reaction was monitored by thin layer chromatography (TLC) using 100% ethyl acetate as eluant (starting material relative front (Rf) = 0.01, product Rf = 0.40). The reaction was quenched by cooling to 0°C, followed by addition of 40 ml methanol and stirring for 15 min. The reaction mixture was then evaporated to a minimum under reduced pressure, and subsequently redissolved in 100 ml methylene chloride. The mixture was then neutralized with 5% aqueous sodium bicarbonate while stirring until the aqueous layer remained at pH 8. The layers were then separated and the aqueous layer extracted a second time with fresh methylene chloride. The organic layers were then combined, dried over sodium sulfate, filtered and evaporated to give 14.2 g crude DMT-dU. This was purified by silica gel chromatography using a step gradient from 49.9% ethyl acetate : 49.9% hexane : 0.2% triethylamine to 99.8% ethyl acetate : 0.2% triethylamine. Fractions containing the product were combined and evaporated to give 10.2 g (87.7%) DMT-dU or product **1** (Scheme 1). ¹H NMR (d₆-DMSO): δ 11.3 (s, 1H, NH), 7.63 (d, 1H, *J* = 7.53, H-6), 7.37 (d, 2H, *J* = 7.87, DMT), 7.31 (m, 2H, DMT), 7.24 (m, 5H, DMT), 6.90 (m, 4H, DMT), 6.14 (m, 1H, H-1'), 5.37 (d, 1H, *J* = 7.87, H-5), 5.32 (d, 1H, *J* = 4.79, 3'-OH), 4.27 (m, 1H, H-4'), 3.87 (m, 1H, H-3'), 3.74 (s, 6H, DMT-OCH₃s), 3.23 (m, 1H, H-5'), 3.17 (m, 1H, H-5''), 2.18 (m, 2H, H-2', H-2'').

b) Synthesis of 5'-O-DMT-3'-O-toluoyl-2'-deoxyuridine (DMT-dU-Tol)—DMT-dU (500 mg, 0.942 mmol) was added to a 100 ml flask with 5 ml dry pyridine under argon with stirring. Once dissolved, 150 μl of p-toluoyl chloride (1.13 mmol) was added and the mixture stirred for 3 h at room temperature under argon. The reaction was monitored by TLC using 100% ethyl acetate as the eluant (starting material Rf = 0.40, product Rf = 0.75). The product was recovered by diluting the mixture with 20 ml methylene chloride and carefully washing with 20 ml saturated sodium bicarbonate. The organic layer was then separated, dried over sodium sulfate, filtered and evaporated to yield 720 mg crude product that was then purified by silica gel chromatography using 49.9% ethyl acetate: 49.9% hexane: 0.2% triethylamine as eluant. The pure product fractions were pooled and evaporated to yield 460 mg (75%) of product **2** (Scheme 1). ¹H NMR (d₆-DMSO): δ 11.3 (br s, 1H, N3-H), 7.89 (d, 2H, *J* = 6.84, p-toluoyl), 7.69 (d, 1H, *J* = 7.87, H-6), 7.36 (m, 4H, DMT + p-toluoyl), 7.29 (m, 2H, DMT), 7.24 (m, 5H, DMT), 6.87 (m, 4H, DMT), 6.23 (m, 1H, H-1'), 5.50 (m, 1H, H-3'), 5.47 (d, 1H, *J* = 7.87, H-5), 4.23 (m, 1H, H-4'), 3.72 (s, 6H, DMT-OCH₃s), 3.41 (m, 2H, H-5', H-5''), 2.59 (m, 2H, H-2', H-2''), 2.40 (s, 3Hs, p-toluoyl CH₃s).

c) Synthesis of 2-hydroxy-3-butene-1-(p-toluenesulfonate)—This synthesis was based on methods used by Martinelli et al (41). Dibutyltin oxide (2.82 g, 11.35 mmol) was added to 1,2-dihydroxy-3-butene (R,S mixture) (10 g, 113.5 mmol) in methylene chloride (200 ml). Toluenesulfonyl chloride (21.6 g, 113.5 mmol) and triethylamine (16 ml, 113.5 mmol) were added and the mixture stirred at room temperature overnight. Water (50 ml) was added and the layers separated. The aqueous layer was extracted twice with methylene chloride (50 ml each) and the combined organic layers were washed with water and saturated NaCl solution (100 ml each), dried over sodium sulfate, filtered and evaporated to give **3** (Scheme 2) as an oil that crystallized upon cooling (26.8 g, 97% yield). ^1H NMR (CDCl_3) δ 7.78 (d, J = 8.27, 2H, Tos-ortho Hs), 7.33 (d, J = 8.07, 2H, Tos-meta Hs), 5.73 (m, 1H, H-3), 5.34 (d, J = 17.15, 1H, H-4a), 5.21 (d, J = 10.49, 1H, H-4b), 4.37 (m, 1H, H-2), 4.03 (dd, J = 3.43, 1H, H-1b), 3.89 (dd, J = 7.26, 1H, H-a), 2.50 (s, 1H, OH), 2.41 (s, 3H, Tos- CH_3).

d) Synthesis of 2-O-t-butyltrimethylsilyl-3-butene-1-(p-toluenesulfonate)—2-Hydroxy-3-butene-1-(p-toluenesulfonate) (130 mg, 0.537 mmol) was added to 500 μl anhydrous dimethylformamide under argon. Anhydrous potassium carbonate (74.2 mg, 0.537 mmol) was added and the mixture stirred under argon for 5 min. Tert-butyldimethylsilyl chloride (89 mg, 0.59 mmol) was then added and the mixture stirred under argon at room temperature overnight. The reaction was assayed by TLC (20% ethyl acetate/hexane). The reaction products showed over an 80% yield (R_f = 0.85). The mixture was then filtered of solids, washed with methylene chloride, evaporated to a minimum and purified by flash chromatography column (10-20% gradient ethyl acetate/hexane) to give **4** (86.7 mg, 45.7%) along with 25 mg recovered **3** (19.2%) (Scheme 2). ^1H NMR (CDCl_3) δ 7.78 (d, 2H, J = 7.76, Tos-ortho Hs), 7.34 (d, 2H, J = 7.76, Tos-meta Hs), 5.71 (m, 1H, H-3), 5.31 (d, 1H, J = 17.22, H-4 trans), 5.17 (d, 1H, J = 10.13, H-4 cis), 4.34 (m, 1H, H-2), 3.91 (m, 1H, H-1a), 3.82 (m, 1H, H-1b), 2.45 (s, 3H, Tos- CH_3), 0.86 (s, 9H, t-Butyl-Si- CH_3 s), 0.04 (s, 3H, Si- CH_3), 0.03 (s, 3H, Si- CH_3).

e) Synthesis of 5'-O-DMT-3'-O-toluoyl -N3-(2-O-tert-butyldimethylsilyl (tBDMSi)-3-butene) - 2'-deoxyuridine, (DMT-dU-(Bu-OSi)-Tol)—DMT-dU-Tol (350 mg, 0.54 mmol) was dissolved in 5 ml anhydrous dimethylsulfoxide in a 20 ml flask under argon with stirring. Anhydrous potassium carbonate (100 mg) was added and the mixture stirred under argon for 10 min before adding 2-O-t-butyltrimethylsilyl-3-butene-1-(p-toluenesulfonate) (200 mg, 0.562 mmol). The mixture was sealed and stirred under argon for 8 days. The crude mixture was added directly to a silica gel column packed in 25% ethyl acetate/hexane and eluted by step gradient to 33% ethyl acetate/hexane resulting in a yield of 215 mg of product **5** (47%) (Scheme 3). ^1H NMR (CDCl_3) δ 7.94 (d, 2H, J = 8.06, p-toluoyl-ortho Hs), 7.69 (m, 1H, H-6), 7.30 (d, 2H, J = 8.06, p-toluoyl-meta Hs), 7.27 (m, 5H, DMT-Bz), 7.17 (d, 4H, J = 8.79, DMT-ortho Hs), 6.84 (d, 4H, J = 8.42, DMT-meta Hs), 6.34 (m, 1H, H-1'), 5.86 (m, 1H, butenol H-3), 5.81 (d, 1H, J = 8.06, H-5), 5.57 (m, 1H, H-3'), 5.24 (d, 1H, J = 17.58, butenol H-4 trans), 5.12 (d, 1H, J = 9.16, butenol H-4 cis), 4.55 (m, 1H, butenol H-2), 4.26 (s, 1H, H-4'), 4.17 (m, 1H, butenol H-1a), 4.01 (s, 2H, H-5', 5''), 3.83 (m, 1H, butenol H-1b), 3.81 (s, 3H, DMT- OCH_3), 3.80 (s, 3H, DMT- OCH_3), 2.59 (m, 1H, H-2'), 2.50 (m, 1H, H-2''), 2.44 (s, 3H, p-toluoyl- CH_3), 0.85 (s, 9H, silyl t-butyl CH_3 s), -0.01 (s, 3H, silyl CH_3), -0.04 (s, 3H, silyl CH_3).

f) Synthesis of 5'-O-DMT-2'-deoxyuridine-N3-(2-O-tBDMSi-3-butene), (DMT-dU-Bu-OSi)—DMT-dU-(Bu-OSi)-Tol (190 mg, 0.228 mmol) was dissolved in 10 ml anhydrous methanol in a sealed 50 ml flask under argon. To this was added 276 mg anhydrous potassium carbonate and the suspension stirred under argon for 3 h. TLC (49.9% ethyl acetate: 49.9% hexane: 0.2% triethylamine) showed complete deprotection. The reaction was quenched with 40 ml 0.5 M sodium phosphate buffer (pH 5.5) and quickly extracted with 100 ml methylene

chloride. The methylene chloride fraction was dried over sodium sulfate, filtered and evaporated. The crude product was purified by preparative CycloGraph TLC using a 25% ethyl acetate to 50% ethyl acetate (in 1% ethylamine/hexane) step gradient. The product was monitored by UV 254 nm and collected to yield 160 mg of **6** (98%) (Scheme 3). HPLC analysis showed the product to be greater than 99% pure. ¹H NMR (CDCl₃) δ 7.53 (m, 1H, H-6), 7.27 (m, 5H, DMT-Bz), 7.18 (d, 4H, *J* = 7.69, DMT-ortho Hs), 6.83 (d, 4H, *J* = 7.69, DMT-meta Hs), 6.17 (m, 1H, H-1'), 5.85 (m, 1H, butenol H-3), 5.77 (d, 1H, *J* = 7.69, H-5), 5.23 (d, 1H, *J* = 17.21, butenol H-4 trans), 5.11 (d, 1H, *J* = 10.26, butenol H-4 cis), 4.59 (m, 1H, H-3'), 4.53 (m, 1H, butenol H-2), 4.15 (m, 1H, butenol H-1a), 4.03 (s, 1H, H-4'), 3.94 (m, 1H, butenol H-1b), 3.84 (m, 2H, H-5', 5''), 3.81 (s, 6H, DMT-OCH₃s), 2.40 (m, 2H, H-2', 2''), 0.84 (s, 9H, silyl *t*-butyl CH₃s), -0.01 (s, 3H, silyl CH₃), -0.06 (s, 3H, silyl CH₃).

g) Synthesis of 5'-O-DMT-2'-deoxyuridine-N3-(2-O-*t*BDMSi-3-butene)-3'-O-(O-cyanoethyl-N,N-diisopropyl), (DMT-dU-BuOSi-CE) phosphoramidite—DMT-dU-Bu-OSi (112 mg, 0.16 mmol) **6** was dissolved with 1 ml dry methylene chloride in a 20 ml flask under argon. To this was added simultaneously via syringe, while stirring under argon, 71 μl (0.224 mmol) neat bis-N,N,N',N'-tetra-isopropyl-O-cyanoethyl-phosphine and 448 μl (0.192 mmol) tetrazole solution in tetrahydrofuran (30 μg/μl). The solution was stirred for 1 h under argon at room temperature. TLC (29.9% ethyl acetate: 69.9% hexane: 0.2% triethylamine, pre-run TLC plates) showed that the reaction had proceeded to completion. The mixture was extracted with ethyl acetate and 5% sodium bicarbonate, and the ethyl acetate fraction further extracted with saturated aqueous NaCl. The ethyl acetate fraction was then dried over sodium sulfate, filtered and evaporated. The crude product was purified using a preparative TLC CycloGraph system pre-wetted and run with the same TLC solvent above. Pure product fractions were pooled to yield 95 mg pure product **7** (67%) (Fig. 1: Scheme 3). ¹H NMR (300MHz, CD₃CN) δ 7.63 (m, 1H, H-6), 7.42 (m, 2H, DMT-ortho Hs), 7.25 (m, 7H, DMT-ortho Hs and Bz), 6.85 (m, 4H, DMT-meta Hs), 6.19 (m, 1H, H-1'), 5.84 (m, 1H, butenol H-3), 5.41 (m, 1H, H-5), 5.23 (d, 1H, *J* = 17.3, butenol H-4 trans), 5.10 (d, 1H, *J* = 10.5, butenol H-4 cis), 4.60 (m, 1H, H-3'), 4.53 (m, 1H, butenol H-2), 4.05 (m, 2H, butenol H-1a and H-4'), 3.76 (s, 3H, DMT-OCH₃), 3.75 (s, 3H, DMT-OCH₃), 3.62 (m, 1H, butenol H-1b), 3.60 (m, 2H, H-5', 5''), 3.33 (m, 2H, isopropyl CH₃s) 2.63 (t, 1H, OCH, cyanoethyl), 2.52 (t, 1H, OCH, cyanoethyl), 2.42 (m, 1H, H-2'), 2.27 (m, 1H, H-2''), 1.93 (m, 2H, CH₂CN), 1.16 (m, 6H, isopropyl CH₃s), 0.82 (s, 9H, silyl *t*-butyl CH₃s), -0.03 (s, 3H, silyl CH₃), -0.10 (s, 3H, silyl CH₃). ³¹P NMR (121.5 MHz, CD₃CN) δ 147.3, 147.2.

h) Oligodeoxynucleotide Synthesis and Purification—Two oligodeoxynucleotides (12-mer 5'-GCTAGCXAGTCC-3' and 32-mer 5'-ACCATGCCTGCAAGAAAXTAAGCAATGATCGCC-3') were synthesized incorporating N3-(3-butene-2-O-*t*-butyldimethylsilyl)-2'-deoxyuridine in place of a 2'-deoxycytidine at site **X** using phosphoramidite chemistry and standard oligodeoxynucleotide synthesis procedures. Oligodeoxynucleotides were synthesized at the 1.0 μM scale using a PerSeptive Biosystems Expedite 8909 DNA synthesizer with off-line coupling of the DMT-dU-BuOSi-CE phosphoramidite by placing a hold in the sequence program at the coupling step and introducing the modified nucleoside by mixing of the phosphoramidite (10 mg/100 μl anhydrous acetonitrile) and tetrazole (3 mg/100 μl anhydrous acetonitrile) solutions across the synthesis cassette via syringes. The coupling was carried out for 20 min with occasional mixing, followed by placement of the cassette back on the synthesizer, washing with acetonitrile and continuation of the sequence synthesis program. Standard phosphoramidites were used for all other coupled bases and the final 5'-dimethoxytrityl group was left on. Oligodeoxynucleotide cassettes were treated using standard cleavage from the support (1 h concentrated ammonia at room temperature) and deprotection of the resulting oligodeoxynucleotide/ammonia solution (60°C for 16 h). Following deprotection, an aliquot of the crude sample was subjected to analytical

HPLC analysis (Waters Delta Pak C-18, 0-20% acetonitrile gradient in 0.1 M ammonium formate (pH 7.2) for 20 min, at 1 ml/min flow rate) and the bulk of the product was evaporated to a minimum on a Savant SpeedVac system. The 5'-dimethoxytrityl containing full-length oligodeoxynucleotides were then purified on C-18 Sep Pak cartridges. A sample of this material was also analyzed by HPLC using the same system with a shallower gradient (4-10% acetonitrile, 20 min). Purification of the base-protected oligodeoxynucleotides after DMT removal was conducted on a preparative Hamilton PRP column using a 5 ml/min flow rate and gradient of 2-10% acetonitrile in 0.1 M ammonium formate (pH 7.2).

i) Deprotection of t-BDMSi from the oligodeoxynucleotides—The remaining t-butyldimethylsilyl protecting groups on the butenol side chain on N3 adducted deoxyuridine oligodeoxynucleotides were removed using 200 μ l of a 67/33 mixture of anhydrous 1.0 M tetrabutylammonium fluoride/neat triethylammonium hydrofluoride solution overnight at 0-4° C, followed by Nap-10 purification against 0.1 M sodium phosphate buffer (pH 7.2) to remove residual tert-butyldimethylsilyl fluoride and reagents. This was necessary since triethylammonium hydrofluoride alone was too acidic, causing noticeable degradation of the oligodeoxynucleotides, whereas tetrabutylammonium fluoride deprotection alone failed to achieve greater than 60% deprotection. All products were analyzed by HPLC and MALDI-TOF mass spectrometry. The observed pre and post desilylation mass spectrometric values (versus calculated values) were 3789.4 (3789.7) and 3675.4 (3675.7) for the 12-mer and 9951.6 (9951.8) and 9836.5 (9837.7) and for the 32-mer, respectively, within 0.012% of instrument accuracy.

Characterization of the 12-mer oligodeoxynucleotide containing the BD N3-dU adduct

a) Capillary Gel Electrophoresis (CGE)—Electrophoretic analyses were carried out using a Beckman P/ACE MDQ instrument system (using 32 Karat software, version 5.0) monitored at 260 nm on a 31.2 cm $\times$ 100 μ m eCAP capillary, with samples applied at 10 kV and run at 9 kV. The capillary was packed with 100-R gel (for single-stranded DNA) using the Tris-borate buffer system containing 7 M urea.

b) Enzymatic Hydrolysis—The 12 mer oligodeoxynucleotide 5'-GCTAGCXAGTCC-3' (0.3 A_{260} units) was dissolved in 30 μ l of 10mM Tris-HCl buffer (pH 7.0) containing 10 mM $MgCl_2$ and incubated with DNase I (8 units, Promega), snake venom phosphodiesterase I (0.02 units, Sigma), and alkaline phosphatase (1.7 units, *E. coli*; Sigma) at 37°C for 24 h. The mixture was analyzed by reversed phase HPLC. The adducted nucleosides were identified by comparison with authentic samples based on retention times, co-injection, and ultraviolet spectra. HPLC analyses of the enzymatic digestion were performed on a Beckman HPLC system (32 Karat software version 3.1, pump module 125) with a diode array UV detector (module 168) monitoring at 260 nm using a Waters YMC ODSAQ columns (250 mm $\times$ 4.6 mm i.d., 1.5 ml/min) with H_2O and CH_3CN using the following gradient: 1-10% acetonitrile over 15 min, 10-20% acetonitrile over 5 min, hold for 5 min, 100% acetonitrile over 3 min, hold for 2 min, and then to 1% acetonitrile over 3 min.

c) DNA Sequencing—The 12 mer oligodeoxynucleotide 5'-GCTAGCXAGTCC-3' (0.03 A_{260} units) in 24 μ l ammonium hydrogen citrate buffer (pH 9.4) containing 20 mM $MgSO_4$ was incubated at 37°C with 2 milliunits of snake venom phosphodiesterase I. Aliquots (4 μ l) were taken before enzyme addition and at 1, 8, 18, 28, and 38 min time points after enzyme addition. The aliquots were combined and kept frozen until the analysis. The combined aliquots were desalted using a Millipore C_{18} Ziptips and eluted directly onto a MALDI plate in 3-hydroxypicolinic acid containing ammonium hydrogen citrate (7 mg/ml). MALDI-TOF analysis gave a sequencing ladder from the 3' $\rightarrow$ 5' direction. An identical analysis was performed using 12 milliunits of bovine spleen phosphodiesterase II in 24 μ l of 20 mM

ammonium acetate buffer (pH 6.6) that provided a complementary sequencing ladder from the 5' → 3' direction.

Construction of circular ss pMS2 DNAs containing the BD N3-dU adducts

The methodologies for introducing site-specific DNA lesions into the ss pMS2 vector (42) were performed as described by Kanuri et al., (43) and Fernandes et al., (44). In brief, ss pMS2 DNA (59 pmol) was annealed to a 58-mer scaffold (295 pmol), and the annealed product was digested with *EcoRV* to release the hairpin loop structure of ss pMS2 DNA. This procedure results in the creation of a precise gap in the DNA. The scaffolding DNA was designed such that 22 nucleotides on the 5' end and 24 nucleotides on the 3' end were complementary to the two termini of the digested ss pMS2. The 58-mer also had a central sequence that was complementary to the 12-mer oligodeoxynucleotide sequence 5'-GCTAGCXAGTCC-3' where X is control dC or BD N3-dU. To ensure that scaffolding DNA was not used as a substrate for replication in COS-7 cells, all thymines were substituted by uracil in the 58-mer scaffold. The BD N3-dU 12-mer and the control 12-mer were phosphorylated at the 5' end using T4 DNA kinase (5 units/pmol) and then ligated (325 units of T4 DNA ligase/pmol) into the partially duplex pMS2 vector. The ligated DNAs were then purified using Centricon 100 concentrators (to remove unligated 12-mers) and recovered by ethanol precipitation. The products obtained were treated with uracil DNA glycosylase for 1 h to severely damage the scaffold DNA and prevent it from being used as a primer for (-)- strand replication.

Site-specific mutagenesis in COS-7 cells

The overall strategy for replication and mutagenic analyses was as previously described (43, 44). COS-7 cells were grown to approximately 70% confluency and transfected with 1 µg of control or BD N3-dU modified DNAs using lipofectin. The transfection medium was replaced after 24 h with DMEM medium containing 10% fetal bovine serum. Following replication for 48 h, the COS-7 cells were harvested and their progeny plasmid recovered as a Hirt supernatant (45). In order to ensure removal of any input ss DNA and progeny associated with the original ss pMS2 vector, the DNAs so obtained were treated with 1 unit of S1 nuclease in a 60 µl reaction mixture, and with 20 units of *EcoRV* in a 70 µl reaction mixture.

The Hirt supernatant DNAs were used to transform *E. coli* DH5α cells via electroporation and the resultant transformants were selected on LB plates containing 100 µg/ml ampicillin. To detect the presence of mutants, the transformant colonies were individually picked, and grown overnight at 37°C in 96 well plates containing ampicillin supplemented LB medium. These cultures were then transferred, via a 48 pin replicator, onto Whatman 541 filter papers on ampicillin-containing LB plates in 4 replicates (one for each probe), for both the adducts and the control. The *E. coli* on filter papers were then incubated overnight at 37°C to form distinct colonies that were then lysed and differentially hybridized using the four probes, 5'-GATGCTAGCNAGTCCATC-3', where N refers to G, C, A or T.

In vitro replication using BD N3-dU containing 32-mer template

In vitro polymerase reactions were carried out using 14-mer oligodeoxynucleotide primers annealed to the template, 5'-ACCATGCCTGCAAGAAXTAAGCAATGATCGCC-3', where X represents the site of the BD N3-dU adducts or control dC. Primer oligodeoxynucleotides were first phosphorylated with T4 polynucleotide kinase using [γ -³²P] ATP and purified using P-6 Bio-Spin columns supplied with Tris-HCl buffer (pH 7.4). The ³²P-labeled primers were then mixed with either adducted or non-adducted template oligodeoxynucleotides at a molar ratio of 1:2 in 25 mM Tris HCl buffer (pH 7.6), 50 mM NaCl, heated at 90°C for 2 min, and cooled to room temperature overnight. To confirm the completion of primer annealing, aliquots were assayed on a 7.5% native PAGE. The Kf reaction mixture contained 5 nM primer/template DNA in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol and

dNTPs at a final concentration of 100 μ M and were incubated for 15 min at room temperature with increasing concentrations (0.0005 to 0.5 units) of the polymerase (where one unit is defined as the amount of enzyme required to convert 10 nmol of dNTPs to an acid-insoluble material in 30 min at 37°C). The pol δ reaction mixture contained 5 nM primer/template DNA in 40 mM HEPES-KOH (pH 6.8), 10% glycerol, 200 μ g/ml BSA, 6 mM $MgCl_2$, 1 mM dithiothreitol, 70 ng calf thymus PCNA and dNTPs at a final concentration of 100 μ M and was incubated for 15 min at room temperature with increasing concentrations of pol δ from 0.008 to 0.8 units of polymerase. The pol ϵ reaction mixture contained 5 nM primer/template DNA in 50 mM HEPES-KOH (pH 7.5), 100 mM potassium glutamate, 20% glycerol, 200 μ g/ml BSA, 15 mM $MgCl_2$, 10 mM dithiothreitol, 0.03% Triton X-100, 100 μ M each of the dNTPs, and 0.1 unit of the polymerase. These reactions were carried out for increasing time intervals (0, 1, 5, 10, 15, 20 and 30 min) at room temperature. All reactions were terminated by the addition of stop solution consisting of 95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) xylene cyanol, and 0.02% (w/v) bromphenol blue. Reaction products were resolved on a 15% denaturing PAGE and visualized by autoradiography. Quantitative analyses were performed using a PhosphorImager screen and Image-Quant software.

Results

Synthesis and characterization of the BD N3-dU containing oligodeoxynucleotides

Standard protecting group chemistries were utilized in the formation of the 2'-deoxyuridine precursor **2** for alkylation (Scheme 1). For the synthesis of the buten-ol alkylating reagent **4** (Scheme 2), the C1 alcohol was selectively protected in high yield as the p-toluenesulfonate ester **3** using the alphaschelating method of Martinelli, et al. (41). The toluenesulfonate group served as both protections for subsequent silylation of the C2 alcohol to give **4** and as the leaving group for the alkylation reaction at N3 of 2'-deoxyuridine. However, the alkylation reaction of **2** under aprotic conditions was very slow and did not go to completion (47%) (Scheme 3). Even after 8 days, unreacted starting materials were still recoverable. The p-toluoyl group of the C-3' alcohol of alkylated product **5** was readily removed by a protic solution of KOH, and the resulting product **6** was then phosphitylated using the bis-N,N,N',N'-tetraisopropyl-2-O-cyanoethyl-phosphine procedure to give the required phosphoramidite **7** in good yield and purity for DNA synthesis. HPLC analysis of the 12-mer dU-BuOSi oligodeoxynucleotide showed two distinct overlapping species; however, they could not be independently isolated upon further purification. Subsequently, the silyl protecting group was removed by fluoride treatment and Nap-10 purification to give the BD N3-dU 12-mer oligodeoxynucleotide that was used as the R, S mixture in all further experiments. HPLC analysis of the 32-mer dU-BuOSi oligodeoxynucleotide also showed two distinct overlapping species; however, these could not be independently isolated upon further purification. Subsequently, this product was treated and purified as above to remove the silyl protecting group and used as the R, S mixture in all further experiments.

The 12-mer adducted oligodeoxynucleotide used for the construction within the pMS2 shuttle vector was analyzed by capillary gel electrophoresis (CGE) and judged to be >96 % pure (Figure 1A). Under the CGE conditions used, the diastereomers could be partially resolved. Enzymatic digestion of the oligodeoxynucleotide using a mixture of DNase I, snake venom phosphodiesterase I, and alkaline phosphatase, followed by HPLC analysis showed the appropriate nucleosides in the correct molar ratios (Figure 1B). The modified nucleoside was identified by comparison of the retention time with an authentic sample and by co-injection.

The oligodeoxynucleotide was then sequenced by partial enzymatic digestion with snake venom phosphodiesterase I. Aliquots were taken at various time points, combined and then analyzed by MALDI-TOF mass spectrometry. A sequencing ladder was obtained as a result of sequential loss of the nucleotides from the 3' $\rightarrow$ 5' direction (46) (Figure 2A). For

phosphodiesterase I, the digestion was arrested at the adducted nucleotide. A complementary analysis was performed using bovine spleen phosphodiesterase II to determine the sequence from the 5' → 3' direction (Figure 2B). In this analysis, the progress of the phosphodiesterase II was halted one base before the adducted nucleotide. Although the nucleases were unable to digest through the adducted nucleotide on the time scale of these analyses, the results are consistent with the sequence noted.

Replication and Mutagenesis of DNA containing the site-specific N3-dU adducts of butadiene

The single stranded pMS2 shuttle vector was annealed to a scaffold and digested with *EcoRV* to create a gap into which the BD N3-dU adducted or control dC oligodeoxynucleotide was ligated, followed by digestion of the scaffold. These modified vectors were replicated in COS-7 cells and the resultant progeny DNAs were transformed into DH5 α *E. coli* cells. There was no appreciable difference in the absolute number of transformants between the adducted and control DNAs, implying that these lesions may not pose total blocks to replication within cells. These transformants were subjected to differential hybridization (43, 44) using one of the four probes (5'-GATGCTAGCNAGTCCATC-3' where N refers to G, C, A or T). Typical autoradiographic results are shown in Figure 3. The intensely hybridizing cells were scored as positive colonies that contained the progeny DNAs derived from the replication of adduct-containing or control sequences. All experimental procedures were repeated in triplicate and ~ 190 colonies were analyzed for both adduct-containing and control oligodeoxynucleotides. The overall mutation yield for the BD N3-dU adducts was around 97%, while no mutational events were detected with the control dC (Table 1). The predominant mutations were C to T transitions (53.4%) and C to A transversions (32.5%), followed by a much lower frequency of C to G transversions (11%). To verify the correct identification of mutant sequences derived by the differential hybridization method, random colonies that hybridized to specific probes were sequenced using a primer located upstream from the original adducted site. The sequencing data confirmed that the point mutations detected by autoradiography were properly identified and these data did not reveal any alternate replication bypass events such as deletions or frameshifts. These analyses indicate that these lesions primarily (if not exclusively) induce point mutations.

In vitro replication

To assess the effect of the BD N3-dU lesions on *in vitro* replication, a labeled 14-mer primer was annealed to the 32-mer template containing the BD N3-dU adducts or control dC and several polymerases assayed for their ability to replicate past these lesions. The location of the 3' OH was such that the polymerases would incorporate one nucleotide prior to incorporation opposite the lesion. Replication past the adducts was first tested with Kf. On the nondamaged DNA substrate, an efficient extension of the primers was observed (Figure 4, lanes 2-5). In contrast, on the BD N3-dU containing substrate, after one nucleotide was incorporated, indicating efficient loading and polymerization further synthesis was greatly reduced, suggesting that the BD N3-dU adducts posed a near complete block to further replication. No formation of the full-length size products was detected (Figure 4, lanes 7-10).

Mammalian replicative polymerases δ and ϵ were also examined for their ability to bypass these lesions. In the presence of pol δ and PCNA, the primer was extended by one nucleotide but further polymerization opposite the BD N3-dU adducts was greatly reduced (Figure 5A, lanes 6-8). However, partial bypass of the lesions was observed (Figure 5A, lane 8) under conditions that allowed nearly complete replication of the nondamaged DNA template (Figure 5A, lane 4). PCNA was added to this reaction since it is a pol δ processivity factor known to stimulate the bypass of certain DNA lesions (γ -hydroxypropano deoxyguanosine (43) and thymine dimers (47)) even on DNA templates that are not capped at their termini to restrict the release of the PCNA. Interestingly, the lesions posed an extreme block for replication by yeast

pol δ ². In kinetic experiments using pol ϵ , primer extension was completely blocked one nucleotide prior to and at the site of the lesions under conditions that supported polymerization on unmodified templates (Figure 5B). The same results were observed with increasing concentrations of pol ϵ that enabled complete polymerization on the unmodified templates².

Discussion

Research in BD-mediated carcinogenesis has included the identification of those DNA adducts that may induce mutagenesis. Site-specific methodologies have made it possible to evaluate the effect of individual DNA adducts on replication as compared to general BD exposure studies. Of the various BD-derived DNA monoadducts analyzed through these methods, the two most highly mutagenic adducts found were the N1-inosine adducts (38) and the BD N3-dU adducts as shown in this study. The N1-inosine adducts, like the N3-dU adducts are deamination products following its epoxidation by EB (30). Interestingly, the N1 inosine adducts exhibited a stereospecific difference in their overall mutation yield. While the mutagenic frequencies for the R N1-deoxyinosine adducts were 59%, those of the S N1-deoxyinosine adducts were 90% (38). Since the BD N3-DU adducts are overall highly mutagenic, both isomers should exhibit similar mutation yields, even though the pattern of mutations may be different with each isomer. Separate synthesis of the R and S diastereoisomers of the adducts are currently underway to test this hypothesis.

Nucleotide incorporation studies using exonuclease-deficient Kf with N3-hydroxyethyl uracil have revealed this adduct to be mutagenic, implicating it in the induction of mainly C to T transitions and C to A and C to G transversions to a minor extent (48). This is similar to the trend of mutations measured with the BD N3-dU adducts (Table 1). These data suggest that adduction at the N3 position of uracil renders it mutagenic irrespective of the exact identity of the parent epoxide. Structural data on the N3 BD-dU adducts are not yet available, but would give a better understanding of the relationship of the positioning of this adduct within DNA to explain the incorporation of the incorrect nucleotides opposite these lesions during replication in cells. This has been demonstrated in the case of other adducts of BD like the N1 deoxyinosine adducts (49, 50) in which the *syn* conformation of the adducts at the glycosyl bond could explain its induction of A to G mutations.

Assays to detect the formation of BD N3-dU adducts following *in vivo* exposure to butadiene gas or activated metabolites have not yet been carried out and thus, it is an open question whether these lesions form at an appreciable rate in humans. It is reasonable to speculate that they may be present at low levels as was seen in the case of 3-hydroxypropyl uracil, the only uracil DNA lesion to be detected *in vivo*. This adduct was found at 0.02 lesions/10⁶ nucleotides and at 0.02% of the corresponding 7-hydroxypropyl guanine (51). However, the data presented herein on the extremely high mutagenic frequency associated with the replication of these lesions suggest that such an investigation is well warranted.

In addition to the N3 BD-dU adducts that are the C1 adducts formed from the reaction of deoxycytidine with EB, the C2 N3 BD-dU adducts were also detected (35). However the deoxycytidine form of their precursors was not detected apparently due to more rapid rates of deamination as compared to the C1 N3 BD-dU adducts. Both regioisomers, i.e. the C1 and C2 adducts are formed from reactions of EB with deoxyguanosine (26, 31, 52). This also holds true for reactions of EB with N1 deoxyadenosine that can give rise to both C1 and C2 N1 deoxyadenosine adducts, C2 N1 inosine adduct or can undergo Dimroth rearrangement to give rise to C1 and C2 N⁶ deoxyadenosine regioisomers (30). Similarly, both C1 and C2 N3 deoxyadenosine adducts are formed from *in vitro* reactions of EB with adenine (31). Inhalation

²unpublished data.

exposure studies revealed that the C2 N7 guanine adduct derived from the R enantiomer was the major adduct found in the rat liver (53). Both C1 and C2 adducts were also detected in the testis and lung of mice (54). Thus, the C2 adducts are as important as the C1 adducts. Since these adducts are formed to both carbons associated with the epoxy group, the chemistry of EB seems to more resemble that of styrene oxide than that of saturated aliphatic epoxides that react mainly with the terminal carbon of the epoxide (55)

The BD N3-dU adducts are formed from the N3 (2-hydroxy-3-buten-1-yl) deoxycytidine adducts that are unstable under physiological conditions. The deoxycytidine adducts have a high deamination rate with short half-lives of 2.3 and 2.5 h, whereas their deaminated products are stable for up to 168 h (35). Similarly 3-(2-hydroxyethyl)-2'-deoxycytidine derived from bis(2-chloroethyl)nitrosourea is unstable under physiological conditions and undergoes deamination to 3-(2-hydroxyethyl)-2'-deoxyuridine with a half-life of approximately 5 h (56). Thus these uracil adducts are readily formed and may remain persistent in DNA. Other epoxides which yield a uracil product on reaction with cytidine or calf thymus DNA include propylene oxide (57), ethylene oxide (58), 2-cyanoethyleneoxide (59), acrylonitrile, (60) epichlorohydrin, (61) and glycidol (62). It is suggested that once the epoxide attacks the N3 of deoxycytidine, the hydroxyalkyl side chain intramolecularly catalyzes the hydrolysis of the adjacent imine C=NH, bond. As a result an unstable carbinolamine intermediate is formed that loses ammonia to form the uracil adduct (60).

Our studies indicate that mammalian pol ϵ and bacterial Kf are severely blocked upon encountering these adducts. They are also blocking to pol δ , although some bypass activity was observed. *In vivo*, these adducts may be recognized and bypassed more efficiently by specialized translesion polymerases like polymerase eta, iota, zeta and kappa that are implicated in the bypass of certain DNA adducts (63-66). Studies are currently underway to test this hypothesis.

In summary, we have synthesized and established the mutagenic effect of the BD N3-dU adducts. They are stable adducts that are blocking to replicative and repair polymerases and mutagenic in mammalian cells. These data thus suggest the importance of the BD N3-dU adducts as crucial lesions contributing to BD-carcinogenesis.

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Figure 1. Analysis of 12-mer oligodeoxynucleotide 5'-GCTAGCXAGTCC-3'. (A) Capillary gel electrophoresis profile. (B) HPLC profile of the enzymatic hydrolysate. The HPLC trace of an authentic sample of the adducted nucleoside is superimposed.

Figure 2.
MALDI-TOF analysis of partial exonuclease digestion of 12-mer oligodeoxynucleotide 5'-GCTAGCXAGTCC-3'. (A) 3' → 5' sequencing using phosphodiesterase I. (B) 5' → 3' sequencing using phosphodiesterase II.

Figure 3.

Autoradiographs displaying the result of mutagenic replication past the BD N3-dU adducts in COS-7 mammalian cells. Single-stranded pMS2 vector containing the BD N3-dU adducts or control dC was transfected into COS-7 cells and replicated for 48 h. The progeny plasmids were then recovered from the COS-7 cells and used to transform DH5a *E. coli* cells. Individual transformants were picked and grown in 96-well plates containing LB with ampicillin. The resultant colonies were transferred in four replicates onto Whatman 541 filter papers, one for each of the four probes G, C, A, and T, and were differentially hybridized.

Figure 4.

Primer extension reactions catalyzed by Kf on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a -2 primer. The DNA substrates (5 nM) were incubated in the presence of all four dNTPs (100 μ M) without or with increasing concentrations (0.0005, 0.005, 0.05 and 0.5 units) of Kf (lanes 2-5 and 7-10) for 15 min at room temperature. The position of the 14-nucleotide primer is indicated. U* indicates the position of the modified uracil on the template.

Figure 5.

Primer extension reactions catalyzed by calf thymus pol δ and human pol ϵ on the BD N3-dU adducted template. Control dC or BD N3-dU adducted 32-mer DNA templates were annealed to a -2 primer. (A) The DNA substrates (5 nM) were incubated in the presence of all four dNTPs (100 μ M) without or with increasing concentrations (0.008, 0.08 and 0.8 units) of pol δ , in the presence of 70 ng of calf thymus PCNA for 30 min at room temperature. (B) Time course experiments (at time intervals of 0, 1, 5, 10, 15, 20 and 30 min) were carried out with the DNA substrates (5 nM) at room temperature in the presence of all four dNTPs (100 μ M) with 0.1 units of pol ϵ . The positions of the 14-nucleotide primers and the 32-nucleotide full-length products are indicated. U* indicates the position of the modified uracil on the template.

Scheme 1.
Synthesis of 5'-DMT-3' p-Tol-2'deoxyuridine

Scheme 2.
Synthesis of 2-O-t-butyltrimethylsilyl-3-butene-1-(p-toluenesulfonate)

5'-DMT-N3-(3-buten-2-O-t-butyldimethylsilyl-1-yl)-2'-deoxyuridine-3'-O-(N,N,-diisopropyl-O-cyanoethyl)-phosphoramidite **7**

Scheme 3.

Synthesis of 5'-DMT-N3-(3-buten-2-O-t-butyldimethylsilyl-1-yl)-2'-deoxyuridine-3'-O-(N,N,-diisopropyl-O-cyanoethyl)-phosphoramidite

Table 1

Mutation Yield of the BD N3-dU adducts in COS-7 mammalian cells

	No. of colonies	Non mutagenic (%)	C to G (%)	C to A (%)	C to T (%)	Total Mutation Yield (%)
Single-stranded vector pMS2 containing						
Control dC	199	100	0	0	0	0
BD N3-dU adducts	191	3.1	11	32.5	53	96.9