Synthesis and Damage Specificity of a Novel Probe for the Detection of Abasic Sites in DNA

Hiroshi Ide,*,‡ Ken Akamatsu,‡ Yoshiharu Kimura,‡ Kenji Michiue,‡ Keisuke Makino,‡ Ayumi Asaeda,§ Yasuhiko Takamori,§ and Kihei Kubo§

Department of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan, and Department of Veterinary Radiology, School of Veterinary Medicine, College of Agriculture, University of Osaka Prefecture, Gakuen-cho 1-1, Sakai, Osaka 593, Japan

Received February 18, 1993; Revised Manuscript Received May 18, 1993

ABSTRACT: The abasic site (apurinic/apyrimidinic site) is the most common lesion in DNA and is suggested to be an important intermediate in mutagenesis and carcinogenesis. We have recently reported a novel assay for the detection and quantitation of abasic sites in DNA [Kubo, K., Ide, H., Wallace, S. S., & Kow, Y. W. (1992) *Biochemistry 31*, 3703–3708]. In this assay, the aldehyde group in an abasic site is first modified by a probe bearing a biotin residue, called the Aldehyde Reactive Probe (ARP) and then the tagged biotin is quantified by an ELISA-like assay. However, in the previous study, ARP was prepared only in a crude form, and no solid chemical data concerning the structure and specificity of ARP were reported. In this study, an improved method for the preparative synthesis of ARP has been established, and its structure has been unambiguously characterized using spectroscopic means. In order to elucidate the specificity of ARP to DNA damages, ARP was incubated with a variety of damaged bases or nucleosides and the reaction mixtures were analyzed by HPLC. Of the 14 compounds tested for their reactivity to ARP, 2-deoxyribose (a model compound for an abasic site) and 5-formyluracil reacted with ARP. Interestingly, compounds bearing a formamide group such as formamidopyrimidine and deoxyribosylformamide did not react with ARP, indicating that ARP is specific to damages having an alkyl or allyl aldehyde group. Furthermore, the ability of ARP synthesized by the defined chemical route to detect abasic sites has been substantiated using natural DNA containing abasic sites. Potential applications and limitations of the ARP assay are discussed.

Abasic sites (apurinic/apyrimidinic sites) are common lesions in DNA and are considered to be important intermediates in mutagenesis and carcinogenesis (Loeb & Preston, 1986; Kamiya *et al.*, 1992). These lesions are produced by spontaneous hydrolysis of the N-glycosidic bond under physiological conditions (Lindahl & Nyberg, 1972), as well as by chemical modification of bases by potent carcinogens or alkylating agents that destabilize the N-glycosidic bond (Tarpley *et al.*, 1982; Foster *et al.*, 1983; Osborne & Merrifield, 1985; Lawley & Brookes, 1963; Singer, 1976). Modification of base or sugar moieties by certain antibiotics (Bose *et al.*, 1980; Rabow *et al.*, 1986) and ionizing radiation (von Sonntag, 1987) also promote the formation of abasic sites. Abasic sites are generally restored in the repair pathway initiated by AP endonucleases in cells (Friedberg, 1985). If unrepaired, abasic sites are strong blocks to DNA synthesis and constitute lethal lesions (Schaaper & Loeb, 1981; Sagher & Strauss, 1983; Moran & Wallace, 1985; Hevroni & Livneh, 1988; Laspia & Wallace, 1989). It has also been shown that translesion DNA synthesis occurs at an abasic site with a low frequency, resulting in mutation. In this case, purine nucleotides, particularly dAMP, are preferentially incorporated opposite the lesion (Boiteux & Laval, 1982; Schaaper *et al.*, 1983; Kunkel, 1984; Randall *et al.*, 1987; Takeshita *et al.*, 1987; Lawrence *et al.*, 1990).

In view of the biological significance of abasic sites, a number of methods have been developed to detect and quantitate abasic sites in DNA. These include assays utilizing alkali elution (Brent *et al.*, 1978), DNA unwinding (Kohn *et al.*, 1981; Birnboim & Jevcak, 1981), ^{32}P-post-labeling (Weinfeld *et al.*, 1990), and modification of abasic sites by [^{14}C]-methoxyamine (Talpaert-Borle & Liuzzi, 1983; Liuzzi & Talpaert-Borle, 1988) or *O*-(nitrobenzyl)hydroxylamine (Kow, 1989; Chen *et al.*, 1992). We have recently reported a novel assay for the detection and quantitation of abasic sites (Kubo *et al.*, 1992). In this assay, the aldehyde group in an abasic site is specifically modified by a biotin-tagged reagent, called the Aldehyde Reactive Probe (ARP), and the bound biotin tag is quantitated colorimetrically by an ELISA-like assay using an avidin–biotin–horseradish peroxidase conjugate. The sensitivity of ARP assay is comparable with that of currently existing methods for quantitation of abasic sites, and more importantly, it obviates the need for radioactive materials, which most of the methods require (Brent *et al.*, 1978; Kohn *et al.*, 1981; Birnboim & Jevcak, 1981; Weinfeld *et al.*, 1990; Talpaert-Borle & Liuzzi, 1983; Liuzzi & Talpaert-Borle, 1988). However, in our previous study, ARP was conveniently prepared in a crude form by carbodiimide-catalyzed coupling of biotin hydrazide and *O*-(carboxymethyl)hydroxylamine and was not unambiguously characterized. This method of preparation of ARP was acceptable for preliminary studies, but it is not suitable for wide application of this assay, which would require a large quantity of ARP in a chemically pure form. In this article, we report a procedure for the preparative synthesis of ARP following a chemically defined route, and we define the specificity of this reagent for DNA damages, which is critical for the accurate quantitation of abasic sites. The specificity of ARP has been studied extensively using chemically synthesized base and nucleoside damages.

* Author to whom correspondence should be addressed
‡ Kyoto Institute of Technology.
§ University of Osaka Prefecture.

0006-2960/93/0432-8276$04.00/0

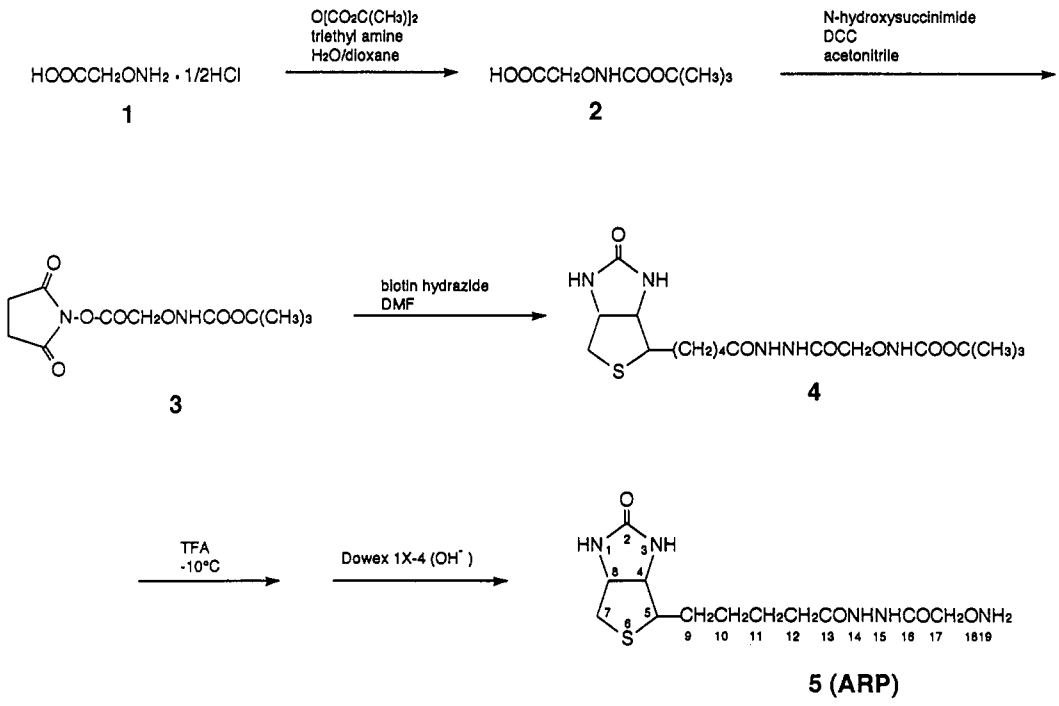


FIGURE 1: Scheme for the synthesis of ARP.

EXPERIMENTAL PROCEDURES

Chemicals. *O*-(Carboxymethyl)hydroxylamine hemihydrochloride, 2-deoxyribose (dR), and four normal 2′-deoxyribonucleosides (dA, dG, dC, and dT) were purchased from Nacalai. Di-*tert*-butyl dicarbonate, *N*,*N*′-dicyclohexylcarbodiimide, and *N*-hydroxysuccinimide were from Wako. Biotin hydrazide and 5,6-dihydrothymine (DHT) were obtained from Sigma. Triethylamine, 1,4-dioxane, and acetonitrile were distilled before use. Modified bases and nucleosides (also see Figure 2) were synthesized following published procedures, and their structures and purities were confirmed by ^{1}H and ^{13}C NMR and HPLC analysis (data not shown): 4,6-diamino-5-formamidopyrimidine (FAPY-A) (Cavalieri & Bendich, 1950); thymidine glycol (dT-GLY) (Iida & Hayatsu, 1971; Ide *et al.*, 1987); 5-formyluracil (FU) (Ressner *et al.*, 1976); 7,8-dihydro-8-oxoadenosine (8-oxoA) (Ikehara & Maruyama, 1975); 7,8-dihydro-8-oxoguanosine (8-oxoG) (Lin *et al.*, 1985). *N*-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)formamide (dFA) (Shida *et al.*, 1991) and 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (dHMH) were gifts from Drs. T. Shida and J. Cadet (unpublished results), respectively.

Instruments. ^{1}H and ^{13}C NMR spectra were measured at 300 and 75.5 MHz, respectively, using a Shimadzu/GE QE300 spectrometer. Chemical shifts for ^{1}H NMR are in ppm (δ) relative to tetramethylsilane (TMS), and those for ^{13}C are relative to dimethyl sulfoxide-d_6 (δ = 39.5 ppm). IR spectra were measured on a JASCO FT/IR-5300 spectrophotometer.

High-performance liquid chromatography (HPLC) analysis was carried out using a Shimadzu liquid chromatograph consisting of two LC-6A pumps with a gradient mixing unit, an SPD-M6A photodiode array detector, an SCL-6B system controller. HPLC data including UV spectra of eluates (200–350 nm) were collected every 4 s and analyzed by an NEC PC 9801 RA computer. A Wakosil 5C18-200T column (4 mm × 15 cm) was used throughout this study, and the column temperature was maintained at 50 °C by a column oven. Gradient elution was performed by building up a linear gradient starting with solvent A [H_2O/acetonitrile, 98:2 (v/v)] and applying solvent B [H_2O/acetonitrile, 60:40 (v/v)] up to 100% for 30 min with a flow rate of 0.5 mL/min. LC–MS analysis was performed using Shimadzu HPLC system similar to that described above, except that the column eluents were introduced into a Shimadzu TSP 100 detector operating in the thermospray ionization mode and the elution solvent contained 0.1 M ammonium acetate (pH 7.0).

Synthesis of ARP. ARP was prepared by applying standard *t*-Boc chemistry coupled with an activated ester method used in peptide synthesis (Figure 1) (Carpino, 1957; Anderson *et al.*, 1963a).

N-(tert-Butoxycarbonyl)-O-(carboxymethyl)hydroxylamine (**2**). *O*-(Carboxymethyl)hydroxylamine hemihydrochloride (**1**; 7.0 g, 64 mmol) and triethylamine (8.1 g, 80 mmol) were dissolved in water (35 mL). Di-*tert*-butyl dicarbonate (15.3 g, 70 mmol) in dioxane (35 mL) was added, and the reaction mixture was stirred for 2 days at room temperature. Water (150 mL) was added to the mixture and the solution was extracted with ethyl acetate (3 × 200 mL). The aqueous phase was cooled at 0 °C, adjusted to pH 2 with 5 N HCl, and extracted by ethyl acetate (3 × 150 mL). The combined ethyl acetate was washed with precooled 5% HCl (3 × 100 mL) and then with water saturated with NaCl (3 × 100 mL). The ethyl acetate phase was dried over Na_2SO_4 and evaporated to yield a crude product, which was recyrstallized from ethyl acetate/*n*-hexane to give 8.35 g (68%) of white powder: IR (cm^{-1}) 1118, 1230, 1242, 1719, 1743, 2980, 3370; ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H, CH_3), 4.26 (s, 2H, CH_2), 10.11 (br s, 1H, NH), 12.8 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 28.0 (CH_3), 72.0 (CH_2), 80.1 [$C(CH_3)_3$], 156.5 (NHCO), 170.2 (COOH).

N-Hydroxysuccinimide Ester of **2** (**3**). **2** (8.0 g, 41.9 mmol) and *N*-hydroxysuccinimide (3.0 g, 46.1 mmol) were dissolved in dry acetonitrile (140 mL), and dicyclohexylcarbodiimide (9.51 g, 46.1 mmol) in dry acetonitrile (25 mL) was added with stirring. The reaction mixture was stirred at room temperature overnight, and precipitated dicyclohexylurea was removed by filtration. The filtrate was evaporated leaving an oily product. The product was dissolved in hot carbon

tetrachloride (500 mL) and filtered. The filtrate was kept at 4 °C for 2 days to yield 7.81 g (65%) of white crystals: IR (KBr, cm^{-1}) 1095, 1201, 1730, 1782, 1818, 2980, 3340; ^1H NMR (DMSO-d_6) δ 1.42 (s, 9H, CH_3), 2.84 (s, 4H, CH_2-CH_2), 4.83 (s, 2H, CH_2O), 10.37 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 25.5 (CH_2CH_2), 27.9 (CH_3), 69.9 (CH_2O), 80.6 [$C(CH_3)_3$], 156.6 (NHCO), 165.2 ($NCOCH_2$), 170.0 ($OCOCH_2$).

Biotin Hydrazide–2 Conjugate (4). 3 (4.51 g, 15.7 mmol) was dissolved in dimethylformamide (40 mL), and biotin hydrazide (3.68 g, 14.2 mmol) was added. The reaction mixture was stirred for 4 h at room temperature and then at 50 °C for 1 h to complete the reaction. The suspension of biotin hydrazide became clear as the reaction proceeded. The solvent was removed *in vacuo*, leaving a viscous residue. The residue was extracted with boiling ethyl acetate (3 × 800 mL). The extracts were combined and left at 4 °C to give fine gels. The gels were collected by filtration and dried *in vacuo* to yield 4.62 g (75%) of 4: IR (KBr, cm^{-1}) 1165, 1256, 1478, 1698, 2932, 2980, 3231; ^1H NMR (DMSO-d_6) δ 1.2–1.7 (m, 6H, H_9, H_{10}, H_{11}), 1.41 [s, 9H, CH_3 (*t*-Boc)], 2.14 (t, J = 7.1 Hz, 2H, H_{12}), 2.58 (d, J = 12.3 Hz, 1H, H_{7a}), 2.83 (dd, J = 5.1, 12.3 Hz, 1H, H_{7b}), 3.10 (m, 1H, H_5), 4.15 (m, 1H, H_4), 4.27 (s, 2H, H_{17}), 4.31 (m, 1H, H_8), 6.39 and 6.46 (br s, 2H, H_{14}, H_{15}), 9.82 (br s, 2H, H_1, H_3), 10.19 (br s, 1H, H_{19}); ^{13}C NMR (DMSO-d_6) δ 25.1 and 28.0 (C_9, C_{10}, C_{11}), 27.9 [CH_3 (*t*-Boc)], 32.9 (C_{12}), 39.9 (C_7), 55.5 (C_5), 59.2 (C_8), 61.1 (C_4), 73.5 (C_{17}), 80.6 [$C(CH_3)_3$ (*t*-Boc)], 156.7 [CO (*t*-Boc)], 162.8 and 166.7 (C_2, C_{13}), 171.0 (C_{16}) (for the atom numbering scheme, see Figure 1); MS(FAB) 432 (MH^+).

ARP (5). 4 (4.50 g, 10.4 mmol) was cooled at –10 °C, and trifluoroacetic acid (25 mL) precooled at –10 °C was added with stirring. The solution was kept at –10 °C for 10 min and then at room temperature for 30 min with stirring. Trifluoroacetic acid was removed by evaporation at room temperature to give a viscous material (ARP·trifluoroacetic acid salt). The ARP·trifluoroacetic acid salt was dissolved in water (120 mL), and Dowex 1X-4 resin (OH^- form) was added with stirring until the pH of the solution became 7. The resin was removed by filtration and washed with water (50 mL). The resin was stirred further in water (150 mL) for 2 h to recover ARP adsorbed to the resin, and this was repeated again. The filtrates were combined and evaporated to dryness, yielding a white powder of ARP (2.70 g, 78%): IR (KBr, cm^{-1}) 1049, 1262, 1480, 1696, 2938, 3248; ^1H NMR (DMSO-d_6) δ 1.2–1.7 (m, 6H, H_9, H_{10}, H_{11}), 2.13 (t, J = 7.2 Hz, 2H, H_{12}), 2.58 (d, J = 12.3 Hz, 1H, H_{7a}), 2.83 (dd, J = 5.0, 12.3 Hz, 1H, H_{7b}), 3.10 (m, 1H, H_5), 4.04 (s, 2H, H_{17}), 4.14 (m, 1H, H_4), 4.32 (m, 1H, H_8), 6.36 (br s, 2H, H_{19}), 6.38 and 6.44 (br s, 2H, H_{14}, H_{15}), 9.71 (br s, 2H, H_1, H_3); ^{13}C NMR (DMSO-d_6) 25.1, 28.0, and 28.1 (C_9, C_{10}, C_{11}), 33.0 (C_{12}), 39.9 (C_7), 55.4 (C_5), 59.2 (C_8), 61.0 (C_4), 73.5 (C_{17}), 162.8 and 168.6 (C_2, C_{13}), 171.1 (C_{16}) (the assignment of the NMR signals was confirmed by ^1H and ^{13}C–^1H COSY); high-resolution MS (FAB) for $C_{12}H_{22}N_5O_4S_1$ (MH^+) calcd 332.1392, found 332.1391. The ^1H NMR spectrum and HPLC analysis revealed that ARP was highly pure, and the level of contaminants was estimated to be less than a few percent (data not shown).

Reaction of ARP with Damaged Bases and Nucleosides. One milliliter of an aqueous ARP solution (2 mM) was added to 1 mL of phosphate buffer (20 mM, pH 7.0) containing a modified base or nucleoside and allowed to react at room temperature. Samples were taken after an appropriate time and analyzed by HPLC or LC–MS as described in Instruments.

DNA. DNA containing abasic sites was prepared by treating calf thymus DNA (Worthington Biochem. Co.) in depurination buffer consisting of NaCl (100 mM) and sodium citrate (10 mM, pH 5.0) at 70 °C. The duration of the heat treatment was varied (0–75 min) to introduce different amounts of abasic sites into DNA.

DNA from HeLa RC355 cells (Kubo *et al.*, 1982) treated by methylmethanesulfonate (MMS) was prepared as follows. HeLa RC355 cells were cultured in Eagle's MEM medium supplemented with 10% fetal bovine serum in a 100-mL dish. The medium was removed by aspiration, and the cells were washed with PBS(–) consisting of NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (4.3 mM), and KH_2PO_4 (1.4 mM). MMS (5 mL; final concentration 0–23 μM) in PBS [PBS(–) + $CaCl_2$ (0.9 mM) and $MgCl_2$ (0.49 mM)] was added to the dish, and the incubation was performed at 37 °C for 1 h. The MMS solution was removed, and the dish was washed with PBS(–) twice. PBS (10 mL) containing 0.02% EDTA was added to the dish, followed by incubation at room temperature for 10 min. The cell suspension was centrifuged at 1500 rpm for 5 min. The supernatant was removed, 1.1 mg/mL of proteinase K solution (Wako) was added with approximately 10 vol of the cell pellet, and the solution was incubated at 37 °C overnight. The proteinase-treated cell suspension was extracted with phenol, followed by chloroform, and dialyzed extensively against TE buffer consisting of Tris-HCl (10 mM, pH 7.5) and EDTA (1 mM). After dialysis, the solution was treated with RNase (Sigma, Type II-A) [10 μL of RNase stock solution (10 mg/mL) per dialyzed solution] at 37 °C for 1 h. The solution was extracted with phenol and then chloroform, and the aqueous phase was dialyzed extensively against TE buffer. After the absorbance was measured at 260 nm to determine the DNA concentration, the sample was appropriately diluted in TE buffer and subjected to the ARP assay.

ARP Assay. The ARP assay was performed following the method reported by Kubo *et al.* (1992), with slight modification. To eliminate traces of potentially reactive aldehyde groups remaining on the well surface of the microtiter plates (Sumilon Elisa plate MS-3696F, Sumitomo Bakelite Co.), 300 μL of 0.1% $NaBH_4$ solution was added to each well, and the plate was incubated for 30 min at room temperature. After the plate was washed with TPBS (PBS + 0.5% Tween 20) 10 times followed by distilled water twice, 200 μL of DNA solution (10 μg/mL) was added to each well. The plate was covered with Parafilm and incubated at 37 °C overnight. In a separate experiment, using [^3H]thymidine-labeled DNA, we found that the amount of DNA bound to each well was 300 ng (data not shown). The plate was washed with TPBS four times, 100 μL of ARP solution (5 mM) was added, and the plate was covered with Parafilm and incubated at 37 °C for 1 h. The plate was washed with TPBS 10 times, and 100 μL of ABC solution [preformed avidin–biotin–enzyme complex prepared by following the instructions of the supplier (Vectastain peroxidase standard kit no. PK-4000, Vector Laboratories, Inc.)] was added to each well. The plate, covered with Parafilm, was incubated at 37 °C for 30 min and washed with TPBS 10 times. Enzyme substrate solution (200 μL) was added to each well. The substrate solution consisted of ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 1 mg/mL], Na_2HPO_4 (51 mM), citric acid (24 mM), and H_2O_2 (0.5 μL of 30% H_2O_2 per 1 mL of the substrate solution). The plate was covered with Parafilm, incubated at

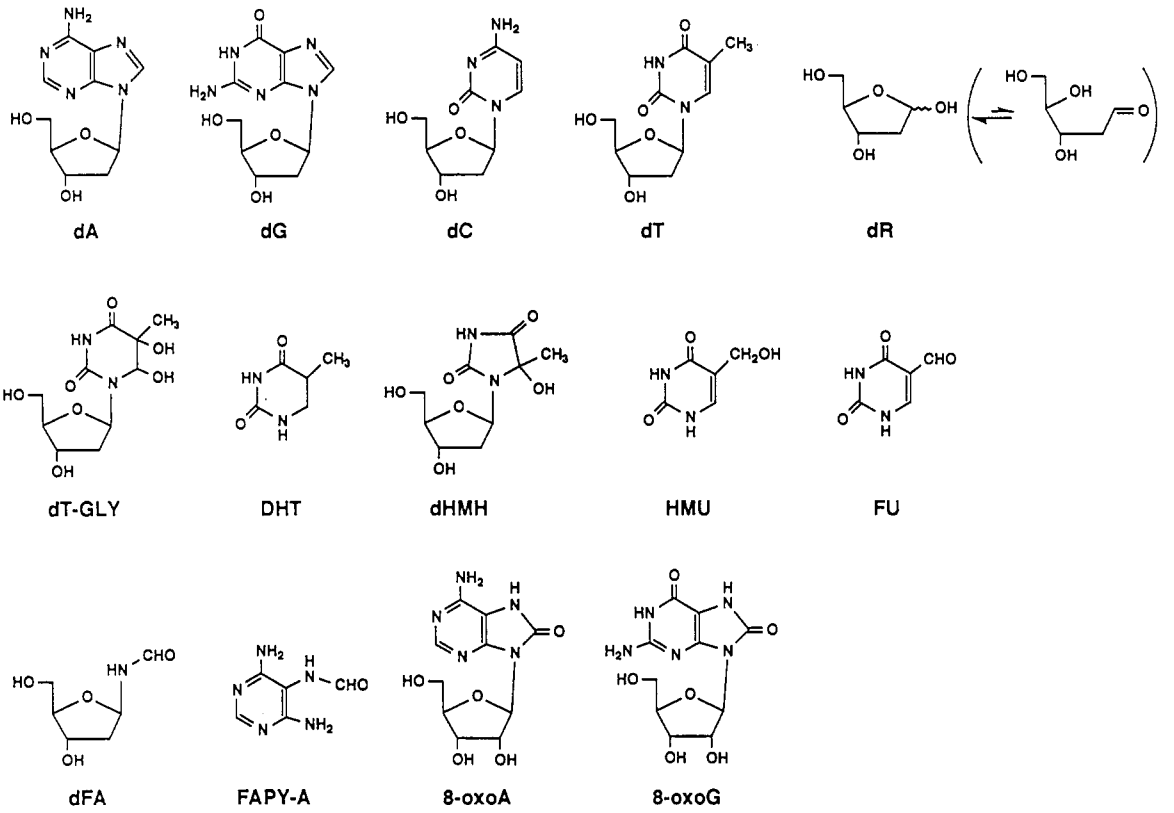


FIGURE 2: Structures of bases and nucleosides tested for their reactivity toward ARP.

room temperature for 2 h, and subjected to OD measurement at 405 nm. All ARP assays were performed in duplicate, and means were calculated on the basis of these data (see Figures 6–8). The scatter of experimental data points was consistently within ±5% of the means.

RESULTS AND DISCUSSION

Synthesis of ARP. ARP was synthesized in four steps (Figure 1) by applying standard *t*-Boc chemistry coupled with an activated ester method used in peptide synthesis (Carpino, 1957; Anderson *et al.*, 1963a). In the first step, the *tert*-butyloxycarbonyl (*t*-Boc) group was introduced to protect the amino moiety of *O*-(carboxymethyl)hydroxylamine. This step is required to avoid self-condensation of the activated ester of *O*-(carboxymethyl)hydroxylamine. The yield of **2** was fair (68%) and was not as high as that reported for glycine (89%) (Tarbell *et al.*, 1972). An attempt to improve the yield of **2** using 2-[(*tert*-butyloxycarbonyl)thio]-4,6-dimethylpyridine as a *t*-Boc reagent (Nagasawa *et al.*, 1973) failed and the yield remained similar. The *N*-hydroxysuccinimide ester of the protected *O*-(carboxymethyl)hydroxylamine (**3**) was obtained in 65% yield in the second step, which is comparable to those reported for *t*-Boc amino acids (Anderson *et al.*, 1963b). In the last two steps, biotin hydrazide was conjugated with the activated ester, and the *t*-Boc group was removed by acidolysis using trifluoroacetic acid. Although the overall yield of the four reactions was very low (26%), the present reaction scheme should be practically acceptable in light of the fact that biotin hydrazide, which is the most costly material in this synthesis, is employed only in the last two steps, and ARP was obtained in 56% yield in the last two reactions.

The structure of ARP was confirmed by ^1H and ^{13}C NMR and high-resolution MS measurements. By comparison with ^1H NMR spectra of biotin and biotin hydrazide (not shown), it is evident that the protons of the methylene (H_{17}; see Figure 1 for the numbering scheme), terminal NH_2 (H_{19}), and NHNH (H_{14} and H_{15}) groups are present in ARP, as well as a biotin moiety. The carbonyl (C_{16}) and methylene (C_{17}) carbons derived from *O*-(carboxymethyl)hydroxylamine were also verified by the ^{13}C NMR spectrum of ARP. The assignments of the NMR signals were further supported by two-dimensional NMR measurements including ^1H and ^1H–^{13}C COSY.

Specificity of ARP to DNA Damages. In a previous publication (Kubo *et al.*, 1992), we showed that ARP reacts with the aldehyde groups of abasic sites in DNA prepared by heat/acid depurination or enzymatic removal of damaged bases. However, it is probably rare for DNA molecules of interest to contain solely abasic sites as damages. For example, reactive oxygens such as OH radicals generated by aerobic metabolism (Fridovich, 1978; Ames, 1983, 1987) or ionizing radiation (Teoule & Cadet, 1978; von Sonntag, 1987) react with many DNA constituents to produce not only abasic sites but also other DNA lesions. Thus, elucidation of the influence of coexisting damages on the ARP assay is essential for the accurate quantitation of abasic sites in damaged DNA. In addition, high concentrations of hydroxylamine and methoxyamine react with normal nucleic acid bases to form adducts (Phillips & Brown, 1967; Cashmore *et al.*, 1971; Chang, 1973; Piper & Clark, 1974). Since ARP has the same functional group (ONH_2) as hydroxylamine and methoxyamine to tag abasic sites with biotin residues, nonspecific reaction of ARP with normal DNA constituents may interfere with the ARP assay by increasing the background signal. For these reasons, we have screened a number of damaged DNA constituents as well as normal nucleosides to determine whether they react with ARP.

Figure 2 shows the structures of DNA damages and four normal 2′-deoxyribonucleosides tested for their reactivity toward ARP. Nine out of the ten listed damages are known

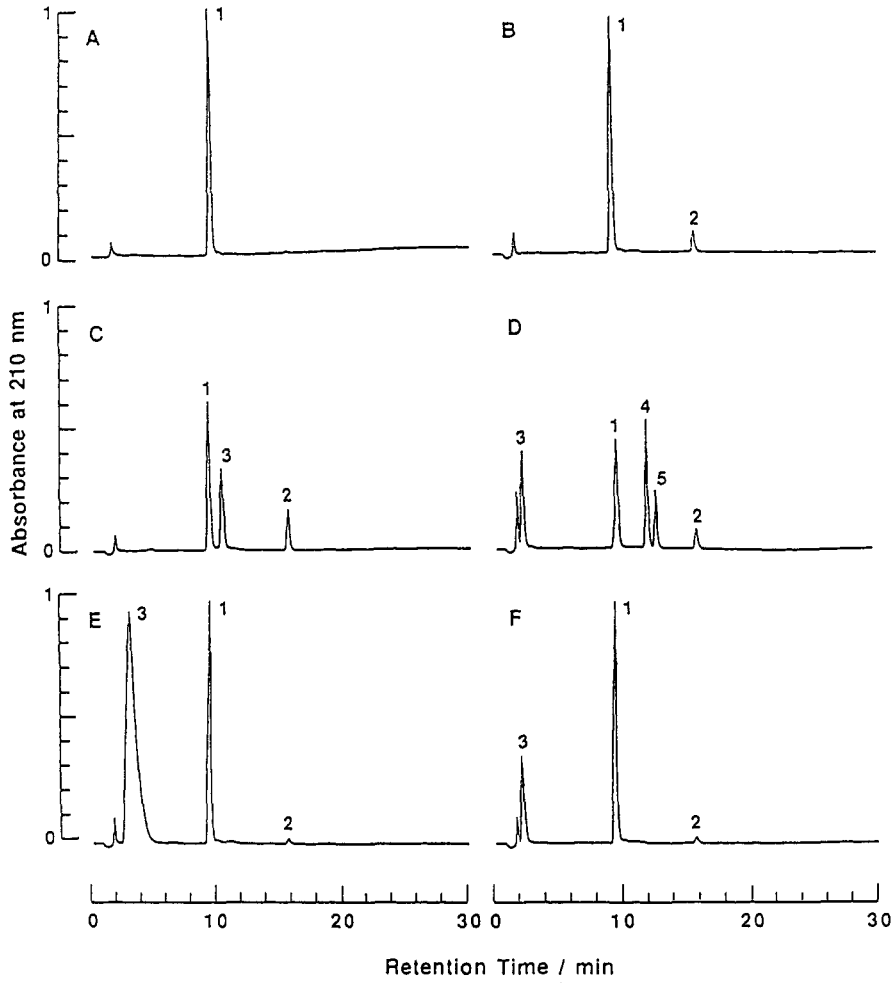


FIGURE 3: HPLC elution profiles of the reaction mixtures of ARP and damaged bases or nucleosides. ARP (1 mM) was incubated in the absence (B) or presence (C–F) of a base or nucleoside (1 mM) in phosphate buffer (10 mM, pH 7.0) for 4 days at room temperature. The samples were analyzed by HPLC as described in the Experimental Procedures. (A) ARP solution immediately after preparation. (B) ARP solution incubated without additive. (C) ARP + 2-deoxyribose (dR). (D) ARP + 5-formyluracil (FU). (E) ARP + 4,6-diamino-5-formamidopyrimidine (FAPY-A). (F) ARP + *N*-(2′-deoxy-β-D-*erythro*-pentofuranosyl)formamide (dFA). Throughout this figure, peaks 1 and 2 are ARP and its decomposition product, respectively. Peak 3 in C corresponds to the ARP–dR adduct, while those in D, E, and F correspond to FU, FAPY-A, and dFA, respectively.

to be produced by OH radicals (Teoule & Cadet, 1978; von Sonntag, 1987), and 5,6-dihydrothymine (DHT) is a radiolysis product of thymine under anaerobic conditions (Nishimoto *et al.*, 1983a,b; Hubbard *et al.*, 1989). The bases and nucleosides were incubated with ARP in phosphate buffer (pH 7.0) at room temperature for 4 days, and the reaction mixtures were analyzed by reversed-phase HPLC. ARP was also treated in a similar manner in the absence of a base or nucleoside. Figure 3A,B shows typical HPLC chromatograms of ARP solutions immediately after preparation and after incubation for 90 h, respectively. ARP slowly reacted by itself to give a product with retention time 15.8 min (Figure 2B, peak 2), and its conversion was about 7% under these conditions (also see Figure 4). This product was also formed in the presence of additives, but the yield varied. According to LC–MS analysis, the molecular weight of the reaction product appears to be 372 assuming a protonated form (MH^+), but no further attempt was made to elucidate the structure and mechanism of formation of the reaction product of ARP.

Of the 14 compounds tested for their reactivity toward ARP, 2-deoxyribose (dR) and 5-formyluracil (FU) reacted with ARP. HPLC profiles for 2-deoxyribose and 5-formyluracil incubated with ARP are shown in Figure 3C,D, respectively. 2-Deoxyribose gave a product that eluted as peak 3 (Figure 3C). The molecular weight of the product determined by LC–MS (MH^+ 448) agreed with that expected for a condensation product between ARP and 2-deoxyribose [M = ARP(331) + deoxyribose(134) – H_2O(18) = 447, MH^+ 448]. In the case of 5-formyluracil, two reaction products (Figure 3D, peaks 4 and 5) were detected by HPLC analysis, in addition to the decomposition product of ARP (peak 2). The two products in elution peaks 4 and 5 had the same molecular weights on the basis of LC–MS analysis (MH^+ 454), corresponding to a product derived from condensation between the formyl group of 5-formyluracil and the amino group of ARP [M = ARP(331) + formyluracil(140) – H_2O(18) = 453, MH^+ 454]. Figure 5 shows the UV spectra of the 5-formyluracil–ARP adducts obtained by post analysis of the three-dimensional HPLC data. The UV spectra of both adducts were similar, but the absorption maxima were shifted to longer wavelengths relative to that of the parent 5-formyluracil, implying that modification occurred in the formyl group while the 5,6 doulbe bond was intact. From these MS and UV data, we have concluded that 5-formyluracil reacts with the hydroxylamine moiety of ARP to form an oxime. The products in peaks 4 and 5 are tentatively assigned to *E* and *Z* configurational isomers with respect to the C=N bond formed by the condensation reaction. In order to compare the reactivities of 2-deoxyribose and 5-formyluracil with ARP, the time courses of the reactions were also followed by HPLC

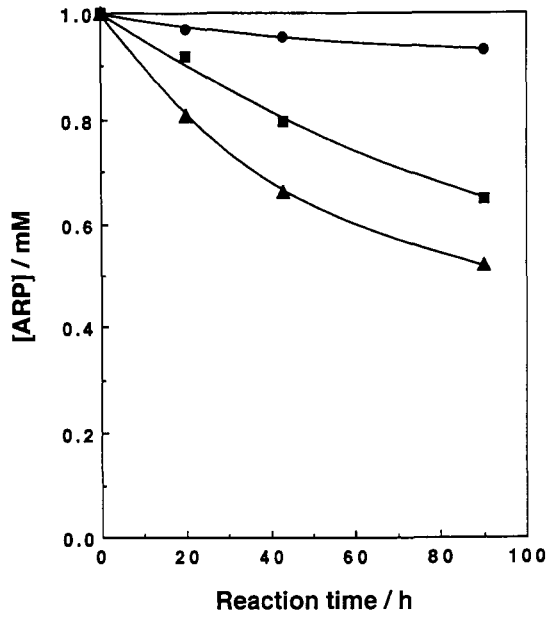


FIGURE 4: Time course of the conversion of ARP in the absence of additive (●) and the presence of 2-deoxyribose (■) or 5-formyluracil (▲). ARP (1 mM) was incubated in the absence or presence of 2-deoxyribose (1 mM) or 5-formyluracil (1 mM) in phosphate buffer (10 mM, pH 7.0) at room temperature. Samples were taken at the indicated times, and the conversion of ARP was determined by HPLC as described in Experimental Procedures.

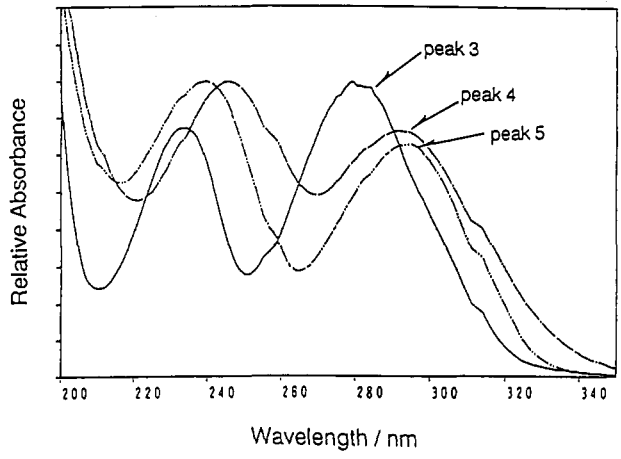


FIGURE 5: UV spectra of 5-formyluracil and ARP–5-formyluracil adducts obtained by postanalysis of HPLC data. Peak 3 corresponds to 5-formyluracil, and 4 and 5 correspond to ARP–5-formyluracil adducts. The HPLC elution profile of these peaks is shown in Figure 3D.

(Figure 4). According to the conversion rate of ARP, the reactivity of 5-formyluracil with ARP is somewhat higher than that of 2-deoxyribose.

There was no sign of adduct formation between normal 2′-deoxyribonucleosides and ARP in HPLC analysis, showing that normal nucleosides do not react with ARP under the present conditions. This was also the case for thymidine damages, including thymidine glycol (dT-GLY), 5-(hydroxymethyl)uracil (HMU), 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (dHMH), 5,6-dihydrothymine (DHT), and 7,8-dihydro-8-oxopurines including 7,8-dihydro-8-oxoadenosine (8-oxoA) and 7,8-dihydro-8-oxoguanosine (8-oxoG). Interestingly, 4,6-diamino-5-formamidopyrimidine (FAPY-A), identified as a imidazole ring fission product of adenine, and *N*-(2′-deoxy-β-D-*erythro*-pentofuranosyl)formamide (dFA), identified as a thymine ring fragmentation product, both have a formamide group but

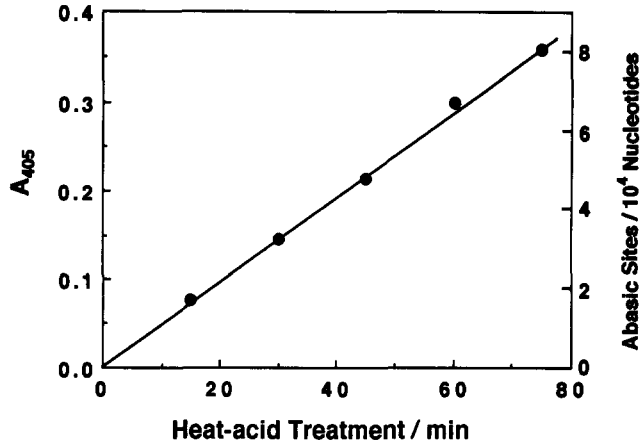
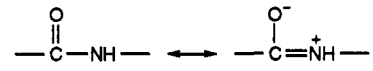


FIGURE 6: Detection of abasic sites in calf thymus DNA by the ARP reagent. Calf thymus DNA was depurinated by acid/heat treatment for the indicated time and subjected to the ARP assay. The conditions for the acid/heat treatment and ARP assay are described in Experimental Procedures. The ARP signal (A_{405}) was converted to the number of abasic sites (right ordinates) on the basis of the reported depurination rate of acid/heat-treated calf thymus DNA (Kow, 1989; Kubo *et al.*, 1992).

they did not react with ARP at all. As shown in Figure 3E,F, no adduct formation was detected in HPLC analysis with these formamide compounds. It is widely accepted that the amide bond has a partial double-bond character, as shown by the following resonance structures (Pauling, 1960). This also reduces the partial positive charge on the carbonyl carbon (March, 1985). Accordingly, the aldehyde group of the above two compounds becomes less susceptible to nucleophilic attack by the amino group of ARP.



Detection of Abasic Sites in DNA. In order to substantiate the ability of ARP synthesized by the defined chemical route to detect abasic sites in DNA, two types of natural DNA substrates containing abasic sites were subjected to the ARP assay. Calf thymus DNA containing abasic sites was prepared by acid/heat depurination. Chromosomal DNA was also extracted from HeLa cells treated with methylmethanesulfonate (MMS). In the latter, the N-glycosidic bond become labile upon alkylation, resulting in facilitated formation of abasic sites (Friedberg, 1985; Loeb & Preston, 1986). The ARP assay was performed as described in the Experimental Procedures. The ARP signal (absorbance at 405 nm) was proportional to the duration of the acid/heat treatment of calf thymus DNA (Figure 6), as well as the concentration of MMS used for the treatment of HeLa cells (Figure 7). These data umambiguously demonstrate that ARP synthesized here can quantitatively detect abasic sites in natural DNA. Since acid/heat treatment of calf thymus DNA for 9.3 min generates about 1 abasic site/10^4 nucleotides under the conditions used (Kow, 1989; Kubo *et al.*, 1992), the ARP signal obtained for DNA extracted from MMS-treated HeLa cells was further standardized using the signal derived from acid/heat-treated calf thymus DNA (Figure 7, right ordinates). Consistent with the previous results on the sensitivity of the ARP assay (Kubo *et al.*, 1992), the data in Figure 7 show that at least 1 abasic site/10^4 nucleotides can be detected by ARP assay. This sensitivity roughly translates into 10 fmol of abasic sites on the basis of the amount of DNA bound to each well (300 ng).

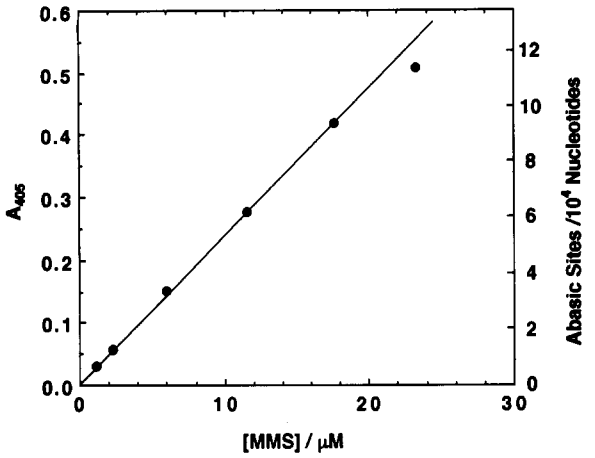


FIGURE 7: Detection of abasic sites in chromosomal DNA extracted from HeLa RC355 cells treated with methylmethanesulfonate (MMS). HeLa RC355 cells were treated with the indicated concentrations of MMS. Chromosomal DNA was extracted from the cells and subjected to the ARP assay. The ARP signal (A_{405}) was converted to the number of abasic sites (right ordinates) on the basis of the ARP signal obtained for acid/heat-treated calf thymus DNA, from which the depurination rate was previously estimated (Kow, 1989; Kubo *et al.*, 1992).

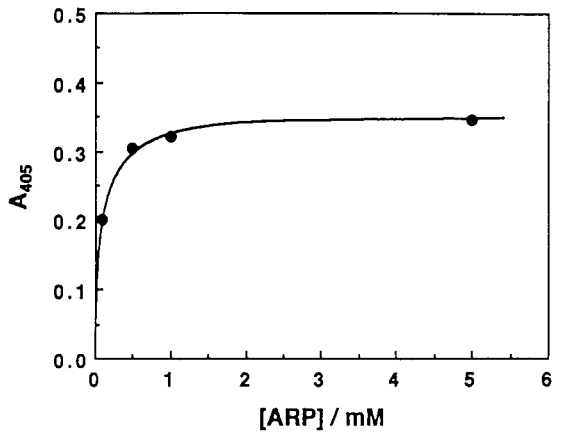


FIGURE 8: Dependence of the ARP signal (A_{405}) on the concentration of ARP. Calf thymus DNA containing abasic sites was prepared by acid/heat treatment for 60 min and plated. The ARP assay was performed as described in Experimental Procedures, except that the concentration of ARP was varied.

In our previous protocol for the ARP assay, the concentration of ARP to be incubated with DNA was not defined since ARP was obtained as a crude product in solution and its purity was not determined. To determine the optimum ARP concentration for maximizing the ARP signal and minimizing the consumption of ARP, the ARP assay was performed with varying concentrations of ARP (0.1–5 mM). The ARP signal for depurinated DNA increased with increasing concentration of ARP and then reached a plateau at 5 mM ARP (Figure 8). These data indicate that potentially reactive sites in the test DNA are modified almost quantitatively if ARP is present at 5 mM under these conditions. Therefore, we have concluded that the optimum concentration of ARP is 5 mM under the present assay conditions.

The reaction between ARP and 2-deoxyribose proceeded unexpectedly slowly (Figure 4), yet the modification of abasic sites in DNA by ARP was completed in 1 h (Figure 8): plated DNA was incubated with ARP (5 mM) for 1 h in the ARP assay. Since equimolecular amounts of ARP and 2-deoxyribose are consumed in the reaction between these compounds, it is estimated from Figure 4 that 0.2 mM 2-deoxyribose reacted with ARP after 40 h of incubation. This amount approximately translates into 5 nmol of 2-deoxyribose reacted with ARP/h/mL. In contrast, only 10–100 fmol of abasic sites was present in each well in the DNA assay. Thus, the rate for the reaction between ARP and 2-deoxyribose should be high enough to account for the complete modification of abasic sites in DNA bound to each well.

In summary, ARP, a novel probe for the detection and quantitation of abasic sites in DNA, has been synthesized on a preparative scale following a defined chemical route, and its structure has been unambiguously characterized by spectroscopic methods. ARP is reasonably stable in aqueous solution in the absence of a reactant. The reaction of ARP is strictly restricted to DNA damages containing an alkyl or allyl aldehyde group. In this sense, the ARP signal can be directly correlated with the number of abasic sites in DNA when they are produced in the following manner: (i) Abasic sites derived from spontaneous depurination (Lindahl & Nyberg, 1972) or chemical modifications of bases that destabilize the N-glycosidic bond. The latter includes alkylation (Lawley & Brookes, 1963; Singer, 1976) and bulky adduct formation involving benzo[*a*]pyrene, aflatoxin B_1, and 2-(acetylamino)fluorene (Tarpley *et al.*, 1982; Foster *et al.*, 1983; Osborne & Merrifield, 1985). (ii) Abasic sites resulting from enzymatic removal of modified bases by DNA *N*-glycosylases (Weiss & Grossman, 1987; Wallace 1988; Asahara *et al.*, 1989; Doetsch & Cunningham, 1990). However, particular care is necessary when the ARP assay is performed on DNA where both abasic sites and products bearing an alkyl or allyl aldehyde group are simultaneously produced. For example, radiolysis of DNA results in the formation of such products including 5-formyluracil and altered sugars (Teoule & Cadet, 1978; von Sonntag, 1987). Elucidation of the contribution of these individual products to the ARP signal is a subject of future study.

ACKNOWLEDGMENT

We thank Drs. Toshio Shida and Jean Cadet for the generous gifts of dFA and dHMH, respectively, and Drs. Susan S. Wallace and Yoke W. Kow for helpful discussions. We are also grateful to Mr. Seisuke Tanaka, Dr. Masanori Sato, and Dr. Ryohei Yamaoka for assistance with LC–MS analysis.

REFERENCES

Ames, B. N. (1983) *Science 221*, 1256–1264.
Ames, B. N. (1987) *Ann. Intern. Med. 107*, 526–545.
Anderson, G. W., Zimmerman, J. E., & Callahan, F. (1963a) *J. Am. Chem. Soc. 85*, 3039.
Anderson, G. W., Zimmerman, J. E., & Callahan, F. (1963b) *J. Am. Chem. Soc. 86*, 1839–1842.
Asahara, H., Wistort, P. M., Bank, J. F., Bakerian, R. H., & Cunningham, R. P. (1989) *Biochemistry 28*, 4444–4449.
Birnboim, H. C., & Jevcak, J. J. (1981) *Cancer Res. 41*, 1889–1892.
Boiteux, S., & Laval, J. (1982) *Biochemistry 21*, 6746–6751.
Bose, K. K., Tatsumi, K., & Strauss, B. (1980) *Biochemistry 19*, 4761–4766.
Brent, T. P., Teebor, G. W., & Duker, N. J. (1978) in *DNA Repair Mechanisms* (Hanawalt, P. C., Friedberg, E. C., & Fox, C. F., Eds.) pp 19–22, Academic Press, New York.
Carpino, L. A. (1957) *J. Am. Chem. Soc. 79*, 4427–4331.
Cashmore, A. R., Brown, D. M., & Smith, J. D. (1971) *J. Mol. Biol. 59*, 359–373.
Cavalieri, L. F., & Bendich, A. (1950) *J. Am. Chem. Soc. 72*, 2587–2594.

Chang, S. E. (1973) *J. Mol. Biol. 75*, 533–547.
Chen, B. X., Kubo, K., Ide, H., Erlanger, B. F., Wallace, S. S., & Kow, Y. W. (1992) *Mutat. Res. 273*, 253–261.
Doetsch, P. W., & Cunningham, R. P. (1990) *Mutat. Res. 236*, 173–201.
Fridovich, I. (1978) *Science 201*, 875–880.
Friedberg, E. C. (1985) *DNA Repair*, W. H. Freeman, New York.
Foster, P. L., Eisenstadt, E., & Miller, J. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2695–2698.
Hevroni, D., & Livneh, Z. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5046–5050.
Hubbard, K., Ide, H., Erlanger, B. F., & Wallace, S. S. (1989) *Biochemistry 28*, 4382–4387.
Ide, H., Melamede, R. J., & Wallace, S. S. (1987) *Biochemistry 26*, 964–969.
Iida, S., & Hayatsu, H. (1971) *Biochim. Biophys. Acta 240*, 370–375.
Ikehara, M., & Maruyama, T. (1975) *Tetrahedron 31*, 1369–1372.
Kamiya, H., Suzuki, M., Komotsu, Y., Miura, H., Kikuchi, K., Sakaguchi, T., Murata, N., Masutani, C., Hanaoka, F., & Ohtsuka, E. (1992) *Nucleic Acids Res. 20*, 4409–4415.
Kohn, K. W., Ewing, R. G. A., Erikson, L. C., & Zwelling, L. A. (1981) in *DNA Repair: A Laboratory Manual of Research Procedures* (Friedberg, E. C., & Hanawalt, P., Eds.) Vol. 1, Part B, pp 379–401, Marcel Dekker, New York.
Kow, Y. W. (1989) *Biochemistry 28*, 3280–3287.
Kubo, K., Koiwai, S., & Morita, K. (1982) *J. Radiat. Res. 23*, 204–217.
Kubo, K., Ide, H., Wallace, S. S., & Kow, Y. W. (1992) *Biochemistry 31*, 3703–3708.
Kunkel, T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1494–1498.
Laspia, M. F., & Wallace, S. S. (1989) *J. Mol. Biol. 207*, 53–60.
Lawley, P. D., & Brookes, P. (1963) *Biochem. J. 89*, 127–138.
Lawrence, C. W., Borden, A., Banerjee, S. K., & LeClerc, J. E. (1990) *Nucleic Acids Res. 18*, 2153–2157.
Lin, T.-S., Chen, J.-C., Ishiguro, K., & Sartorelli, A. C. (1985) *J. Med. Chem. 28*, 1194–1198.
Lindahl, T., & Nyberg, B. (1972) *Biochemistry 11*, 3610–3618.
Liuzzi, M., & Talpaert-Borle, M. (1988) *Int. J. Radiat. Biol. 54*, 709–722.
Loeb, L. A., & Preston, B. D. (1986) *Annu. Rev. Genet. 20*, 201–230.
March, J. (1985) in *Advanced Organic Chemistry*, 3rd ed., Chapter 10, John Wiley & Sons, New York.
Moran, E., & Wallace, S. S. (1985) *Mutat. Res. 146*, 229–241.
Nagasawa, T., Kuroiwa, K., Narita, K., & Isowa, Y. (1973) *Bull. Chem. Soc. Jpn. 46*, 1269–1272.
Nishimoto, S., Ide, H., Nakamichi, K., & Kagiya, T. (1983a) *J. Am. Chem. Soc. 105*, 6740–6741.
Nishimoto, S., Ide, H., Wada, T., & Kagiya, T. (1983b) *Int. J. Radiat. Biol. 44*, 585–600.
Osborne, M., & Merrifield, K. (1985) *Chem. Biol. Interact. 53*, 183–195.
Pauling, L. (1960) in *The Nature of the Chemical Bond*, 3rd ed., Chapter 8.4, Cornell University Press, Ithaca, NY.
Phillips, J. H., & Brown, D. M. (1967) in *Progress in Nucleic Acid Research and Molecular Biology* (Davidson, N., & Cohn, W. E., Eds.) Vol. 7, p 349, Academic Press, New York.
Piper, P. W., & Clark, B. F. C. (1974) *Nucleic Acids Res. 1*, 45–55.
Rabow, J., Stubbe, J., Kozarich, J. W., & Gerlt, J. A. (1986) *J. Am. Chem. Soc. 108*, 7130–7131.
Randall, S. K., Eritja, R., Kaplan, B. E., Petruska, J., & Goodman, M. F. (1987) *J. Biol. Chem. 262*, 6864–6870.
Ressner, E. C., Fraher, P., Edelman, M. S., & Mertes, M. P. (1976) *J. Med. Chem. 19*, 194–196.
Sagher, D., & Strauss, B. (1983) *Biochemistry 22*, 4518–4526.
Schaaper, R. M., & Loeb, L. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1773–1777.
Schaaper, R. M., Kunkel, T. A., & Loeb, L. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 487–491.
Shida, T., Iwaori, H., & Sekiguchi, J. (1991) *Nucleic Acids Res., Symp. Ser. 25*, 97–98.
Singer, B. (1976) *Nature (London) 264*, 333–339.
Takeshita, M., Chang, C.-N., Johnson, F., Will, S., & Grollman, A. P. (1987) *J. Biol. Chem. 262*, 10171–10179.
Talpaert-Borle, M., & Liuzzi, M. (1983) *Biochim. Biophys. Acta 740*, 410–416.
Tarbell, D. S., Yamamoto, Y., & Pope, B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 730–732.
Tarpley, W. G., Miller, J. A., & Miller, E. C. (1982) *Carcinogenesis 3*, 81–88.
Teoule, R., & Cadet, J. (1978) in *Effects of Ionizing Radiation on DNA. Physical, Chemical and Biological Aspects* (Hutterman, J., Kohnlein, W., Teoule, R., & Bertinchamps, A. J., Eds.) pp 171–203, Springer-Verlag, Berlin.
von Sonntag, C. (1987) in *The Chemical Basis of Radiation Biology*, pp 221–294, Taylor and Francis, London.
Wallace, S. S. (1988) *Environ. Mol. Mutagen. 12*, 431–477.
Weinfeld, M., Liuzzi, M., & Paterson, M. C. (1990) *Biochemistry 29*, 1737–1743.
Weiss, B., & Grossman, L. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 60*, 1–34.