Nucleosides, Nucleotides & Nucleic Acids

SYNTHESIS OF THE FULLY PROTECTED PHOSPHORAMIDITE OF THE BENZENE-DNA ADDUCT, N^2- (4-HYDROXYPHENYL)-2'-DEOXYGUANOSINE AND INCORPORATION OF THE LATER INTO DNA OLIGOMERS

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Running Title:

Synthesis and Insertion of N^2-4-HOPh-dG into DNA

Abbreviations: HQ, hydroquinone; *p*-BQ, *p*-benzoquinone; N^2-4-HOPh-dG, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

Abstract

N^2-(4-Hydroxyphenyl)-2'-deoxyguanosine-5'-*O*-DMT-3'-phosphoramidite has been synthesized and used to incorporate the N^2-(4-hydroxyphenyl)-2'-dG (N^2-4-HOPh-dG) into DNA, using solid-state synthesis technology. The key step to obtaining the xenonucleoside is a palladium (Xantphos-chelated) catalyzed N^2-arylation (Buchwald-Hartwig reaction) of a fully protected 2'-deoxyguanosine derivative by 4-isobutyryloxybromobenzene. The reaction proceeded in good yield and the adduct was converted to the required 5'-*O*-DMT-3'-*O*-phosphoramidite by standard methods. The latter was used to synthesize oligodeoxynucleotides in which the N^2-4-HOPh-dG adduct was incorporated site-specifically. The oligomers were purified by reverse-phase HPLC. Enzymatic hydrolysis and HPLC analysis confirmed the presence of this adduct in the oligomers.

Keywords Benzene DNA adduct; Hydroquinone; *p*-Benzoquinone; Buchwald-Hartwig reaction; N^2-(4-Hydroxyphenyl)-2'-deoxyguanosine

INTRODUCTION

Humans are exposed to benzene from different sources such as automobile exhaust fumes, cigarette smoke and in industries involved in rubber manufacture, crude oil, gasoline, and chemical manufacturing. Benzene has been linked to a number of chronic conditions of the haematopoietic system of exposed individuals, due to its metabolism in the liver and bone marrow, to more reactive species.[1] These chronic effects include leucopenia, thrombocytopenia, pancytopenia, anemia, and myelodysplastic syndrome. More importantly, benzene is a recognized human leukemogen.[2,3] Several epidemiologic studies have established a relationship between occupational exposure to benzene and human leukemia, especially acute myelogenous leukemia (AML).[4,5]

Inhaled benzene is first metabolized in the liver, where it is converted by cytochrome P450-2E1 to benzene oxide, then to phenol, hydroquinone (HQ) and other phenolic metabolites.[6,7] These compounds may travel to the bone marrow, the main target organ of benzene, where they are oxidized to genotoxic quinones such as *p*-benzoquinone (*p*-BQ) and *o*-BQ by the high levels of myeloperoxidase (MPO) in this tissue (Figure 1). The mutagenic potential of benzene and its major metabolites such as HQ and *p*-BQ has been extensively investigated.[8] Studies using mammalian systems have shown that benzene metabolites induce base substitution and deletion in various cell lines, animal tissues, and blood from exposed individuals.[8]

The precise mechanisms underlying the benzene-induced mutagenesis remain poorly understood. There are numerous reports dealing with the detection of covalent DNA adducts by HQ and *p*-BQ *in vitro*.[8,9] These two compounds are stable metabolites derived from benzene, and *p*-BQ is also present in a number of drugs and chemical substances.[10-12] Both metabolites form exocyclic benzetheno adducts with dG, dA and dC.[13-17] Although they were not detected

in tissues such as the bone marrow by ^{32}P-postlabeling,[18,19] their *in vivo* formation by similar mechanisms to those seen in *in vitro* reactions is expected, given that the yields of these adducts were high in the latter. Using site-directed mutagenesis, we recently showed that all three *p*-BQ adducts are highly mutagenic in *S. cerevisiae* by predominantly causing deletion mutations.[20] We have also shown that these *p*-BQ adducts, when present in DNA, are substrates for human apurinic/apyrimidinic (AP) endonuclease (APE1), which directly incises the phosphodiester bond 5' to the adduct.[21-24]

Although benzene-derived DNA adducts have been identified *in vitro*, the *in vivo* detection of such adducts has been a subject of controversy. Using ^{32}P-postlabeling, Bauer *et al* showed the formation of several DNA adducts in the livers of rabbits treated with benzene.[25] Later, the Bodell group discovered *in vivo* adducts in a human promyelocytic (HL-60) cell line[18,26] and bone marrow of mice[19] that were treated with benzene, HQ, or *p*-BQ. They found that the DNA adducts formed after benzene administration in mouse bone marrow are identical to those produced in HL-60 or mouse bone marrow cells treated with HQ or *p*-BQ. Moreover, the observed induction of toxicity in the bone marrow was paralleled by formation of DNA adducts.[19] By ^{32}P-postlabeling, the principal adduct found in the cells targeted by benzene has the same chromatographic properties as N^2-(4-hydroxyphenyl)-2'-deoxyguanosine-3'-phosphate.[18,26] This adduct can also be formed *in vitro* by allowing guanosine 3'-phosphate to react with *p*-BQ.[18,26] To date, the biochemical properties and biological importance of this adduct await investigation.

In order to understand the molecular mechanism by which HQ and *p*-BQ exert their mutagenic effects, it is important to study their principal adduct formed *in vivo*, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine (N^2-4-HOPh-dG), regarding its potential effects on DNA

replication and repair. Such studies would be greatly facilitated with oligonucleotides containing a site-specific adduct. Previously, we have synthesized the phosphoramidites of exocyclic *p*-BQ-dA, *p*-BQ-dC and one of the *p*-BQ-dG adducts and incorporated them site-specifically into oligomeric DNA.[27,28] In this work, we report for the first time the synthesis of N^2-4-HOPh-dG-phosphoramidite and its incorporation into defined DNA oligonucleotides.

RESULTS AND DISCUSSION

Previously, Pongracz and Bodell[26] reported the synthesis of N^2-(4-hydroxyphenyl)-2'-deoxyguanosine-3'-phosphate as the 3'-phosphate. We have now successfully synthesized the fully-protected N^2-(4-hydroxyphenyl)-2'-deoxyguanosine-3'-phosohoramidite **7**, by a completely new method (Scheme 1) which gave better yields. Our approach is similar to that used previously for the synthesis and incorporation of the 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) adducts into DNA in which the key procedure is a Buchwald-Hartwig (BH) reaction.[29,30] Compound **1** when coupled with 4-isobutyryloxybromobenzene **2** under the conditions of the Buchwald-Hartwig reaction gave compound **3** in 80% yield. Removal of the 6-*O*-benzyl group from the purine moiety was then accomplished by hydrogenolysis over a palladium catalyst to give compound **4,** which, after purification by column chromatography, was obtained in 88% yield. The silyl protecting groups of compound **4** were removed by HF in pyridine, to give compound **5** in 76% yield after purification. The electrospray mass spectrum of this compound showed an intense protonated molecular ion at *m/z* 430 $(M+H)^+$ and the natriated species at *m/z* at 452 $(M+Na)^+$ (Figure 2). Reaction of compound **5** with DMT-Cl in pyridine produced compound **6** in 90% yield, which was then allowed to react with 2-cyanoethyl *N*, *N*, *N*, *N*-tetraisopropyl phosphane to produce the fully protected phosphoramidite compound **7**. All of the

above compounds were identified by MS, ^{1}H NMR, ^{13}C NMR and in the case of compound **7,** ^{31}P NMR analysis was used as well (See ^{1}H NMR of **7** in Figure 3). The latter was then used to introduce the xenonucleoside into defined oligonucleotides by DNA synthesizer. The composition of the oligomers was confirmed after enzymatic digestion of the DNA oligomers to nucleosides, followed by HPLC analysis, which showed that the N^2-4-HOPh-dG residual survived the conditions used in the DNA synthesis and the deprotection procedure (Figure 4). The biochemical work is underway to evaluate the mutagenic potential of this adduct as well as possible repair mechanism(s) that acts on it using these oligonucleotides.

EXPERIMENTAL

Caution: *The organic solvents used in this work should be stored and used in a well-ventilated hood.*

Chemicals and Reagents.

All reagents and solvents employed were of commercial grade and were used as such, unless otherwise specified. NMR (^{1}H and ^{13}C) spectra were recorded on a Gemini 300 spectrometer. Samples prepared for NMR analysis were dissolved in $CDCl_3$ or DMSO-d_6. Chemical shifts are reported in ppm relative to TMS in the proton spectra and to the deuterated solvent in the carbon spectra. Mass spectra were recorded on a Micromass Trio in fast atom bombardment (FAB) mode. Thin-layer chromatography (TLC) was performed on silica gel sheets (riedel-deHaën, Sleeze, Germany) containing a fluorescent indicator. Components were visualized by UV light (λ = 254 nm) or by spraying with a solution of phosphomolybdic acid. Flash column chromatography separations were carried out on 60 Å (230-400 mesh) silica gel (Silica-P Flash Silica Gel obtained from Silicycle, Quebec, QC, Canada). All experiments dealing with

moisture or air-sensitive compounds were conducted under dry nitrogen. The starting materials and reagents, unless otherwise specified, were the best commercial grades available (Aldrich, Fluka) and were used without further purification. After purification all new products showed a single spot on TLC analysis.

HPLC. Solvent systems included acetonitrile (solvent A), triethylammonium acetate (0.1 M, pH 7.0, solvent B), and potassium phosphate buffer (0.01 M, pH 4.5, solvent C). System 1: Oligonucleotides were purified using a PRP-1-C18 column (305 X 7 mm, 10 µm, Hamilton Corp.). The initial concentration of solvent A was 10% and solvent B was 90%, then solvent A was increased linearly to 38% over 40 min at a flow rate of 3 ml/min. System 2: This was used for the analysis of the enzyme digest of the oligonucleotides and was performed with a Supelcosil LC-18-DB column (25 x 0.46 cm, 5 µm, Supelco Inc.) using 0% solvent A, 100% solvent C at start. Solvent A was linearly increased to 12% over 30 min, then to 40% over 15 min where it was held for 10 min at a flow rate of 1 ml/min. The HPLC instrument was an Agilent 1100 series.

Ultraviolet spectra were recorded on an Agilent 8453 spectrophotometer using 0.2 cm cuvette. TLC was performed on silica gel 60, F254 plates. Column chromatography was performed using silica gel 60 with elution under pressure.

Chemical Syntheses (Scheme 1).

N^2-(4-Isobutyryloxyphenyl)-O^6-benzyl-3',5'-bis-O-(tert-butyldimethylsilyl)-2'-deoxyguanosine (3). An oven-dried 100 mL two-necked round-bottomed flask was charged with the protected purine nucleoside **1**[31] (1.17 g. 2 mmol), cesium carbonate (0.92 g, 2.8 mmol), $Pd_2(dba)_3$ (230 mg, 0.25 mmol), xantphos (480 mg, 0.8 mmol), 4-isobutyryloxybromobenzene (**2**, 0.724 g, 2.98 mmol) and toluene (15 mL) under a nitrogen atmosphere. This mixture was stirred for 30 min at

room temperature, later heated at 80°C for 6 h. It was cooled to room temperature, the solids were filtered, and the solid cake was washed with ethyl acetate. The filtrate was evaporated to dryness and the residue was purified by silica gel column chromatography using CH_2Cl_2:ethyl acetate (98:2) as the eluant to give pure **3** (1.2 g, 80%). ^{1}H NMR ($CDCl_3$): δ 8.09 (s, 1H), 7.67 (d, 2H), 7.55 (m, 2H),7.45-7.33 (m, 3H), 7.28 (br s, 1H), 7.09 (d, 2H), 6.46 (t, 1H), 5.68 (s, 2H), 4.64 (m, 1H), 4.08 (m, 1H), 3.88-3.85 (m, 2H), 2.87 (m, 1H), 2.72-2.39 (m, 2H), 1.40 (s, 3H), 1.38 (s, 3H), 0.98 (s, 9H), 0.97 (s, 9H), 0.16 (s, 6H), 0.15 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 175.43, 160.15, 155.26, 152.81, 145.11, 137.79, 137.35, 136.08, 128.03, 127.68, 127.60, 121.24, 119.37, 116.09, 87.35, 83.61, 77.42, 77.00, 76.57, 71.64, 67.79, 62.53, 41.01, 33.80, 25.68, 25.47, 18.67, 18.09, 17.67, -4.94, -5.06, -5.66, -5.77. ESI (M+H) 748.

N^2-(4-Isobutyryloxyphenyl)-3',5'-bis-O-(tert-butyldimethylsilyl)-2'-deoxyguanosine (4). To a solution of **3** (1.2 g, 1.6 mmol) in ethyl acetate (50 mL) was added a 10% palladium-on-carbon (150 mg) catalyst. The flask was evacuated (50 Torr) and flushed with hydrogen three times. The mixture was shaken under hydrogen for 16 h at 50 psi, filtered through a pad of Celite to remove the catalyst, and then concentrated under reduced pressure. The residue was purified by column chromatography using 3% methanol in methylene chloride to obtain the pure debenzylated product **4** as a glassy solid (0.930 gm 88%). ^{1}H NMR ($CDCl_3$): δ 11.76 (br s, 1H), 9.75 (br s, 1H), 8. 14 (d, 2H), 8.09 (s, 1H), 7.10 (d, 2H), 6.41 (t, 1H), 4.63 (m, 1H), 4.07 (m, 2H), 2.87 (m, 1H), 2.52 (m, 2H), 1.40 (s, 3H), 1.38 (s, 3H), 1.00 (s, 9H), 0.98 (s, 9H), 0.18 (s, 6H), 0.16 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 175.54, 158.83, 150.62, 149.57, 146.05, 136.46, 136.31, 121.33, 121.02, 118.16, 87.64, 84.04, 71.76, 62.75, 41.71, 34.09, 25.92, 25.71, 18.94, 18.35, 17.90, -4.68, -4.82, -5.41, -5.53. ESI (M+H) 658.

Preparation of N^2-(4-isobutyryloxyphenyl)-2'-deoxyguanosine (5). To an ice-cold solution of **4** (0.9 g, 1.37 mmol) in pyridine (20 mL) was added HF/Py (2.2 mL, 70% of HF in Py from Aldrich Chemical Co.) over a period of 3 min. The mixture was stirred at room temperature overnight, then poured into ice-cold water (100 mL) containing $NaHCO_3$ (6.5 g) and stirred for 2 h. The reaction mixture was evaporated to dryness and co-evaporated with methanol three times and the residual material was adsorbed onto silica gel using methanol as a solvent. It was purified by silica gel using 15% methanol in methylene chloride to obtain the pure **5** as a white solid (450 mg, 76%). ^{1}H NMR (DMSO-d_6): δ 10.67 (br s, 1H), 9.01 (s, 1H), 8.03 (s, 1H), 7.61 (d, 2H), 7.09 (d, 2H), 6.195 (t, 1H), 5.27 (br s, 1H), 4.89 (br s, 1H), 3.81 (m, 1H), 3.50 (m, 2H), 2.78 (m, 1H), 2.59 (m, 1H), 2.28 (m, 1H), 1.22 (s, 3H), 1.20 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 175.96, 157.14, 150.09, 149.97, 146.32, 137.18, 122.73, 120.95, 118.97, 88.35, 83.64, 71.31, 62.29, 33.97, 19.38. ESI (M+H) 429.

N^2-(4-Isobutyryloxyphenyl)-5'-O-(4.4'-dimethoxytrityl)-2'-deoxyguanosine (6): To a solution of **5** (0.428 g, 1 mmol) in pyridine (15 mL) was added DMT chloride (0.440 g, 1.3 mmol) as a solid and the mixture was stirred at room temperature for 3 h. TLC (CH_2Cl_2: MeOH: 85:15) of the mixture showed the reaction to be ~90% complete. The reaction was quenched by adding methanol (5 mL) and the mixture was stirred for 30 min at room temperature, and then concentrated under reduced pressure. To the residue was added aqueous $NaHCO_3$ (25 mL) and the mixture was extracted with methylene chloride. The methylene chloride extract was dried over anhydrous $MgSO_4$, filtered and concentrated. The crude product was purified by column chromatography using TEA-treated silica gel and CH_2Cl_2: MeOH (95:5) as the eluant to give pure **6** (550 mg, 90% yield based on consumed starting material). The column was further eluted with 15% methanol in methylene chloride to recover the unreacted starting material (70 mg). ^{1}H

NMR ($CDCl_3$): δ 10.01 (br s, 1H), 7.75 (s, 1H), 7.58 (d, 2H), 7.41-7.16 (m, 10H), 6.89 (d, 2H), 6.79 (d, 4H), 6.15 (t, 1H), 4.46 (m, 1H), 4.05 (m, 1H), 3.73 (s, 6H), 3.27 (m, 2H), 2.84 (m, 1H), 2.72 (m, 1H), 2.41 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 176.51, 158.38, 150.19, 149.95, 146.30, 144.68, 136.73, 135.90, 135.86, 129.95, 128.13, 127.69, 126.71, 123.31, 121.49, 118.59, 113.00, 86.23, 85.61, 83.98, 71.44, 64.25, 55.09, 39.52, 34.07, 18.83. ESI (M+H) 732.

N^2-(4-Isobutyryloxyphenyl)-5′-O-(4,4’-dimethoxytrityl)-3′-O-[N,N-diisopropylamino-(2-cyanoethoxy)-phosphinyl]-2′-deoxyguanosine (7). Compound **6** (256 mg, 0.35 mmol) was co-evaporated with dry toluene (3 x 10 mL), and the residue was redissolved in dry methylene chloride (10 mL). Tetrazole (28 mg, 0.40 mmole) was then added followed by 2-cyanoethyl *N*,*N*,*N*,*N*-tetraisopropylphosphane (147 mg, 0.49 mmole). The reaction mixture was stirred at room temperature for 2 h under nitrogen and then poured into aqueous $NaHCO_3$ (10%, 10 mL). The methylene chloride layer was separated and the aqueous layer was extracted once with methylene chloride (20 mL). The combined methylene chloride extract was dried over Na_2SO_4, filtered and concentrated under reduced pressure to obtain the desired crude product **7** as an oil. The crude residue was purified on a silica gel column pre-treated with triethylamine, using methylene chloride:acetone (3:2) as the eluant. The fractions containing the pure material were collected and concentrated under reduced pressure and the resulting solid was triturated with isorpropyl ether and filtered to give pure **7** as a white solid (240 mg, 73%). ^1H NMR ($CDCl_3$): δ 8.76 (br s, 1H), 8. 02 (br s, 1H), 8.01 (d, 1H), 7.88 (d, 1H), 7.48-7.25 (m, 10H), 7.02 (d, 2H), 6.85 (m. 4H), 6.41 (t, 1H), 4.71 (m, 1H), 4.34 (m, 1H), 3.80 (s, 6H), 3.65 – 3.74 (m, 5H), 3.41 (m, 2H), 3.09 (m, 1H), 2.87 – 2.47 (m, 3H), 1.38-1.16 (m, 18H). ^{13}C NMR ($CDCl_3$): δ 175.62, 158.94, 158.55, 150.92, 149.72, 146.26, 144.52, 136.22, 135.63, 135.53, 130.13, 130.07, 130.02, 128.16, 128.10, 127.85, 126.90, 121.54, 121.26, 118.46, 117.61, 113.16, 86.48, 85.81, 84.15,

74.01, 63.66, 58.40, 58.21, 55.17, 46.13, 43.39, 43.34, 43.27, 43.21, 40.24, 34.12, 24.58, 24.55, 24.51, 20.35, 20.27, 20.18, 18.96. ^{31}P NMR ($CDCl_3$): δ 150.09, 149.60.

Solid-phase synthesis of DNA oligonucleotides. The fully-protected phosphoramidite compound **7** was used to synthesize three different oligonucleotides at the 1 μmole scale: a 22-mer, a 25-mer and a 40-mer using an Applied Biosystem 394 automated DNA synthesizer by standard phosphoramidite chemistry. Phenoxyacetyl (PAC) was used as the *N*-ptotecting group in the synthesis of the DNA oligomers. Because amidite **7** has very limited solubility in acetonitrile, dry methylene chloride (100%) was used to dissolve the amidite **7,** which was used in the DNA synthesis. The coupling time for the amidite **7** was increased to 15 min, and the coupling efficiency at the N^2-4-HOPh-dG step was 97% for all oligonucleotides. The oligonucleotides were then cleaved from the resin and deprotected by means of 28% ammonium hydroxide containing 1% of 2-mercaptoethanol for 2 hours at 65°C. The latter reagent prevents oxidative degradation of the N^2-4-HOPh-dG lesion. The oligonucleotides were purified by reverse phase HPLC on a C-18 column. The sequences of the DNA oligomers synthesized are as follows:

1: 5'-GTAAGCT**X**GATCCTCTAGAGCG-3' (22-mer)

2: 5'-CCGCTA**X**CGGGTACCGAGCTCGAAT-3' (25-mer)

3: 5'-TTGCTTTGTCACCCAGGCT**X**GACTGCAGTGGTACAATCAT-3' (40-mer)

X= N^2-4-HOPh-dG

Enzymatic digestion of the oligomers. The composition of the oligomers was confirmed by enzymatic digestion of the DNA oligomers to nucleotides, which were identified by HPLC analysis. A defined oligonucleotide (1.0 A_{260} unit) was dissolved in 44 μL of deionized water, 0.8 μL of 1M $MgCl_2$, and 3.5 μL of 0.5M Tris-HCl buffer (pH 7.5) and digested with snake

venom phosphodiesterase (2.5 µL) and bacterial alkaline phosphatase (4.0 µL) at 37 ºC for 16 h. The digested mixture was then analyzed on a C-18 reverse-phase HPLC Supelcosil column using solvent system 2 noted above.

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Figure legends

Figure 1: The metabolic route from benzene to the formation of HQ and *p*-BQ. These two stable metabolites may react with guanine in the bone marrow to form N^2- (4-hydroxyphenyl)-2'-deoxyguanosine (N^2-4-HOPh-dG).

Figure 2: Electrospray mass spectrum of N^2-4-HOPh-dG (**5**).

Figure 3: ^{1}H NMR of N^2-(4-hydroxyphenyl)-2'-deoxyguanosine-3'-phosohoramidite (**7).**

Figure 4: (A) HPLC profile of 2'-deoxynucleosides obtained as a result of enzymatic digestion of the N^2-4-HOPh-dG-containing 40-mer. (B) HPLC profile of N^2-4-HOPh-dG.

Schemes

Scheme 1: The synthesis of the fully protected N^2- (4-hydroxyphenyl)-2'-deoxyguanosine-3'-phosphoramidite **7**.

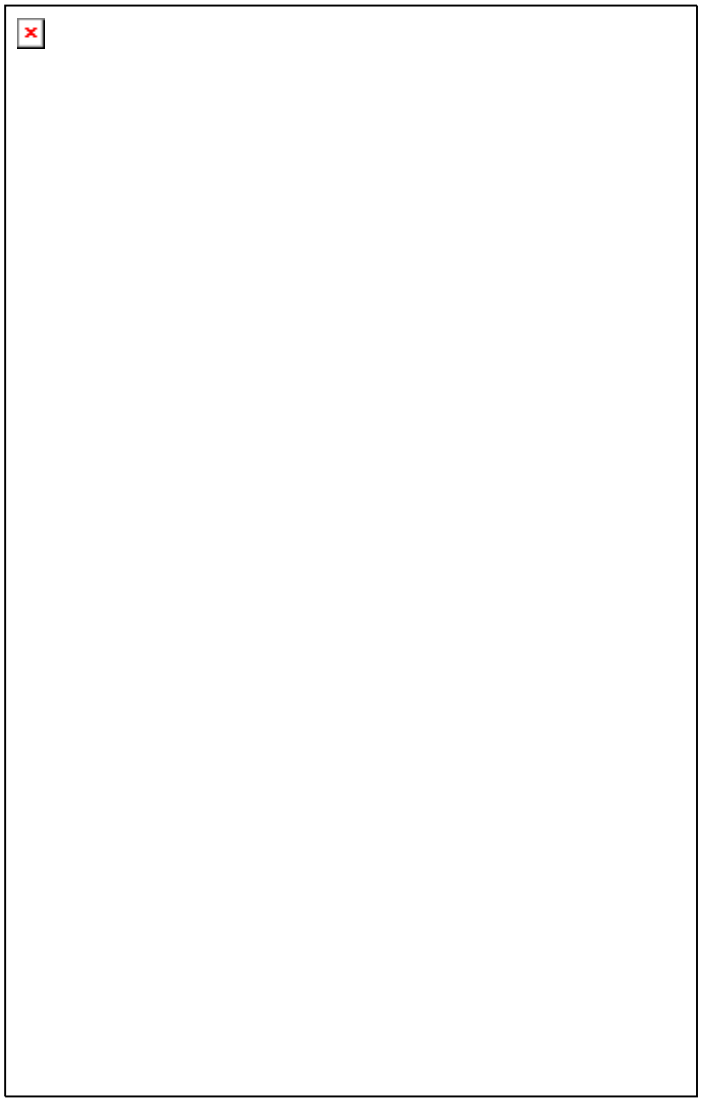


Figure 1

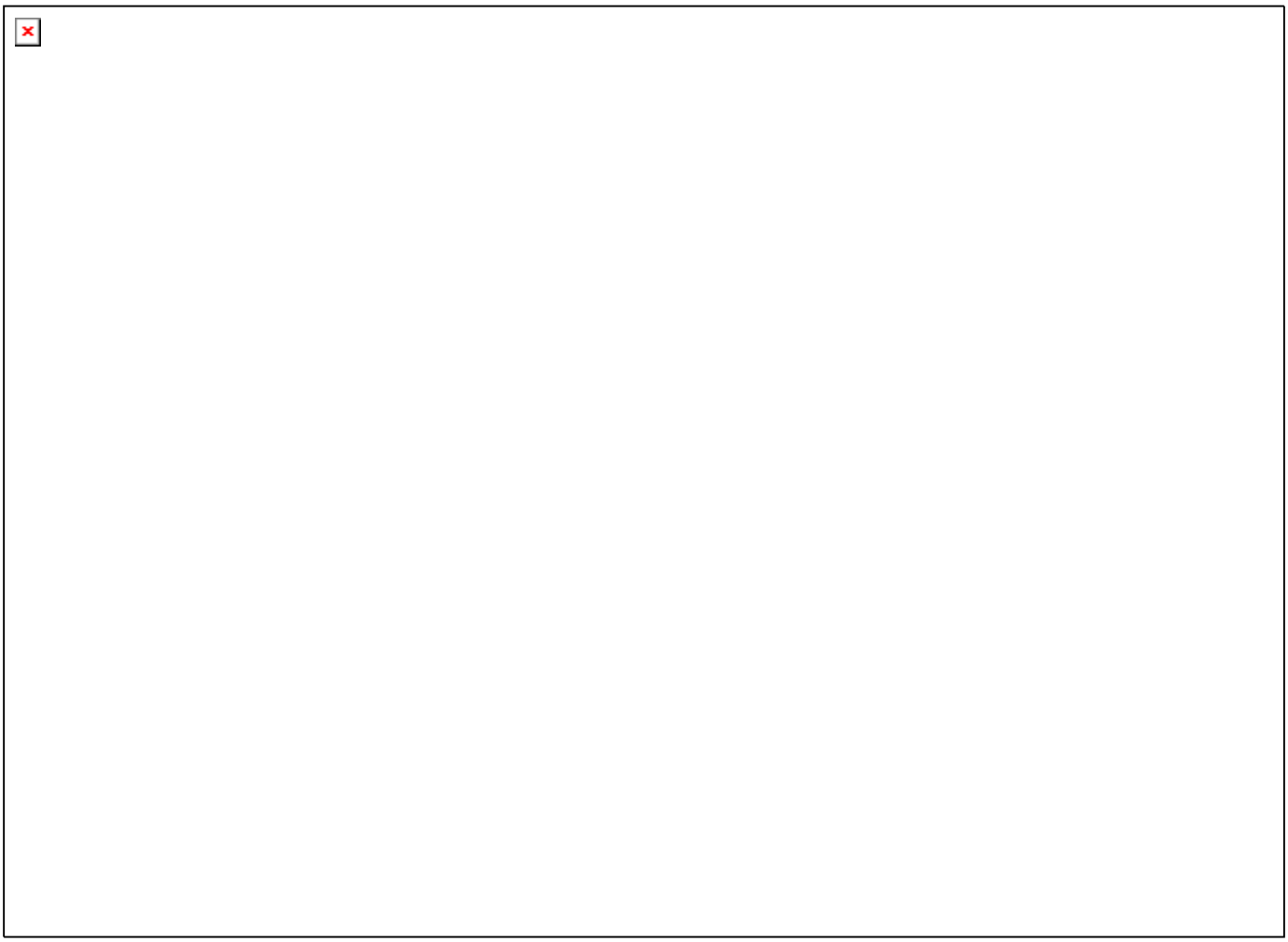


Figure 2

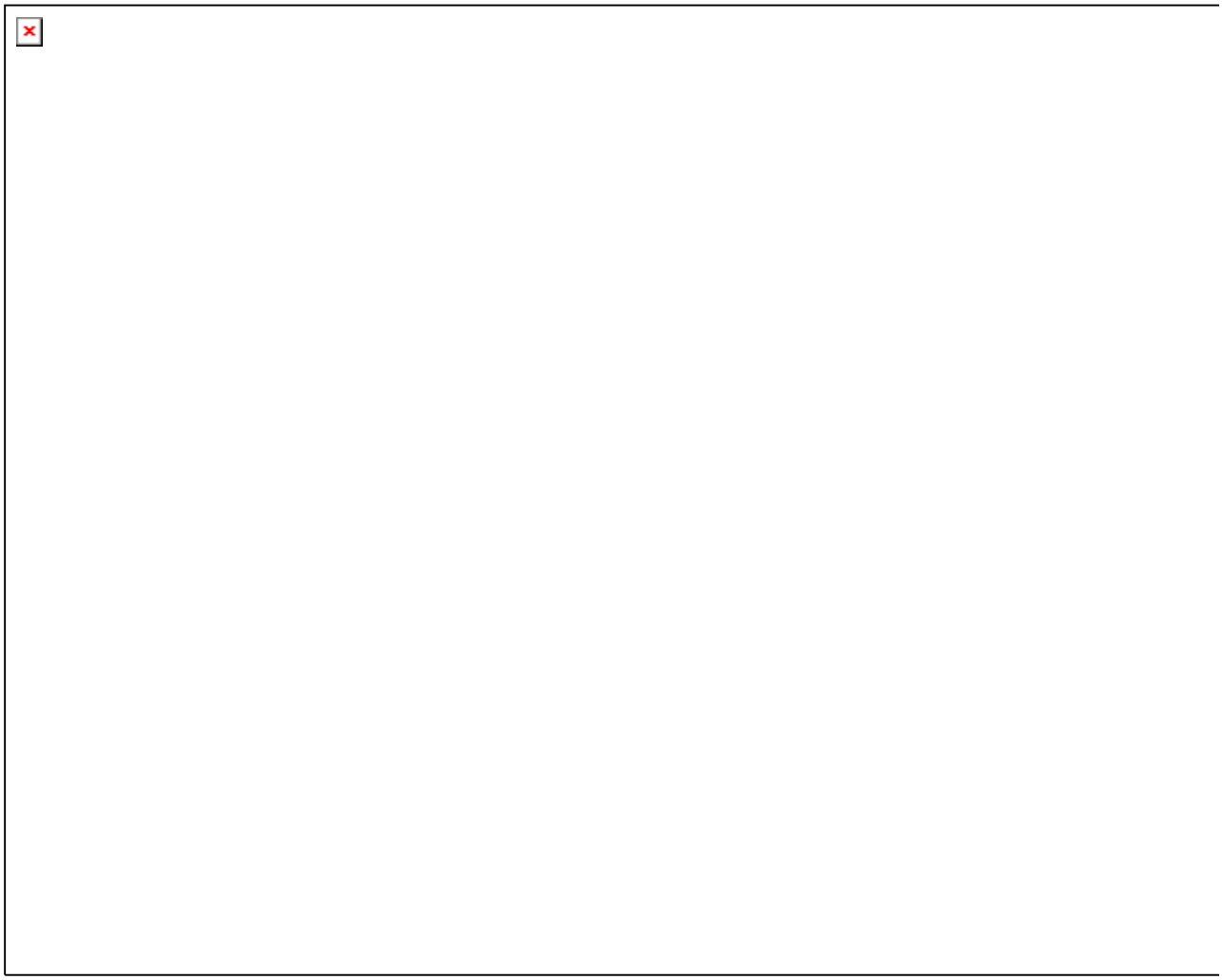


Figure 3

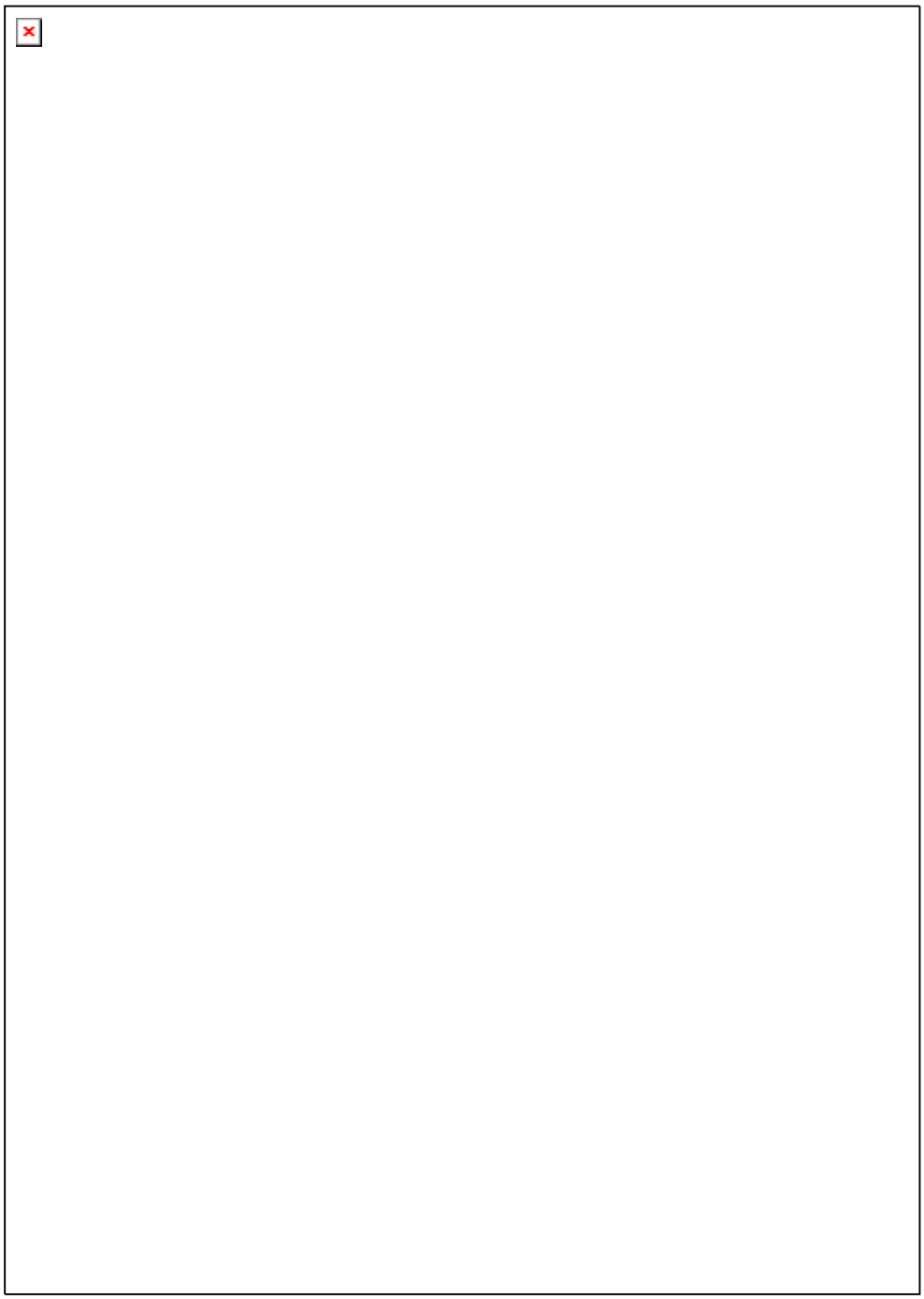
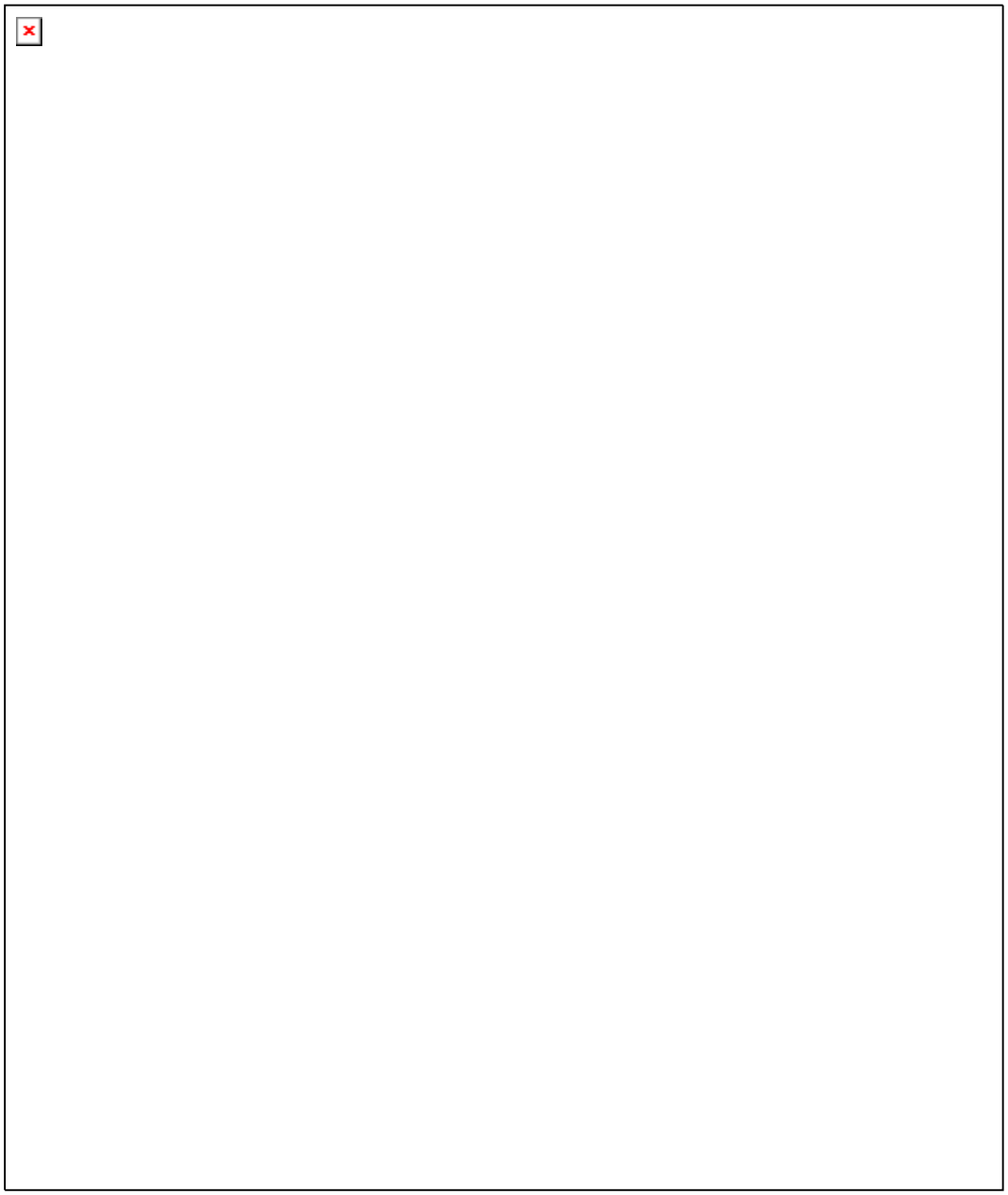


Figure 4



Scheme 1