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Deoxyguanosine Forms a Bis-adduct with *E,E*-Muconaldehyde, an Oxidative Metabolite of Benzene. Implications for the Carcinogenicity of Benzene

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Abstract

Benzene is employed in large quantities in the chemical industry and is a ubiquitous contaminant in the environment. There is strong epidemiological evidence that benzene exposure induces hematopoietic malignancies, especially acute myeloid leukemia, in humans but the chemical mechanisms remain obscure. *E,E*-Muconaldehyde is one of the products of metabolic oxidation of benzene. This paper explores the proposition that *E,E*-muconaldehyde is capable of forming Gua-Gua cross-links. If formed in DNA, the replication and repair of such cross-links might introduce structural defects that could be the origin of the carcinogenicity. We have investigated the reaction of *E,E*-muconaldehyde with dGuo and found the reaction yields two pairs of interconverting diastereomers of a novel heptacyclic bis-adduct having a spiro ring system linking the two Gua residues. The structures of the four diastereomers have been established by NMR spectroscopy and their absolute configurations by comparison of CD spectra with those of model compounds having known configurations. The final two steps in formation of the bis-nucleoside (5-ring → 6-ring → 7-ring) have significant reversibility, which is the basis for the observed epimerization. The 6-ring precursor was trapped from the equilibrating mixture by reduction with NaBH₄. The *anti* relationship of the two Gua residues in the heptacyclic bis-adduct precludes it from being formed in B DNA but the 6-ring precursor could readily be accommodated as an interchain or intrachain cross-link. It should be possible to form similar cross-links of dCyt, dAdo, the ε-amino group of lysine, and *N*-termini of peptides with the dGuo-muconaldehyde monoadduct.

Keywords

benzene; *E,E*-muconaldehyde; dGuo adduct; cross-link; leukemia

1. INTRODUCTION

Benzene is widely dispersed in the environment. It is a component of petroleum and is used extensively in the chemical industry as a solvent and as a building block in the synthesis of more complex organic compounds. It is also a product of incomplete combustion of fossil fuels and organic materials including tobacco. Benzene is a human carcinogen for which epidemiological studies have shown strong correlation of benzene exposure with induction

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ASSOCIATED CONTENT

Supporting Information Available. Listing of HPLC methods; NMR spectra of **1a** and **b**, **2ac** and **2bd**, and **4a–d**; listings of the chemical shifts of **1a–d**, **2ac** and **2bd**, and **4a–d**; HPLC traces of mixtures of **1a–d**, **2ac** and **2bd**, **3ac** and **3bd**, and **4a–d**; UV spectra of **1a–d**, **2ac** and **2bd**, **3ac** and **3bd**, and **4a–d**; CD spectra of **1a**, **1b** and **1cd**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

of hematopoietic malignancies, especially acute myeloid leukemia.^{1–4} No rodent model exists, although feeding studies with rodents have demonstrated induction of other types of tumors. On the basis of these observations, OSHA has restricted workplace exposure to levels less than 1 ppm. Nevertheless, there is evidence that even the 1-ppm threshold may be too high.^{5–7}

The mechanistic basis for the genotoxicity of benzene has been investigated extensively but remains elusive.^{1,8,9} Many mechanisms have been proposed, all initiated by P450 oxidation to form benzene oxide. P450 2E1 has been shown by Valentine et al., via the use of transgenic P450 2E1 knockout mice, to be the major species involved in the metabolism of benzene.¹⁰ Both metabolism and toxicity were greatly reduced in the knockout mouse versus the wild-type. Residual activity in the knockout mouse was attributed to P450 2B1 for which benzene is not a particularly good substrate. Benzene oxide can undergo a variety of transformations including non-enzymatic rearrangement to phenol (NIH shift) and equilibration with oxepin, its valence tautomer. Phenol upon further oxidation yields hydroquinone, catechol, and their quinones. Redox cycling of the hydroquinone-benzoquinone couple will produce hydroxyl radicals, which can cause free radical and oxidative damage to DNA. Benzoquinone can also form adducts with DNA¹¹ and with topoisomerase II.¹² All of these processes may contribute to the carcinogenicity of benzene although animal and epidemiological studies provide no support for phenol being a carcinogen. Furthermore, blood and tissues of animals *not* exposed to benzene contain significant amounts of hydroquinone derived from dietary sources.¹³ Studies on protein adducts of quinone metabolites in the blood and bone marrow of rodents exposed to low doses of ¹⁴C/¹³C-benzene showed that labeled adduct levels were insignificant in comparison with those arising from dietary sources and thus unlikely to increase the risk of leukemia.^{14,15} It should be noted in passing that hydroquinone and other hydroxylated benzenes that arise metabolically from phenol are structurally similar to the flavonoids in fruits and vegetables that are generally regarded as being beneficial antioxidants rather than carcinogens. In summary, although many workers have concluded that hydroquinone, catechol and/or their quinones are the primary initiators of acute myeloid leukemia,¹ there are significant problems with this scenario. In particular, why should benzene be genotoxic at low levels when background levels of the phenolic metabolites are high?

The possibility exists that some metabolite unique to benzene, perhaps formed in only small amounts, plays the critical role in the genotoxicity of benzene. Witz and Goldstein have suggested that muconaldehyde is the causative factor.^{16–19} Muconaldehyde is readily formed from benzene oxide/oxepin by oxidation with a variety of mild oxidants as first shown by Davies and Whitham in 1977.²⁰ It has been proposed that the *in vivo* oxidizing agent is a cytochrome monooxygenase although the precise one has not been identified.²¹ Oxidation of oxepin gives the 2,3-epoxide which spontaneously isomerizes to *Z,Z*- and then *E,Z*-muconaldehyde (Scheme 1). In a reaction catalyzed by amines or glutathione,²² *E,Z*-muconaldehyde ultimately isomerizes to the *E,E* form.²² Whereas there are many natural sources of hydroxylated phenols and their quinones, metabolism of benzene is the only known source of *E,E*-muconaldehyde. *E,E*-muconaldehyde has been detected in liver microsomes incubated with ¹⁴C-benzene.²³

E,E-Muconic acid was first shown to be a urinary metabolite of benzene by Parke and Williams in 1953.²⁴ Subsequently it has been found as a metabolite formed from *E,E*-muconaldehyde²⁵ but not from phenol, catechol or hydroquinone.²⁶ The *E,E* isomer is the only one to be detected in biological systems^{7,24} and has been used as a biomarker for benzene exposure, but a caveat is that it is not uniquely derived from benzene, being formed as well by enzymatic oxidation of sorbic acid, which is a common food preservative.²⁷

It has been argued that the muconaldehydes are too reactive to be able to make their way from the liver to the bone marrow, which is the site of malignancies in acute myeloid leukemia.³ Benzene oxide on the other hand is sufficiently stable that it has been directly identified in the blood of rats administered benzene.²⁸ The lifetime of benzene oxide would appear to be sufficient to permit *in vivo* transport from the liver to other organs via the bloodstream.²⁹ Turteltaub and Mani have postulated that a cytochrome P450 in bone marrow could perform the epoxidation of oxepin; analyses of marrow, liver and blood of rodents exposed to low levels of ¹³C₆-benzene revealed a low capacity, high affinity process in the marrow leading to levels of muconaldehydes (analyzed as *E,E*-muconic acid) in excess of hydroquinone.³⁰ Henderson et al. concluded from rodent studies of oral and inhalation exposure that high doses favor a low affinity, high capacity detoxification pathway, whereas low dose administration leads to toxic metabolites.^{31,32} This observation has been confirmed by other groups.^{25,33–37}

The high cytotoxicity of *E,E*-muconaldehyde complicates evaluation of its mutagenicity and genotoxicity. It is weakly mutagenic in bacterial systems but more mutagenic in V79 Chinese hamster lung fibroblast cells.^{38,39} The primary genetic damage associated with leukemia in benzene-exposed people and various tumors in benzene-exposed rodents involves large-scale damage, including micronuclei, chromosomal rearrangements and aneuploidy.^{3,40,41} Few simple mutations are observed. One possible explanation for this is that the relevant genetic damage involves cross-links. The muconaldehydes, by virtue of their multiple electrophilic sites, should be capable of forming DNA-DNA and DNA-protein cross-links. Both types of cross-links could be the origin of structural alterations observed in the genomes of malignant cells. Indeed, Nakayama et al. in a study in human cells using supF shuttle vectors treated with hydroquinone or muconaldehyde found the *E,E*-muconaldehyde mutational spectrum was unique in its high proportion of tandem mutations, particularly at GG sites.⁴² Witz and coworkers have found evidence for *E,E*-muconaldehyde-mediated cross-links between DNA and proteins. They speculated that these cross-links may be the basis of the genotoxicity of benzene.^{19,43,44} Such cross-links might be involved in the inhibition of human topoisomerase II by *E,E*-muconaldehyde reported by Frantz et al.⁴⁵

Examination of the literature reveals little information about the reaction of *E,E*-muconaldehyde with the constituents of nucleic acids or proteins. Golding and coworkers have studied the chemistry of muconaldehyde with nucleophiles. Their work has focused mainly on the *Z,Z* isomer.^{21,46} A key finding was that reaction of the *Z,Z* isomer with Gua, Ade and their nucleosides under a variety of conditions including 0.1 M pH 7.0 buffer gave pyrrole-type monoadducts; *Z,Z*-muconaldehyde adduction of Guo is shown in Scheme 2. These authors did not report any studies of the reaction of *E,E*-muconaldehyde with nucleosides; they did observe, however, that *E,E*-muconaldehyde yielded a bis-imine upon reaction with 1-propylamine⁴⁶ in contrast to the pyrrole obtained with *Z,Z*-muconaldehyde. The trans double bonds in *E,E*-muconaldehyde promote formation of bis-adducts by protecting against formation of pyrrole-type monoadducts. Golding and his colleagues have also studied the reaction of the muconaldehydes with glutathione.²² The *E,E* isomer reacts with GSH and other thiols to give both mono- and bis-adducts with the latter forming more slowly. The authors propose that the reversibility of GSH addition to the muconaldehydes could allow GSH to act as a transporter of *E,E*-muconaldehyde in biological systems. Latriano et al. demonstrated that the reaction of *E,E*-muconaldehyde with ¹⁴C-labeled dGuo-5'-phosphate in phosphate buffer, pH 7.4 yielded several radioactive products of unknown structure.¹⁷

In recent years we and others have carried out extensive studies of the reactions of simple enals, i.e., acrolein, crotonaldehyde and 4-hydroxynonenal, with nucleosides and DNA.^{47,48}

These enals are bis-electrophiles, being able to form Michael adducts by 1,4-addition and carbinolamines and/or imines by 1,2-addition of nucleophiles. They undergo two-fold reactions with DNA to form cyclic adducts of the bases plus intra- and interchain cross-links. They also form cross-links of DNA with peptides and proteins. The cross-links are less damaging than one might initially expect because they exist primarily as carbinolamines that readily dissociate.

The muconaldehydes have the potential to form bis-1,4-adducts with amines. Such bis-adducts are known to be less reversible^{49–51} and, as a consequence, could be expected to be much more damaging to the genome. For this reason we decided to follow up on Golding's studies and examine the reactions of nucleosides and nucleic acids with muconaldehydes, concentrating on the *E,E* isomer. Our hypothesis of bis-1,4-adducts was, in fact, naïve but in the course of these studies a more complex reaction process was discovered that leads to stable bis-adducts. This paper describes the reaction of dGuo with *E,E*-muconaldehyde to form novel heptacyclic bisnucleosides **1** (Figure 1). It is noteworthy that the condensation produces 4 new stereogenic centers and could potentially yield 16 diastereomers but structural and mechanistic constraints limited the number to four.

The preferred IUPAC name and numbering system for the bis-base is 6,8,10,12,15,17,19,21,23,26-decaazaheptacyclo[14.10.0.0^{1,5}.0^{7,15}.0^{9,13}.0^{18,26}.0^{20,24}]hexacosa-7,9(13),11,18,20(24),22-hexaene-14,25-dione. In the present paper, rather than using this cumbersome numbering system, purine numbering will be used for the eastern (E) and western (W) Gua moieties and the *E,E*-muconaldehyde-derived fragment (M) will be numbered as shown in Figure 1. Subsequently in the paper *E,E*-muconaldehyde will simply be called muconaldehyde.

2. EXPERIMENTAL SECTION

2.1. Materials

Muconaldehyde was prepared from *cis*-1,2-dihydrocatechol (Aldrich) by the method of Golding et al.⁵² and stored at –20 °C. 2'-Deoxyguanosine (Aldrich) was obtained and used as the monohydrate.

2.2. Chromatography

Initial product isolation was carried out by solid phase extraction (SPE) on reverse phase cartridges (Strata-X, 33 µm, Phenomenex) with elution by water-methanol mixtures. HPLC experiments were performed on a Beckman Coulter system comprising a System Gold 126 solvent module and System Gold 168 Diode Array Detector; Beckman Coulter 32 Karat v. 7.0 software was used for the acquisition, processing, and analysis of HPLC data. The diode array detector was configured to simultaneously monitor 254 and 360 nm wavelengths. Analysis and purification were performed using YMC ODS-AQ C-18 columns (250 mm × 4.6 mm i.d. for analysis and 250 mm × 10 mm i.d. for purification). The mobile phase consisted of water and either acetonitrile or methanol. Methods are described in the Supporting Information.

2.3. NMR and CD Spectra

NMR spectra were recorded in anhydrous DMSO-*d*₆ and/or DMSO-*d*₆/D₂O (~90:10, v/v) at 25 °C and referenced to residual DMSO-*d*₅ using 3 mm tubes on a 600 MHz Bruker spectrometer equipped with a cryoprobe. Small differences in chemical shifts of the non-exchangeable protons were observed between the two solvents. ¹³C chemical shift assignments (150 MHz) were derived from HSQC and HMBC spectra. Tabulations of ¹H and ¹³C chemical shifts of **1a–d**, **2ac** and **2bd**, and **4a–d** are in the Supporting Information.

NOE measurements were made with the Bruker selective NOE program SELNOGP using solutions in DMSO-*d*₆ containing 8% D₂O at 25 °C. Under these conditions, the observed NOEs were negative, i.e., they had the same phase as the signal at the irradiation point.

CD spectra were obtained on a JASCO 720 spectropolarimeter at ambient temperature. Samples were dissolved in H₂O-methanol (1:1) to give an absorbance at 260 nm of 0.50–0.55. CD spectra were normalized to absorbance at 260 nm rather than molar absorbance because the absorbance of the compounds does not follow Beer's Law; the molar extinction coefficient decreases with concentration indicating aggregation or stacking in the solvent mixture employed.

2.4. Reactions of Muconaldehyde with dGuo

A variety of reaction conditions were explored (see Results and Discussion) almost all of which led to the same mixture of products in yields ranging from 10–40%; the highest yields were obtained by using a 10:1 molar ratio of dGuo to muconaldehyde. Sonication of freshly mixed reaction mixtures for 10 min at room temperature also improved yields. A typical analytical-scale reaction was carried out with a mixture of 10 µmoles of dGuo and 5 µmoles of muconaldehyde in 2 mL of 50 mM phosphate buffer, pH 7.4, at room temperature or 37 °C. The course of the reaction was followed by HPLC (Method 1). Similarly, a typical preparative reaction employed 50 mg (0.175 mmol) of dGuo and 4.82 mg (0.044 mmol) of muconaldehyde in 30 mL of 50 mM phosphate buffer, pH 7.4. The reaction was sonicated for 10 minutes and allowed to stand at room temperature in the dark for 2 months. One-half of the reaction was further purified and the remainder frozen for future use. Crude purification was accomplished by applying 7.5 mL of the reaction mixture to each of two SPE cartridges (200 mg, prepared and eluted according to the manufacturer's directions). Elution was carried out with methanol-water mixtures. dGuo eluted in the 0% and 5% methanol fractions. Compounds **1a–d** eluted in the 15–25% methanol fractions. The fractions were analyzed by UV (260 nm) and HPLC (Method 2). The fractions containing **1a–d** were combined and lyophilized after removal of most of the methanol by vacuum centrifugation to give 4.67 mg (34%) of crude **1a–d**.

Further purification was performed by preparative HPLC (Method 2). dG-muc-dG isomers **1a** and **1b** could be obtained in >90% purity by repeated chromatography under the same conditions but isomers **1c** and **1d** could not be completely separated. Purified material for UV and mass spectroscopy of **1c** was obtained by collecting only the leading edge of the peak; purified **1d** was obtained from HPLC separation of an equilibrated sample of **1a** (see below). **1a:** Retention time 11.95 min; UV (H₂O-CH₃OH) λ_{max} 256 nm, 285 nm (sh); HRMS (ES+) calcd for C₂₆H₃₀N₁₀O₉ (M + Na⁺) 649.2095; found 649.2098. **1b:** Retention time 12.40 min; UV (H₂O-CH₃OH) λ_{max} 256 nm, 285 nm (sh); HRMS (ES+) calcd for C₂₆H₃₀N₁₀O₉ (M + Na⁺) 649.2095; found 649.2072. **1c:** Retention time 12.78 min; UV (H₂O-CH₃OH) λ_{max} 256 nm, 285 nm (sh); HRMS (ES+) calcd for C₂₆H₃₀N₁₀O₉ (M + Na⁺) 649.2095; found 649.2076. **1d:** Retention time 13.02 min; UV (H₂O-CH₃OH) λ_{max} 256 nm, 285 nm (sh); HRMS (ES+) calcd for C₂₆H₃₀N₁₀O₉ (M + Na⁺) 649.2095; found 649.2100.

2.5. Depurination of dG-muc-dG Diastereomers **1a–d**

Depurinations were performed by heating lyophilized samples of **1a–d** in 0.1 M HCl at 70 °C for 1 hr followed by neutralization with 0.1 M NaOH. Analytical Scale: Reactions were followed by HPLC (Method 3). Preparative Scale: A crude mixture of dG-muc-dG diastereomers (59 A₂₆₀ units) isolated with an SPE cartridge was depurinated in 1.0 mL of 0.1 M HCl as above and purified by preparative HPLC (Method 4) to give bis-bases **2ac** and **2bd**. **2ac:** Retention time 17.00 min; UV (H₂O-CH₃OH) λ_{max} 252 nm, 280 (sh); HRMS (ES+) calcd for C₁₆H₁₅N₁₀O₃ (M + H⁺) 395.1329; found 395.1331. **2bd:** Retention time 18.10

min; UV ($\text{H}_2\text{O}-\text{CH}_3\text{OH}$) λ_{max} 252 nm, 280 (sh); HRMS (ES+) calcd for $\text{C}_{16}\text{H}_{15}\text{N}_{10}\text{O}_3$ ($\text{M} + \text{H}^+$) 395.1329; found 395.1336.

2.6. Reduction of G-muc-G Diastereomers 2ac and 2bd

To a solution of **2ac** in 0.5 mL of water-methanol (1:1) was added excess solid sodium borohydride. After standing for 12 h at ambient temperature, the reaction was neutralized with 5% acetic acid and an aliquot was analyzed by HPLC (Method 6); a single peak at 12.43 min was observed for **3ac**. G-muc-G **2bd** was treated similarly to give **3bd** as a single peak at 13.35 min. The remainder of each sample was purified by preparative HPLC (Method 4). **3ac**: UV ($\text{H}_2\text{O}-\text{CH}_3\text{OH}$) λ_{max} 246 nm, 275 (sh). HRMS (ES+): calcd for $\text{C}_{16}\text{H}_{17}\text{N}_{10}\text{O}_3$ ($\text{M} + \text{H}^+$) 397.1479; found 397.1488. **3bd**: UV ($\text{H}_2\text{O}-\text{CH}_3\text{OH}$) λ_{max} 243 nm, 278 (sh). HRMS (ES+): calcd for $\text{C}_{16}\text{H}_{15}\text{N}_{10}\text{O}_3$ ($\text{M} + \text{H}^+$) 397.1479; found 397.1486.

2.7. Reduction of a crude mixture of dG-muc-dG isomers

Excess solid sodium borohydride (0.77 mg, 0.020 mmol) was added to a solution of crude dG-muc-dGs **1a–d** (SPE purified, 59 A₂₆₀ units) in 3.0 mL of water-methanol (1:1). The mixture was stirred at ambient temperature for 10 h with monitoring by HPLC (Method 5), neutralized with 0.2 M HCl and purified by preparative HPLC (Method 2) to give four diastereomeric products: **4c**: Retention time 10.50 min; UV ($\text{H}_2\text{O}-\text{CH}_3\text{OH}$) λ_{max} 252 nm, 274 nm (sh); HRMS (ES+) calcd for $\text{C}_{26}\text{H}_{33}\text{N}_{10}\text{O}_9$ ($\text{M} + \text{H}^+$) 629.2426; found 629.2435. **4d**: Retention time 11.17 min; UV ($\text{H}_2\text{O}-\text{CH}_3\text{OH}$) λ_{max} 252 nm, 275 nm (sh); HRMS (ES+) calcd for $\text{C}_{26}\text{H}_{33}\text{N}_{10}\text{O}_9$ ($\text{M} + \text{H}^+$) 629.2426; found 629.2435. **4a**: Retention time 12.20 min; UV ($\text{H}_2\text{O}-\text{CH}_3\text{OH}$) λ_{max} 252 nm, 276 nm (sh); HRMS (ES+) calcd for $\text{C}_{26}\text{H}_{33}\text{N}_{10}\text{O}_9$ ($\text{M} + \text{H}^+$) 629.2426; found 629.2434. **4b**: Retention time 13.32 min; UV ($\text{H}_2\text{O}-\text{CH}_3\text{OH}$) λ_{max} 252 nm, 274 nm (sh); HRMS (ES+) calcd for $\text{C}_{26}\text{H}_{33}\text{N}_{10}\text{O}_9$ ($\text{M} + \text{H}^+$) 629.2426; found 629.2427. Individual isomers **1a–d** were reduced on a small scale to confirm the correlation between reduced and unreduced species.

2.8. Effects of pH on the Reaction of dGuo with Muconaldehyde

A stock solution was prepared containing dGuo (10 mg, 0.035 mmol) and muconaldehyde (1.91 mg, 0.017 mmol) in 6 mL of water. The solution was distributed equally among 3 vials and lyophilized. To each vial was added 2 mL of 50 mM potassium phosphate buffer at a different pH values: 6.5, 7.5, and 8.5. The mixtures were stirred at ambient temperature for several days. At intervals, 100 μL aliquots were removed, filtered and analyzed by HPLC (Method 7).

2.9. Equilibration of dG-muc-dG Diastereomers 1a and 1b

Aliquots were removed from solutions of **1a** and **1b** that had been standing in DMSO-*d*₆/D₂O (~90:10, v/v) at ambient temperature for 5 months. The samples were lyophilized and then redissolved in 1.0 mL of potassium phosphate buffer (pH 7.4, 50 mM) and examined by HPLC (Method 8). Aliquots of the aqueous solutions were removed and analyzed periodically over 4 months at which point equilibration appeared essentially complete.

3. RESULTS

3.1. Reaction of dGuo with Muconaldehyde

As a first approach we explored the reaction under aqueous conditions near neutral pH, this being a reasonable approximation of biological milieu. The initial reaction was carried out with equimolar dGuo and muconaldehyde in phosphate buffer (50 mM, pH 7.4) at room temperature. The reactions were monitored by HPLC. Within a few hours several small peaks appeared and additional peaks appeared over the course of several days, several of

which had UV spectra similar to those reported by Latriano et al.¹⁷ ESI-MS of the early mixtures showed the presence of species containing dGuo and muconaldehyde in a 1:1 ratio with and without loss of water; 2:1 species from which 0, 1 and 2 water molecules had been lost were also observed. An interpretation of these results is presented in the Discussion section. In addition, colored, late-eluting species were observed for which the mass spectra were consistent with two muconaldehyde molecules having reacted with one dGuo; these were more prominent when excess muconaldehyde was present.

Our attention quickly turned to exploring reactions in which dGuo was in excess because the products were more likely to have biological relevance. In spite of the mass spectra of these product mixtures being somewhat promising, the complexity of the mixtures held poor prospects for our being able to obtain significant yields of any adducts. As these experiments were underway we reexamined our original reaction mixture after it had been stored at room temperature for 7 weeks and found that the appearance of the chromatogram was much improved. Muconaldehyde had been depleted although dGuo was still present. Many of the initially formed products were diminished or had disappeared entirely. Four main products (**1a–d**) had appeared, which had not been visible in the early chromatograms (Figure 2). They eluted in a group much later than dGuo and muconaldehyde. Products **1a** and **1b** were well resolved but **1c** co-eluted with **1d** on most reverse-phase HPLC columns. Peaks for **1a** and **1c** were of similar intensity and about half again larger than peaks **1b** and **1d**. Molar ratios of dGuo:muconaldehyde varying from 10:1 to 1:2 gave similar results with chromatograms obtained shortly after the start of the experiments showing many peaks, the intensity of which varied somewhat depending on the starting ratio of dGuo and muconaldehyde. Reactions carried out at 37 °C were essentially complete after 4 days; in all cases the final chromatograms were dominated by the four product peaks. With a 10:1 ratio of dGuo to muconaldehyde, the combined yield of the four products approached 40%. In the absence of dGuo *E,E*-muconaldehyde itself is quite stable under the reaction conditions (pH 7.4, room temperature or 37 °C) diminishing < 5% over 4 days; over several months at room temperature, however, it gradually disappears without the appearance of any new UV-absorbing peaks. We conclude that most of the chromatographic peaks arising within hours/days of the start of the reactions with dGuo represent transient species that ultimately give the final bis-nucleosides.

The effect of pH was examined. Reactions were carried out at pH 6.5, 7.5 and 8.5 in 50 mM phosphate buffer at ambient temperature with a 2:1 molar ratio of dGuo to muconaldehyde. The rate of disappearance of muconaldehyde was pH dependent. At pH 8.5, muconaldehyde was depleted within 24 h and after 72 h compounds **1a–d** were the dominant species, whereas they were barely detectable after 72 h at pH 6.5 (Figure 3). Eventually all of the reactions reached the mixture of the four products shown in Figure 2. Based on the HPLC analyses, the yield of **1a–d** was marginally better at pH 7.5.

3.2. Isolation of Bis-nucleosides **1a–d**

For preparation of sufficient material for structural identification, reactions were run in phosphate buffer, pH 7.4, with a dGuo:muconaldehyde ratio of 4:1. A rough separation of the product mixture was made on SPE cartridges using H₂O/methanol with the mixture of **1a–d** eluting in the 15–25% methanol fraction in a combined yield of 30–40%. The LC-ESI mass spectrum (positive-ion) of the **1a–d** mixture gave an [M+H]⁺ signal at *m/z* 627 followed by sequential neutral losses of two deoxyribose fragments (*m/z* 627 - 116 → 511 and 511 - 116 → 395). The negative ion spectrum gave an [M-H]⁻ signal at *m/z* 625.

The mixture was partitioned by preparative HPLC. Compounds **1a** and **1b** were readily isolated in pure form and smaller amounts of **1c** could be obtained in pure form by

sacrificing the trailing side of the peak. Useful quantities of **1d** could not be isolated even by repeated chromatography but eventually small samples of **1d** were obtained by equilibration of **1a** (see below). The quantity of **1d** that could practically be obtained in this manner was only sufficient for UV and MS. Exact mass determinations on the four species were in accord with the empirical formula C₂₆H₃₀N₁₀O₉ (MW 626), establishing that they were, in fact, isomers resulting from the reaction of muconaldehyde with two dGuo residues with loss of one H₂O. They had indistinguishable UV spectra, which were similar to that of dGuo.

During the course of NMR studies (see below), it was discovered that, on long standing at ambient temperature, NMR samples of **1a** and **1b** in DMSO-*d*₆/D₂O became contaminated with **1d** and **1c**, respectively. HPLC examination of samples that had been standing for 5 months revealed that **1a** had become an 89:11 mixture of **1a** and **1d** and **1b** had become a 67:33 mixture of **1b** and **1c**. Equilibration of the isomers was faster in aqueous buffer, pH 7.4. After several weeks at room temperature, **1a** had become a 61:39 mixture of **1a** and **1d**; **1b** had become a 47:53 mixture of **1b** and **1c**.

By careful comparison of ¹H spectra of aged samples of **1a** and **1b** (2–4 months old) with spectra obtained when the samples were fresh, it was possible to deduce the spectra of **1c** and **1d**. No interconversion of **1a** with **1b** and **1c** or of **1b** with **1a** and **1d** was detected either by NMR or HPLC. From these observations we infer that the nucleosides **1a** and **1c** are enantiomeric with respect to their bis-base components and likewise **1b** and **1d**.

3.3. Structural Characterization of the Bis-Nucleosides **1a–d**

Attention was focused on the more readily available bis-nucleosides **1a** and **1b**. Solutions in DMSO-*d*₆/D₂O gave high quality spectra. The spectra showed nine signals for non-exchangeable protons in the bis-bases plus an additional 14 derived from the pair of deoxyribose residues. Of the signals ascribed to the bis-bases, three were singlets and the other six multiplets were in a coupled network. Two downfield singlets (7.93 and 8.00 ppm in **1a** and 7.95 and 7.99 ppm in **1b**) were assigned on the basis of chemical shift to the protons derived from H8 of the constituent Gua moieties showing that they were in non-equivalent environments. The remaining singlet, 6.53 ppm in **1a** and 6.21 ppm in **1b**, had to be in the fragment derived from muconaldehyde. The coupled networks were assigned using COSY and HSQC spectra as CH₂-CH-CH₂-CH spin systems (Figure 4). Isomers **1a** and **1b** were distinguished by the fact that in **1a** the geminal protons in each methylene group were separated by slightly more than 1 ppm whereas in **1b** the separation was less than 0.2 ppm. Detailed analysis of the ¹H spectra of the bis-nucleosides was complicated by the fact that solvent and deoxyribose signals overlapped some of the CH₂-CH-CH₂-CH signals in the 1-D spectra but the location of the obscured signals could be deduced from COSY and HSQC spectra. The chemical shifts of protons on the muconaldehyde fragment of **1c** and **1d** were assigned using a mixture of **1c** and **1d** along with aged samples of **1a** and **1b** in which varying amounts of isomerization had occurred to **1d** and **1c**, respectively. Assignment of signals for **1c** and **1d** was facilitated by the fact that the ¹H spectra of **1a** and **1c** and those of **1b** and **1d** differed only minutely from each other ($\Delta\delta$ values <0.1 ppm). It can be inferred that **1a–d** are configurational not structural isomers with the bis-base portions of **1a** and **1c** and of **1b** and **1d** being antipodal.

The NMR spectra of **1a–d** obtained in anhydrous DMSO-*d*₆ were not analyzed due to the complexity resulting from the presence of two non-equivalent deoxyribose spin systems. Instead, attention was turned to the bis-bases, which retained the spin system derived from the muconaldehyde moiety and promised to yield spectra that would be easier to interpret.

3.4. Bis-Bases **2ac** and **2bd**

Deglycosylation of a mixture of bis-nucleosides **1a–d** gave a 3:2 ratio of two well-separated HPLC peaks for mixtures of **2a** with **2c** and of **2b** with **2d** (Scheme 3). These mixtures are designated as **2ac** and **2bd**. Deglycosylation of the individual bis-nucleosides **1a** and **1c** gave the enantiomeric bis-bases **2a** and **2c**, respectively, which eluted at the position of the first peak. Likewise **1b** and **1d** gave **2b** and **2d**, which eluted at the position of the second peak. This confirmed the previous conclusion as to the stereochemical relationships among **1a–d**. As with the bis-nucleosides, samples of each enantiomeric mixture gradually became contaminated with the other one showing that **2a** was slowly equilibrating with **2d** and **2b** with **2c**.

NMR spectra of **2ac** and **2bd** were acquired in anhydrous DMSO-*d*₆. The spectra closely resembled those of the nucleosides but the absence of deoxyribose moieties provided considerable simplification. Five ¹H signals arising from OH or NH groups were well separated from the CH signals and were identified by exchange with D₂O. The CH₂-CH-CH₂-CH spin system, which had been the hallmark of the bis-nucleosides, was still present and closely resembled the pattern in the bis-nucleosides with the exception that the methines of the spin system were each coupled to exchangeable protons (Figure 5).

The central methine proton was coupled to a signal near 5 ppm, which can be assigned as a hydroxyl group on the basis of chemical shift. The terminal methine proton was coupled to a signal near 7.5 ppm which, can similarly be assigned as an NH group. Three additional exchangeable signals appearing below 7 ppm can be assigned as NH protons; they showed no coupling to other protons. Two of these protons appeared as a very broad signal at ~12.7 ppm and can be assigned to the N9 protons of the Gua residues. The remaining exchangeable proton was a singlet near 8.7 ppm.

The structural information gleaned from the ¹H spectra indicated that four of the six muconaldehyde carbons are present in the CH₂-CH-CH₂-CH fragment which is separated from the singlet methine by a tetrasubstituted carbon. It can be deduced that the four-carbon fragment and the tetrasubstituted carbon comprise a five-membered ring since (1) the mutually coupled terminal methylene protons are only coupled to the central methine and (2) the terminal methine is only coupled to the adjacent methylene group plus an exchangeable proton. The chemical shifts of the singlet at 6.52 ppm in **2ac** and 6.25 ppm in **2bd** suggest that these methine carbons are each linked to the tetrasubstituted carbon and to two nitrogen atoms.

HMBC and HSQC spectra permitted most of the carbon signals to be identified. The combined ¹³C and ¹H data made it possible to assign the heptacyclic structures shown in Figure 6. In spite of large differences between the ¹H spectra of the two isomers, the ¹³C spectra were virtually identical, providing corroboration that the two species were diastereomers not structural isomers. A pair of signals appearing at 135 and 140 ppm for **2ac** and 136 and 138 ppm for **2bd** can be assigned to C8 of the non-equivalent Gua moieties. The carbon signals associated with the muconaldehyde-derived isolated methine (M1, see Figure 1) and the CH₂-CH-CH₂-CH fragment (M3–M6) were identified using HSQC correlations. The quaternary carbon (M2) was assigned to signals at 72 ppm in both diastereomers on the basis of 2-bond couplings in the HMBC spectra.

With **2ac** a 3-bond HMBC correlation was observed between the M1 proton and C2 of Guaw. This correlation does not distinguish between regioisomers having M1 linked to N1 vs N² of Guaw but on mechanistic grounds (see Discussion Section) the muconaldehyde linkage to Guaw can be assigned as M2 to N1 and M1 to N² as shown in Figure 6. The environment of M1-H in **2ab** is further defined by 3-bond HMBC correlations with the M3

carbon at 43 ppm. Neither H-H nor C-H coupling was observed between M1-H and the 2-NH of Guaw, presumably due to NH exchange being too rapid. An HMBC correlation was observed between the M6 proton at 4.64 ppm and a carbon signal at 152 ppm, which is assigned to C2 of Gua_E. This correlation could have resulted from M6 being linked to either N1 or N² of Gua_E but the vicinal ¹H coupling between the M6 proton and the 2-NH of Gua_E observed in the COSY spectrum made it possible to assign the linkages of M6 to N² of Gua_E and, therefore, M1 to N1. The HMBC spectrum of **2bd** had lower signal to noise and failed to show correlations of the muconaldehyde fragment with either Gua_E or Guaw. However, as argued above, the two species (**2ac** and **2bd**) are diastereomers not structural isomers.

3.5. Relative Configurations of **2ac** and **2bd**

For the parent 5-6-5 angularly fused carbocycle equivalent to rings A, C, and B, respectively, of **2**, i.e., only derivatives of the cis-cis and trans-cis isomers have been reported^{53,54} and it is not known whether the cis-trans and trans-trans would be accessible. To examine the possibility that **2ac** and **2bd** were epimers at M1, i.e., cis and trans fusions of rings A and C, they were reduced with excess NaBH₄ (methanol-water (1:1), 16 h, room temperature) to give **3ac** and **3bd**, respectively, in which ring C had opened (Scheme 3). Diastereomers **3ac** and **3bd** were well separated from each other on reverse phase HPLC (See chromatogram on page S8 in the Supporting Information) and eluted in the same order as **2ac** and **2bd**; each had a mass 2 units higher than its parent and the UV spectra were identical. Both reductions would have given the same product if the structural difference between **2ac** and **2bd** had been the configuration of M1.

The ¹³C NMR spectra of **2ac** and **2bd** were virtually identical which also suggests that epimerization is not occurring at a ring junction. In view of the fact that there are four asymmetric sites, three of which (M1, M2 and M6) involve ring junctions, it points to the site of epimerization being the remaining one, i.e., M4. Thus, it is reasonable to conclude that the epimers differ in the orientation of the M4 hydroxyl group.

There are obvious differences between the ¹H spectra of **2ac** and **2bd**. As in the spectra of **1a** and **b**, the chemical shift differences between the geminal protons on M3 and M5 in **2bd** are much smaller than in **2ac** (Figures 6 and 7). The orientation of the M4 hydroxyl relative to the M6 amino group is the basis for this chemical shift difference in the protons on M5. Both functional groups shield proximal protons.⁵⁵⁻⁵⁷ **2ac** can be assigned as having M6-NH on the same face as the M4-OH on the basis of the large chemical shift difference (1.12 ppm) for the geminal pair of protons on M5 reflecting the fact that the 1.33-ppm proton is shielded by both M4-OH and M6-NH. In **2bd** the chemical shifts of the M5 geminal protons differ by only 0.20 ppm. Therefore the M4-OH and M6-NH can be assigned as being trans to each other because M4-OH shields one of the geminal protons and M6-NH the other. The average chemical shifts for the M5 protons are very similar for **2ac** and **2bd**, i.e., 1.89 and 1.79 ppm, respectively.

A similar analysis can be used to define the orientation of M4-OH relative to the carbonyl group of Guaw and thereby the relative configuration of the spiro carbon. The Guaw carbonyl group has a deshielding effect on proximal protons whereas M4-OH is shielding. The average chemical shifts for the M3 protons are 2.47 and 2.60 ppm, respectively, for **2ac** and **2bd**. The Guaw carbonyl group and M4-OH of **2ac** are on opposite faces of ring B leading to a large chemical shift difference (1.05 ppm) between the M3 geminal protons; they are on the same face in **2bd** where, to a large extent, they cancel each other out. Thus in **2ac**, M6-NH, the M5 proton at 1.33 ppm, M4-OH and the M3 proton at 1.96 ppm are on one face of ring B and the Guaw carbonyl group on the other. In **2bd**, M6-NH and M4-OH are on opposite faces of the ring. The difference in chemical shifts of the two protons on M3 is

too small to make relative assignments but the small chemical shift difference between the two protons on M3 is indicative that the shielding/deshielding effects cancel and the M4-OH and the Guaw carbonyl group are proximal to the same M3 proton. Consequently, in **2bd** the carbonyl group of Guaw is on the same face of ring B as M4-OH and opposite M6-NH. This analysis of the effect of the Guaw carbonyl group on chemical shifts of the M3 protons permits the relative configuration of spiro carbon M2 to be assigned as shown in Figure 7 leading to a cis assignment for the A-C ring fusion.

Corroborative evidence for this assignment was obtained from NOE experiments carried out on **2ac** and **2bd**. The spectra were acquired at 25 °C on solutions in DMSO-*d*₆ containing 8% D₂O; the results are summarized in Figure 8. With **2ac**, irradiation of the M1 proton (6.55 ppm) produced a strong NOE to M3-H_{1.94} and a weak one to M3-H_{3.01}. An NOE was also observed to M5-H_{1.34}. NOEs to M4-H and M6-H were not observed. On this basis, M1-H, M3-H_{1.94} and M5-H_{1.34} can be assigned to one face of the ring, i.e., the β face as depicted in Figure 8. This establishes that the A:C and B:C ring fusions of **2ac** are both cis. Irradiation of the M4 methine proton (4.40 ppm) produced NOEs to M3-H_{3.01} and both M5 protons with the NOE to M5-H_{1.34} being stronger than that to M5-H_{2.39}. An NOE was also observed to the M6 methine proton (4.64 ppm). Irradiation of the M6 methine proton (4.64 ppm) gave NOEs to M4-H and M5-H_{2.39}. Therefore, M4-H, M6-H, M3-H_{3.01} and M5-H_{2.39} can all be assigned to the α face of the molecule and the NOEs between M4-H and M6-H establish that M4-OH is β.

A similar analysis of the NOEs for **2bd** makes it possible to assign M1-H_{6.20}, M4-H, M3-H_{2.50} and M5-H_{1.72} to the β face of the molecule and M6-H_{5.00}, M3-H_{2.68}, and M5-H_{1.96} to the α face. It is of particular note that an NOE was observed between M1-H and M4-H establishes that the A:C and B:C ring fusions are both cis and the M4-OH is on the α face of the molecule.

In support of the A-cis-C-cis-B assignment of ring fusions, Ihara et al. have synthesized pentalenene, a trquinane-type sesquiterpene, by a route that passed through a tricyclo[7.3.0.0^{1,5}]dodecanedione intermediate, which is a carbocyclic analog of rings A, C and B. The method of synthesis of this intermediate led to formation of the thermodynamically most stable stereoisomer and the subsequent conversion to pentalenene established that the ring A:C and B:C ring fusions in the intermediate were both cis.⁵³ Furthermore, DFT energy calculations on **2** having cis and trans fusions of rings A and C (carried out without the M4 hydroxyl group) using the 6-31G* basis set with the B3LYP functional indicate that a cis fusion is significantly favored over trans.⁵⁸

3.6. Absolute Configurations of the Diastereomers of **1**

The CD spectra of **1a** and **b** were virtually identical indicating that the overall shapes and major chiral features were the same (See Supporting Information). The CD spectrum of a mixture (57:43) of **1c/1d** was essentially a mirror image of those of **1a** and **1b**. This provides evidence that **1a** and **b** differ only in the orientation of the M4 hydroxyl group since reversing the configuration of one of the ring junctures would be expected to influence the interaction of the Gua residues with each other. It is noteworthy that the configuration of the M4 hydroxyl group has negligible impact on the CD spectra of **1a** and **b**. The application of the Octant Rule to configurational assignment of **1a** and **b** was problematic due to the two Gua chromophores. Assignments of absolute configurations as shown in Scheme 4 were made on the basis of comparison with CD spectra of pyrimidopurinone-type dGuo adducts of 4-hydroxynonenal which had been synthesized by methodology that provided unambiguous assignment of absolute configuration.⁵⁹ Further support for the configurational assignments was obtained from comparison of the CD spectra of **1a** and **b** with those of

tetracyclic 1,N²-ethano-type dGuo adducts of 2,3-epoxy-4-hydroxynonanal which are excellent models for the Guaw-A-C rings in **1**.⁶⁰

Reduction of a mixture of bis-nucleosides **1a-d** with NaBH₄ gave dihydro products **4a-d** (Scheme 4), which were readily separated by HPLC. Reduction of the diastereomers of **1** individually revealed that the order of HPLC elution of the reduced bis-nucleosides had changed to **4c < 4d < 4a < 4b**. Reduction brought about replacement of the M1 singlets seen near 6 ppm in the bis-nucleosides by a pair of vicinal doublets in the region of 3.5 ppm establishing that reduction had cleaved the bond between carbon M1 and N1 of Gua_E. COSY and HSQC spectra showing the presence of the CH₂-CH-CH₂-CH network confirmed that the cyclopentane ring remained intact. Significant changes had occurred in the chemical shifts of the CH₂-CH-CH₂-CH protons such that all four reduced bis-nucleosides had unique spectra.

The reduction brought about a remarkable change in the ¹H spectrum of one deoxyribose in each diastereomer. The two anomeric protons in **1a-d** had had unexceptional chemical shifts near 6.05 ppm. In the reduced species, one of the anomeric protons was strongly shielded giving upfield locations of 5.15, 5.14, 5.51, and 5.61 ppm, respectively, for **4a-d**. The other anomeric proton and the H8 protons of the two dGuo rings had also been shifted upfield but only by 0.1–0.2 ppm. Bis-nucleosides **1a-d** are rigid dihedral species with the two Gua residues extending away from one another. The reduced species have freedom of rotation around both C-N bonds of N² of Gua_E and increased capability for the cyclopentane ring to pucker permitting the Gua residues to stack. The anomeric proton of dGuo_E then lies near the face of Guaw producing the shielding. Apparently, for **4a** and **4b** the anomeric protons of the dGuo_E deoxyribose residues lie in a more highly shielded location than for **4c** and **4d**, i.e., closer to Guaw or the population of the folded conformers may be higher leading to larger upfield shifts.

CD spectra for **4a** and **4b** show negative ellipticity in the 280 nm region and positive ellipticity near 255 nm; **4c** and **4d** show positive ellipticity near 280 nm and negative near 255 nm (Figure 9). As with **1a** and **1b**, the Octant rule can not be reliably used for assignment of the configurations of **4a-d** but the compounds are well-suited for application of Nakanishi's exciton chirality method,^{61,62} taking advantage of the relative orientations of the two chromophores. Molecular models reveal that having *S* and *R* configurations, respectively, for M2 and M6 in **4a** and **4b** leads to a left-handed helical relationship of the two Gua moieties (Figure 10) yielding the observed negative ellipticity for the longest wavelength CD band. Likewise having (*R*)-M2 and (*S*)-M6 in **4c** and **4d** leads to a right-handed relationship and positive ellipticity. This provides confirmation for the absolute configurations of **4a-d** shown in Scheme 4 that had been deduced for **1a-d** above. It can be seen in Scheme 4 that the configurational designations of M2 in **4a-d** are reversed from **1a-d** due to the change of Cahn-Ingold-Prelog priority rules.

Comparison of the CD spectra of the reduced bis-nucleosides with those of B- and Z-DNA is illuminating. The spectra of **4a** and **4b** are similar to that of Z-DNA whereas the spectra of **4c** and **4d** are similar to that of B-DNA.⁶³ This provides additional confirmation of the configurational assignments since Z-DNA has left-handed helicity while B-DNA has right-handed.

4. DISCUSSION

The formation of bis-nucleosides **1a-d** by the condensation of muconaldehyde with dGuo involves a cascade of reactions (Scheme 5). We believe the reaction sequence is initiated by addition of dGuo to a carbonyl group of muconaldehyde forming carbinolamine **5** and then

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cyclization by attack of N1 of dGuo on C2 on the muconaldehyde moiety to give 1,N²-ethano derivative **6** followed by imine formation with a second dGuo to give **8**. We cannot exclude the possibility that the reverse sequence occurs in which **5** first reacts with the second dGuo to form imine **7** followed by cyclization to give 1,N² derivative **8**, or that the two processes occur concurrently. Mass spectroscopic examination of early intermediates provided support for both mono- and bis-adducts. The formation of **8** is analogous to the reactions of dGuo with butene-1,4-dial,⁶⁴ 4-oxo-2-nonenal,⁶⁵ and with 2,3-epoxyaldehydes.^{60,66}

A 1,4-addition of hydroxide ion to **8** yields imine **9**. This could occur either by reaction with water or by an intramolecular attack of the hydroxyl group of **7** leading to tetrahydrofuran **10**, followed by ring-opening of **10** to give **9**. Imine **9** tautomerizes to 1,N²-etheno dGuo **11**, which is in fact an enamine. Attack of the enamine on the imine of **11** creates the cyclopentane ring in **12**. The final ring closure giving **1a-d** occurs by attack of N1 of dGuo_E on the western imine of **12**.

All steps in this reaction sequence are potentially reversible although we only detected reversibility for the final three steps. Specifically, we have found no evidence for the hydroxyl group at M4 ever epimerizing. This means that **9** does not revert to **8** under the reaction conditions we employed. Equilibrium between **1a** and **d** and between **1b** and **c** is evidence that the cyclopentane ring in **12** is opening and reclosing to invert the configuration at quaternary carbon M2. Furthermore, equilibration of **1** with precursor **12** is demonstrated by the reduction of **1** with sodium borohydride that is, in fact, a reduction of **12**. Each diastereomer undergoes reduction with complete stereospecificity, i.e., the reduction of imine **12** by borohydride ion is much faster than the reversion of **12** to **11**. Furthermore, we found no evidence for reduction products that would have arisen from **9** or **11**. Also, we have not observed loss of the dGuo_E residue from **1**, even when NMR samples in wet DMSO-*d*₆ were allowed to stand for more than 2 months at room temperature. Nevertheless, since **1** is in a slow equilibrium with **11**, the possibility exists that Gua_E could be lost under appropriate conditions. Reversibility of the pathway by which diastereomers **1a-d** are formed leads to them being the thermodynamically most favored forms. Cis fusions of ring C with rings A and B have been assigned for **1a-d**. A strong preference for this stereochemistry explains why only four diastereomers are observed even though the presence of 4 stereogenic centers (M1, M2, M4, and M6) would have predicted the formation of as many as 16.

5. CONCLUSIONS

This paper demonstrates that muconaldehyde is able to form stable cross-links between dGuo residues. A central issue is whether muconaldehyde-based bis-dGuo adducts might form in DNA and become the chemical basis for the observed carcinogenicity of benzene. It may be problematic for bis-adducts **1** *per se* to be present as interchain cross-links in either a GpC or CpG sequence context in duplex DNA because the two Gua moieties of **1** are in an *anti* relationship to each other whereas the Gua residues in native DNA are *syn*. Acrolein and mitomycin C form Gua-Gua interchain cross-links in a CpG sequence context; structural studies have shown the guanines retain the relative orientations of native DNA in both cases.^{48,67} Similarly, the two thymines involved in photochemically induced cross-linking by psoralen retain their *syn* relationship.^{68,69} Intrachain cross-links involving **1** in a GpG sequence context will be precluded by geometrical constraints.

Nevertheless, the chemistry elucidated in the present study reveals that imine **12** and the cognate carbinolamine **13** will be in equilibrium with **1** (Scheme 6). Both would be promising candidates for interchain cross-link formation because they would be able to

adopt a syn relationship between the Gua moieties needed for formation of relatively undistorted interchain cross-links. Moreover, **12** and **13** would have the flexibility to form intrachain cross-links. Our observation that the preferred conformation for **4** involves a partially stacked arrangement for the two Gua residues supports the possibility that an intrachain cross-link could form. The intrachain cross-link would be particularly attractive when the configurations of M2 and M6 are as in **4c** and **4d** so that the bis-adduct could conform to a right-hand helix. Muconaldehyde-induced tandem mutations reported by Nakayama et al. were unique to this compound; this observation lends credence to the possibility of muconaldehyde-mediated intrachain cross-links.⁴²

The most attractive type of muconaldehyde cross-links may involve those between DNA and proteins in which the free aldehyde of initially formed muconaldehyde mono-adducts **5** and/or **6** (Scheme 7) reacts with the ε-amino group of lysines to give **14** or analogously with the N-terminus of the protein. Evidence for DNA-protein cross-links has been reported for muconaldehyde-treated HL-60 cells by Schoenfeld et al., who have postulated that these cross-links may contribute to benzene-induced hematotoxicity and leukemogenesis.⁴³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

COSY	correlation spectroscopy
DFT	density function theory
dG-muc-dG	2:1 adducts 1a–d of deoxyguanosine with <i>E,E</i> -muconaldehyde
DMSO	dimethylsulfoxide
ES	electrospray
ESI-MS	electrospray ionization mass spectrometry
G-muc-G	2:1 adducts 2a–d of guanine with <i>E,E</i> -muconaldehyde
HMBC	heteronuclear multiple bond correlation spectroscopy
HSQC	heteronuclear single-quantum correlation spectroscopy
MW	molecular weight
NOE	nuclear Overhauser effect
OSHA	U.S. Occupational Safety and Health Administration
SPE	solid phase extraction

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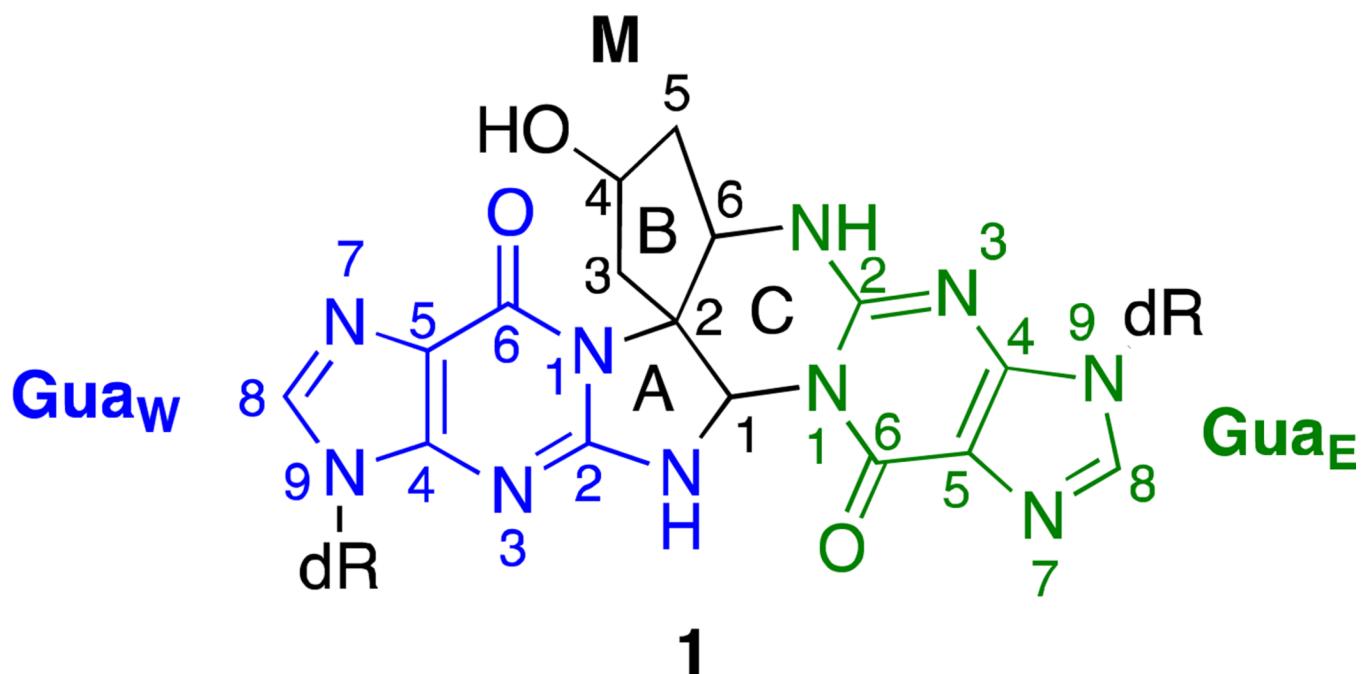


Figure 1.

Numbering and ring designations for the bis-adduct of dGuo with *E,E*-muconaldehyde.

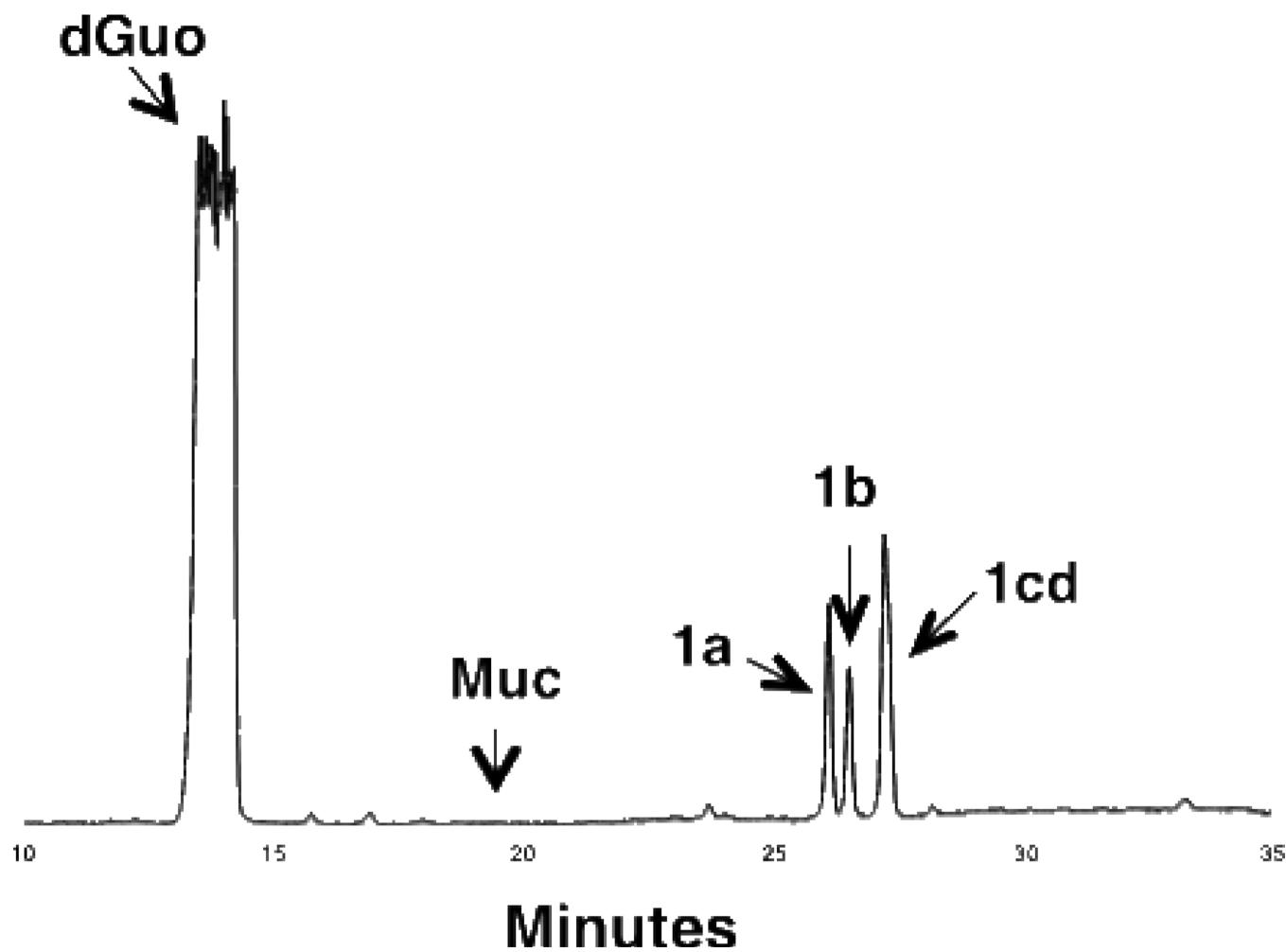
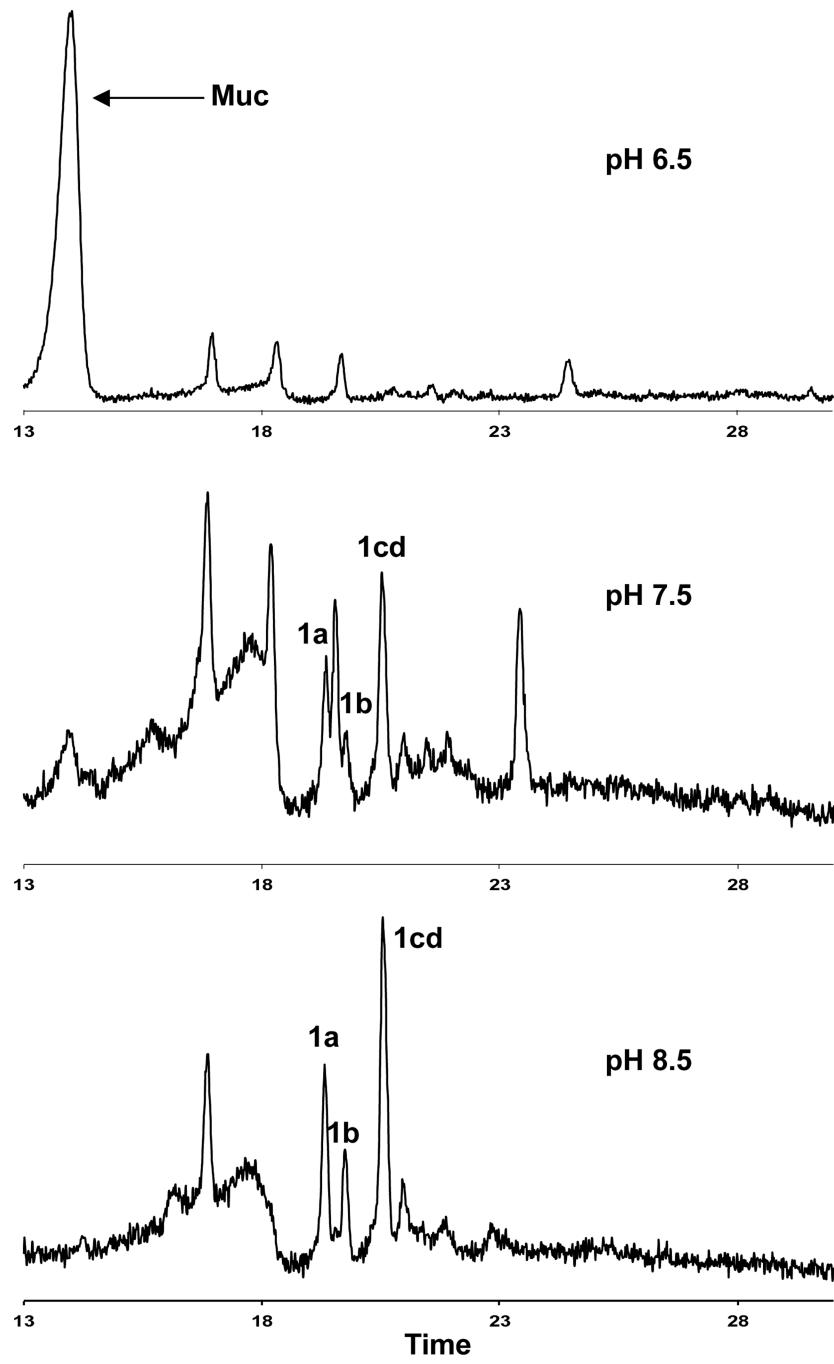


Figure 2.
HPLC chromatogram showing the reaction of dGuo with muconaldehyde in phosphate buffer (0.05 M, pH 7.4) after 7 weeks at ambient temperature.

**Figure 3.**

Effect of pH on the reaction of dGuo with muconaldehyde at ambient temperature. Reaction mixtures contained dGuo (5.8 μ M) and muconaldehyde (2.9 μ M) in potassium phosphate buffer (0.05 M) at pH 6.5, 7.5 and 8.5. Chromatogram shows reaction mixtures after 72 h.

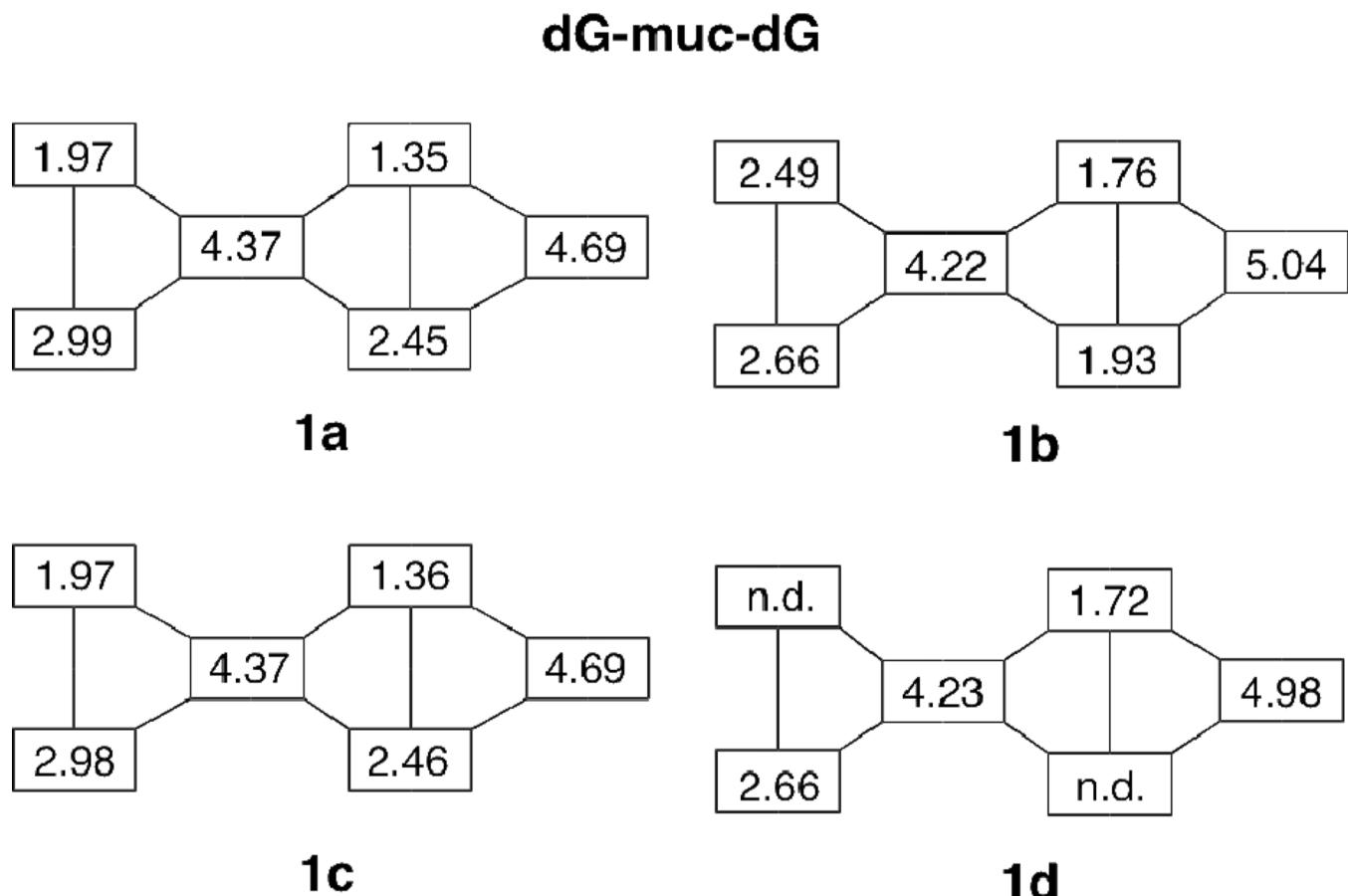
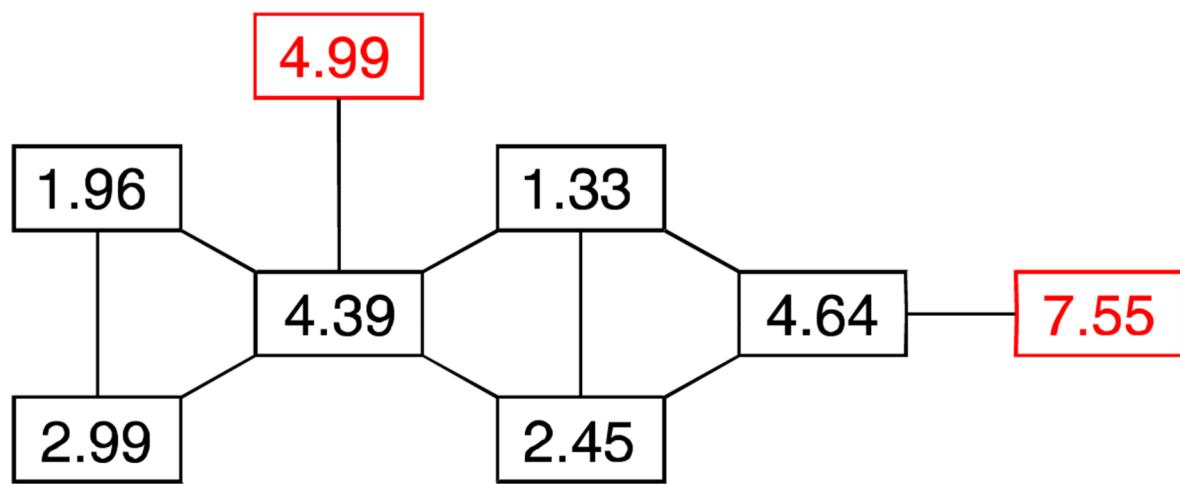
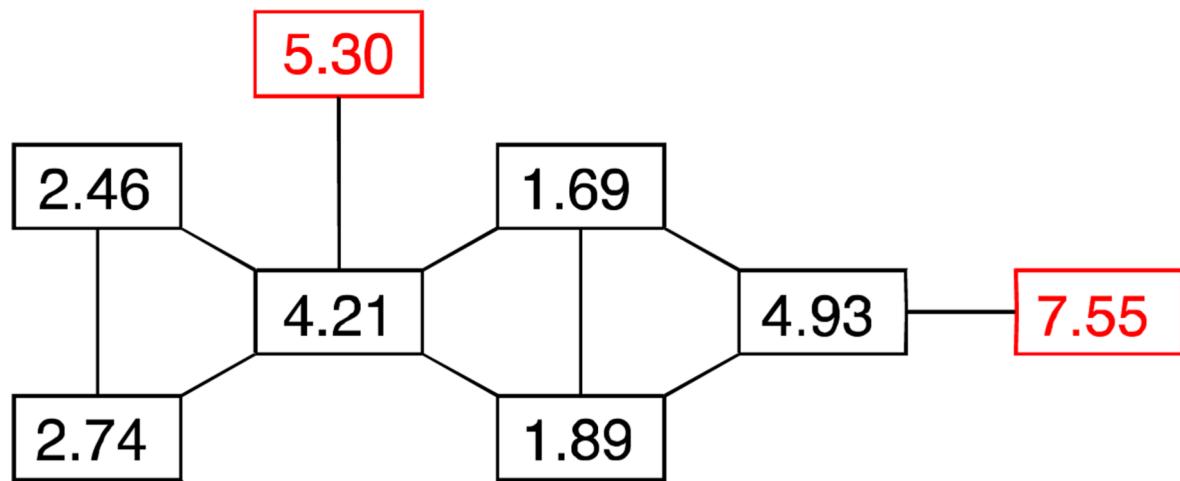


Figure 4.

¹H Chemical shift and coupled networks in compounds **1a–d**. Spectra acquired in DMSO-*d*₆/D₂O (~90:10, v/v).

2ac**2bd****Figure 5.**

^1H chemical shift and coupled networks in compounds **2ac** and **2bd**. Spectra recorded in anhydrous DMSO- d_6 . Chemical shifts (δ) in ppm. Addition of D_2O caused disappearance of the exchangeable protons, which are indicated in red.

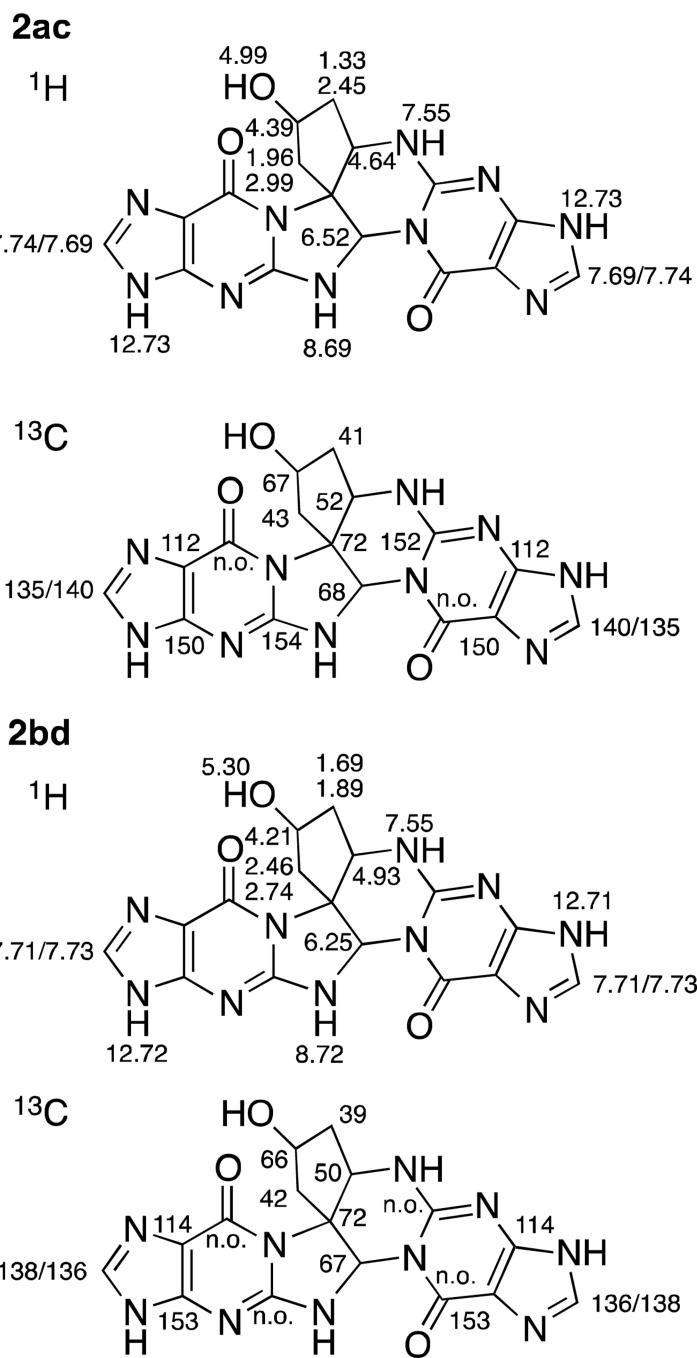


Figure 6.

¹H spectra of **2ac** and **2bd** were recorded in anhydrous DMSO-*d*₆ ¹³C spectra in DMSO-*d*₆/D₂O. Chemical shifts (δ) in ppm n.o. = not observed.

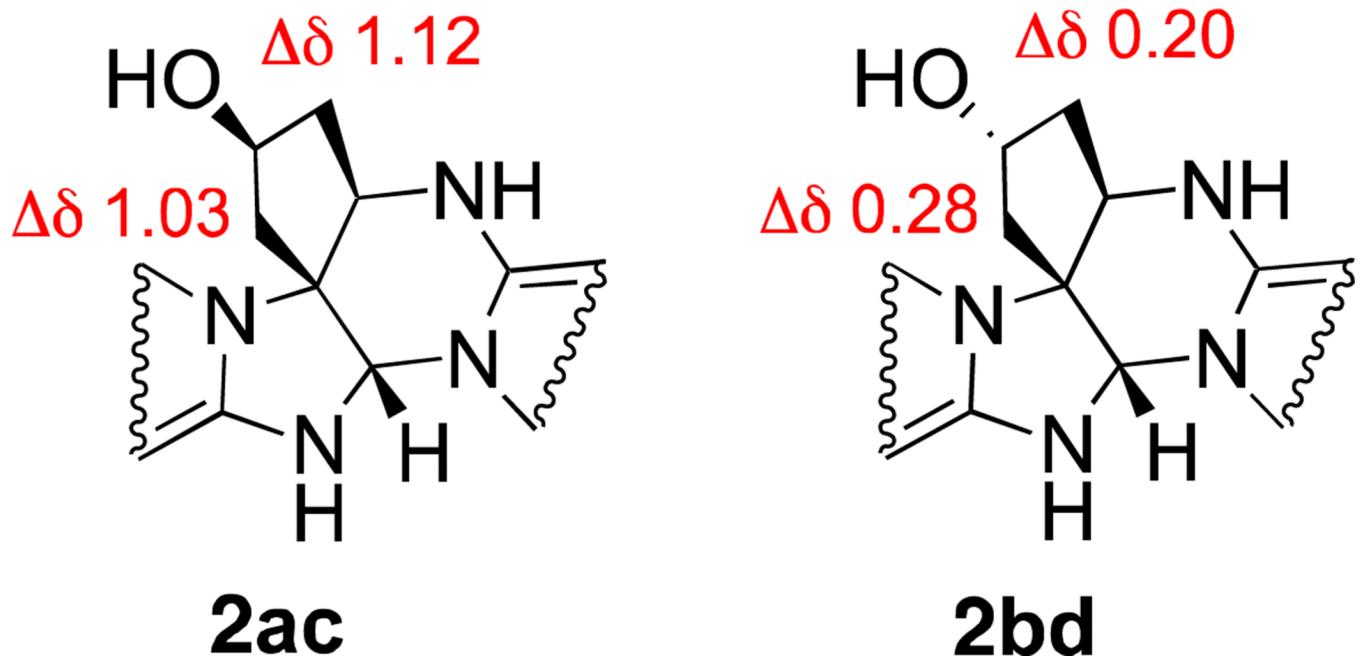
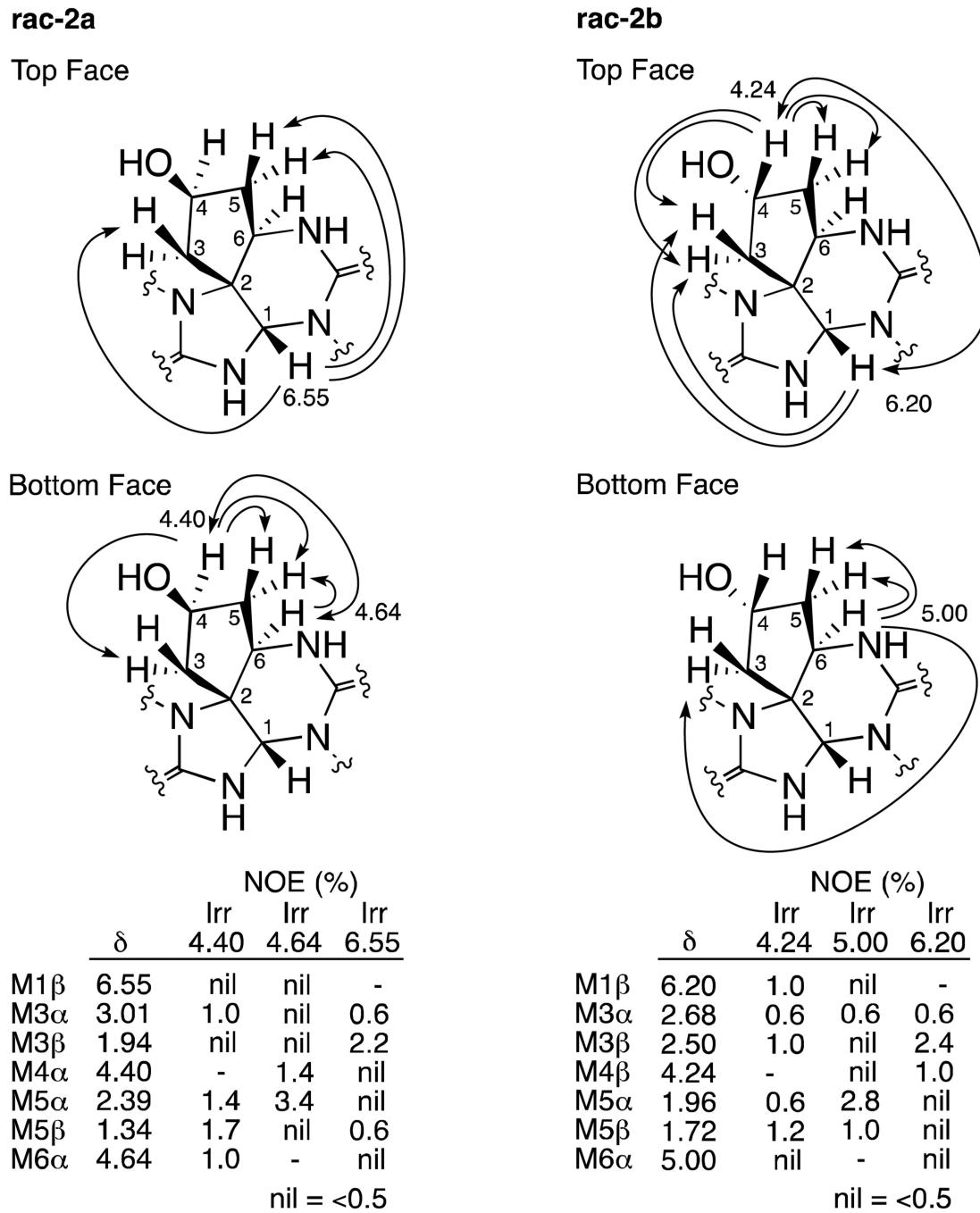


Figure 7.

Chemical shift differences between the M3 and M5 geminal protons of **2ac** and **2bd**. Spectra were recorded in anhydrous DMSO-*d*₆.

**Figure 8.**NOE experiments with **2ac** and **2bd** conducted in DMSO-*d*₆/D₂O (92:8, v/v).

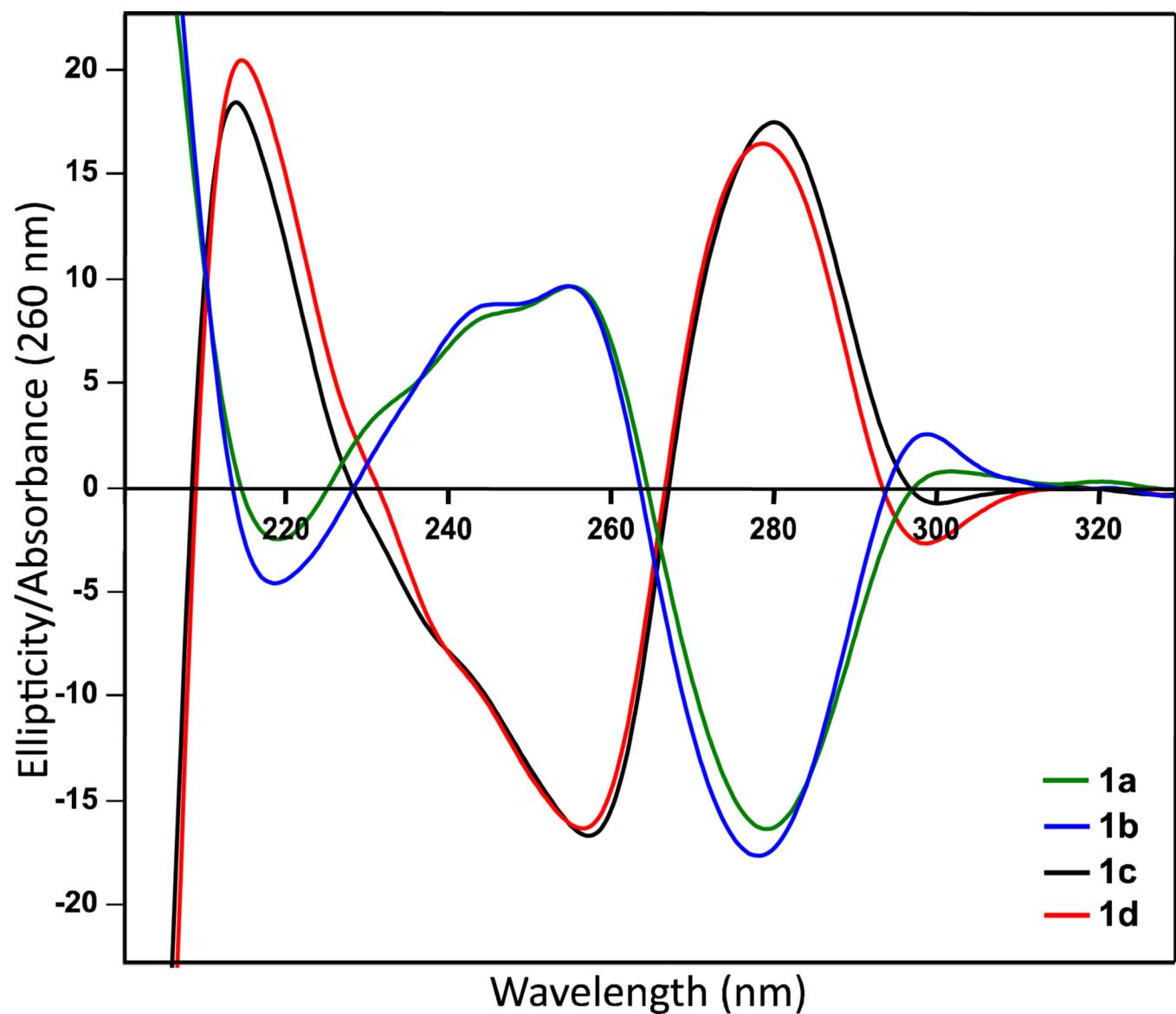
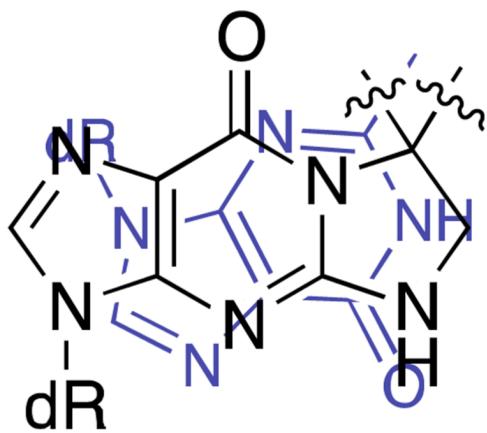
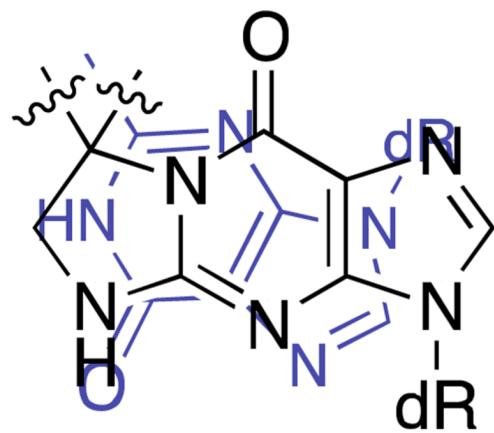


Figure 9.

CD spectra of reduced bis-nucleosides **4a–d** obtained at ambient temperature in water-methanol (1:1, v/v) mixtures.



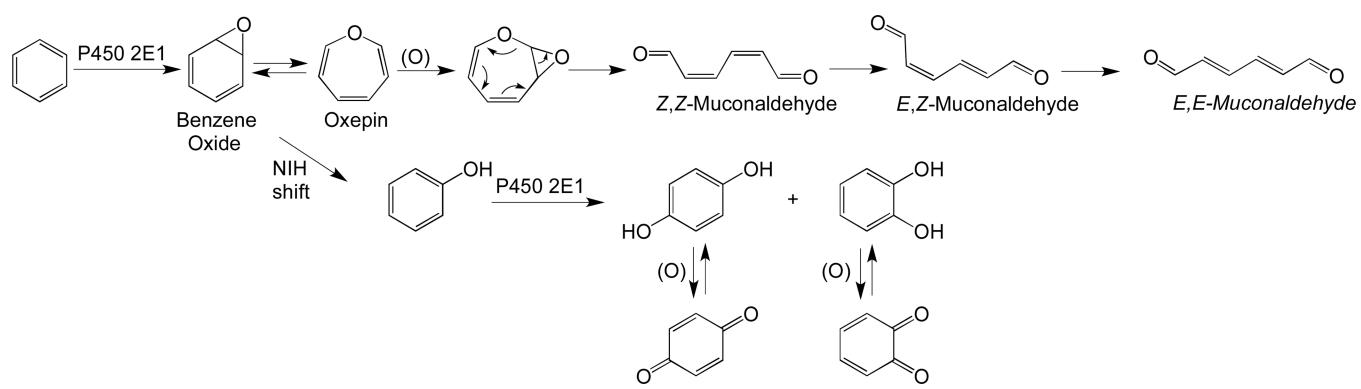
4a and b
left helix



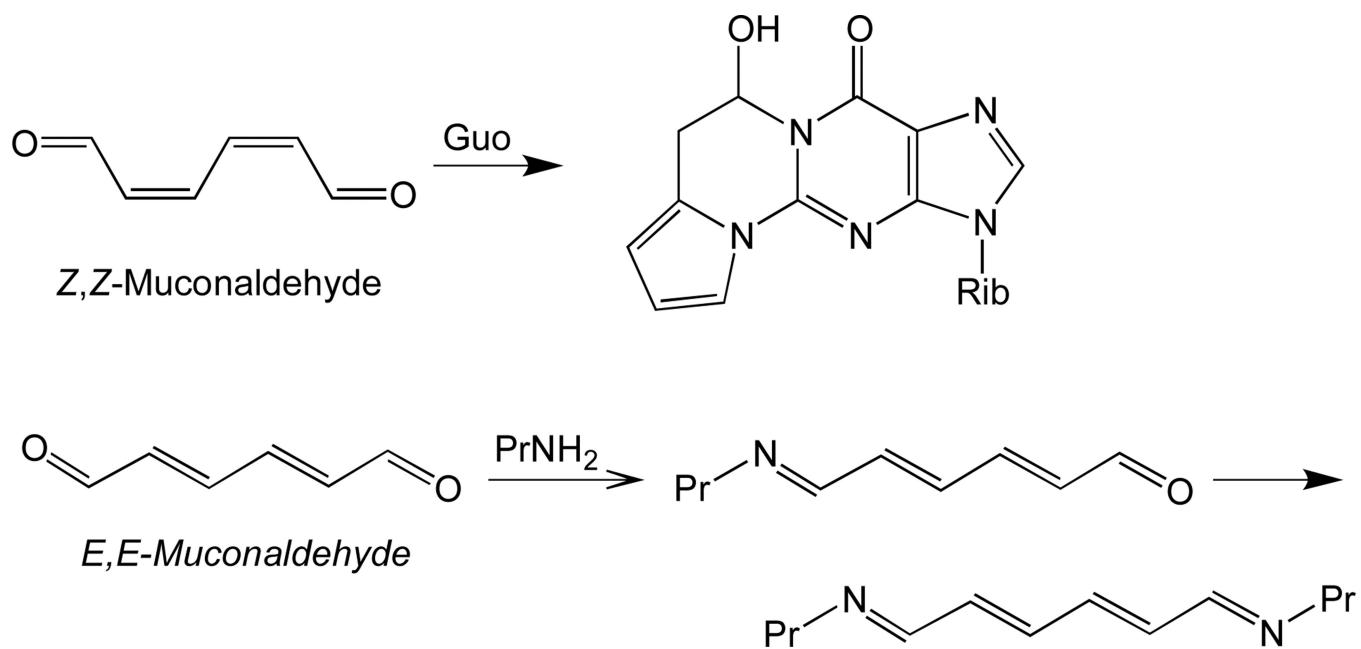
4c and d
right helix

Figure 10.

Depiction of the helical relationship of Gua residues in **4a** and **4b** versus **4c** and **4d**.

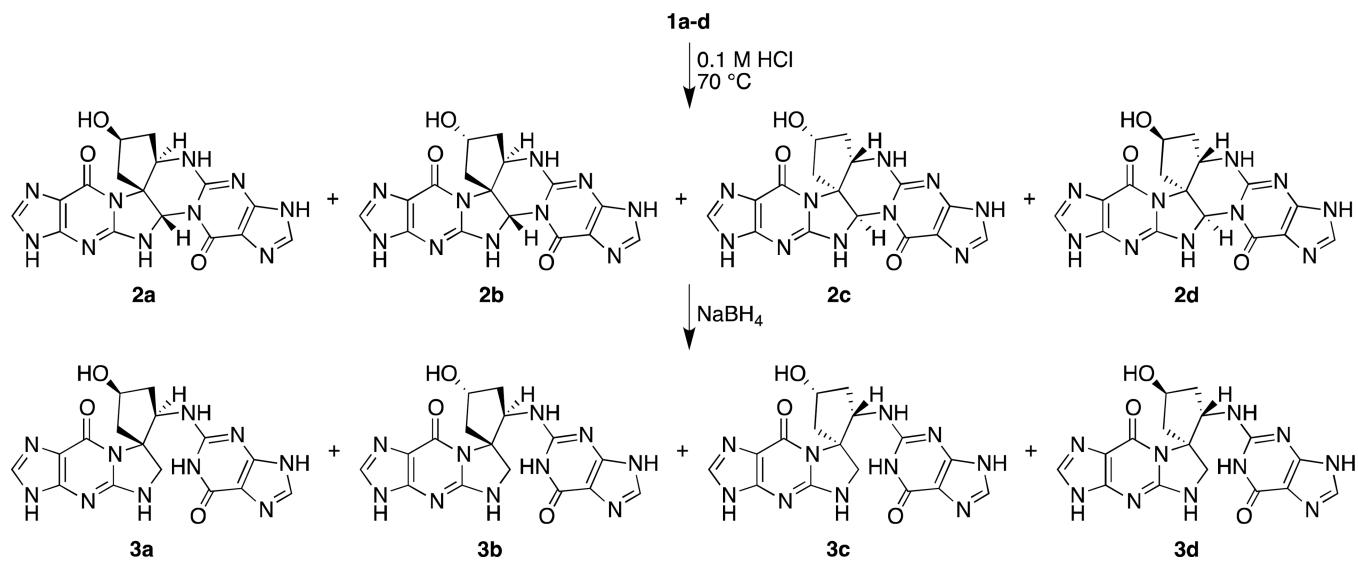


Scheme 1.
Metabolic activation of benzene.



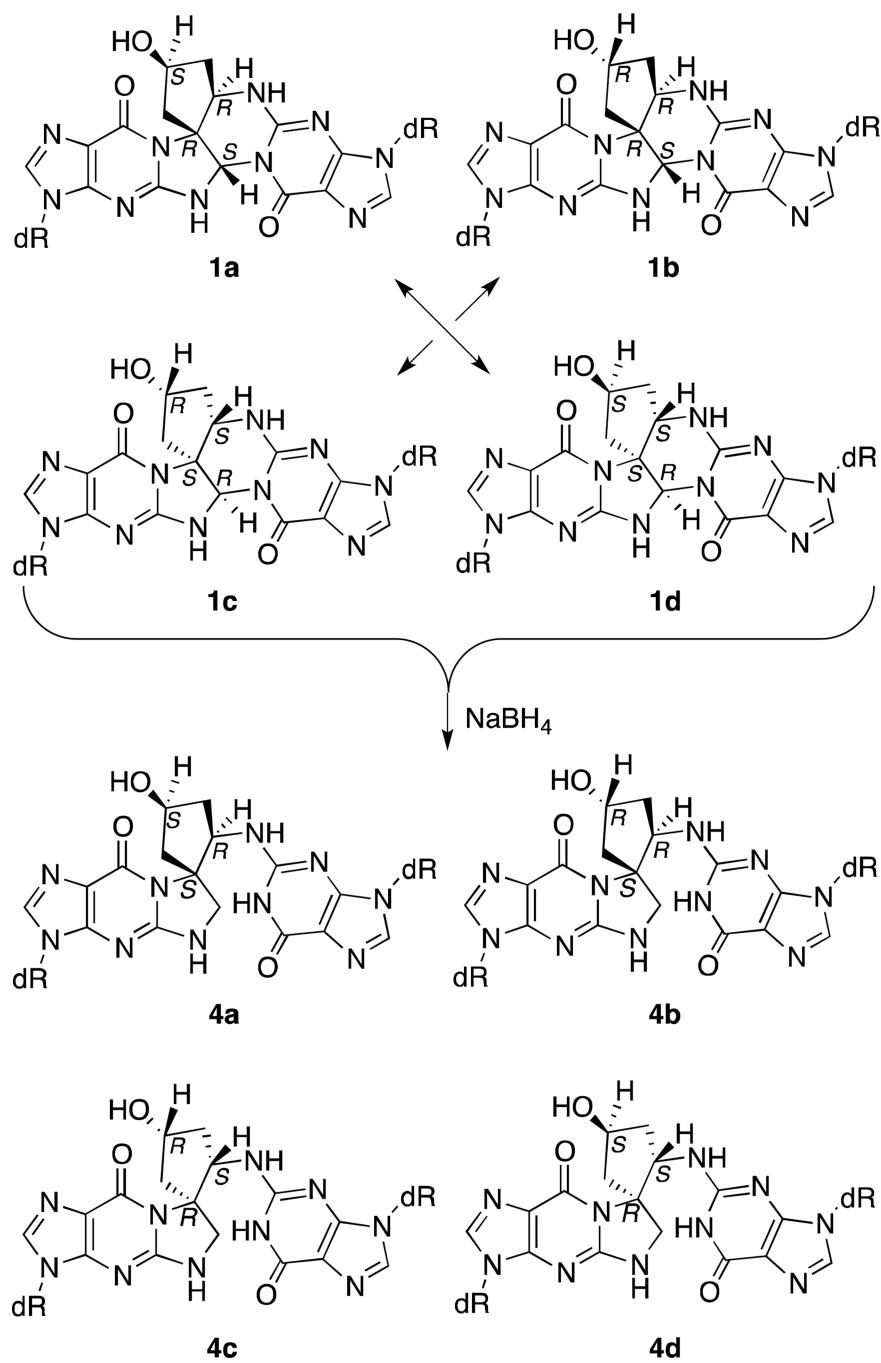
Scheme 2.

Adducts of *Z,Z*- and *E,E*-muconaldehyde characterized by Golding's group.^{21,46}



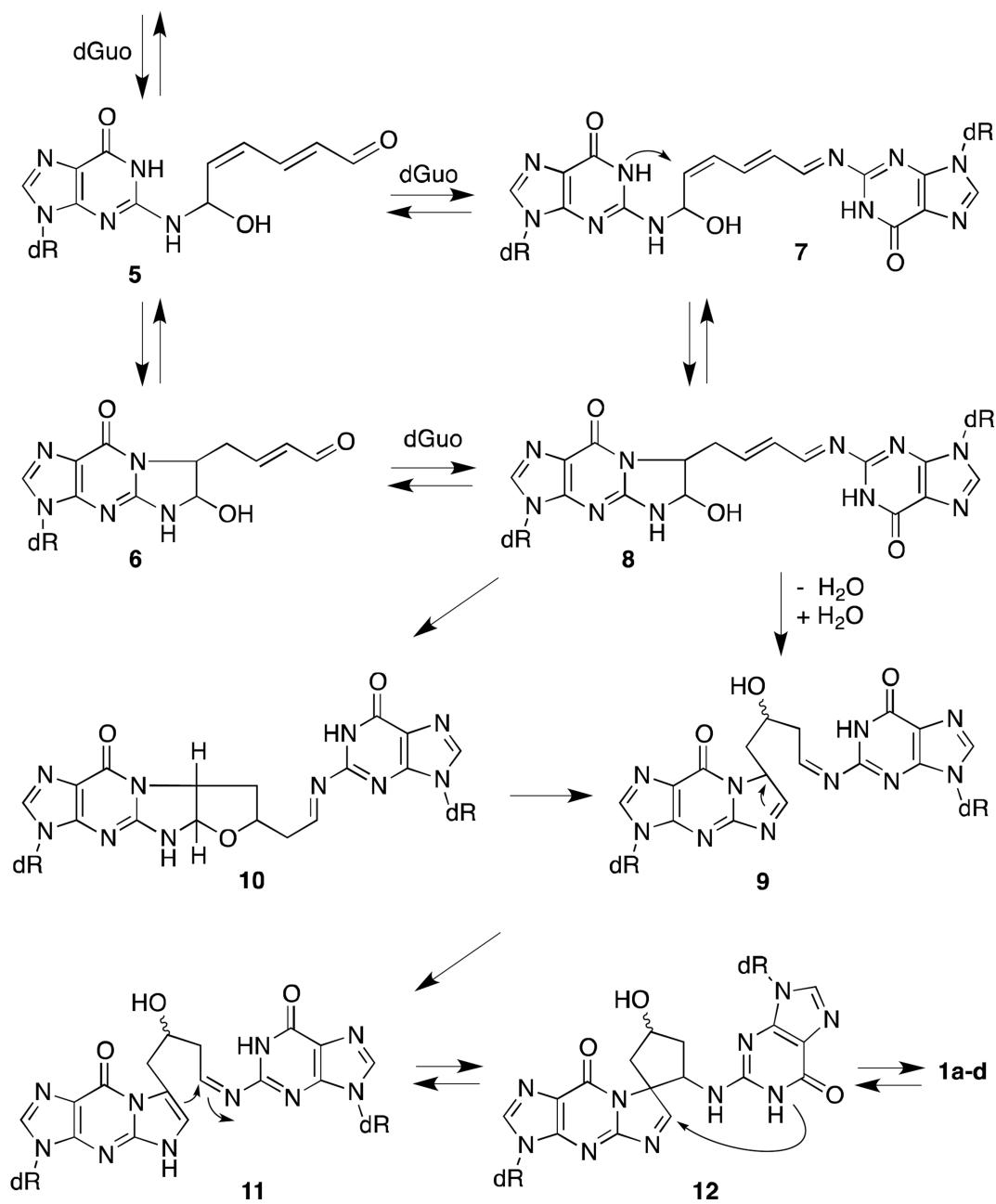
Scheme 3.

Deglycosylation of bis-nucleosides **1a-d** to form the analogous bis-bases **2a-d** and reduction of the bis-bases with **NaBH₄** to form **3a-d**.

**Scheme 4.**

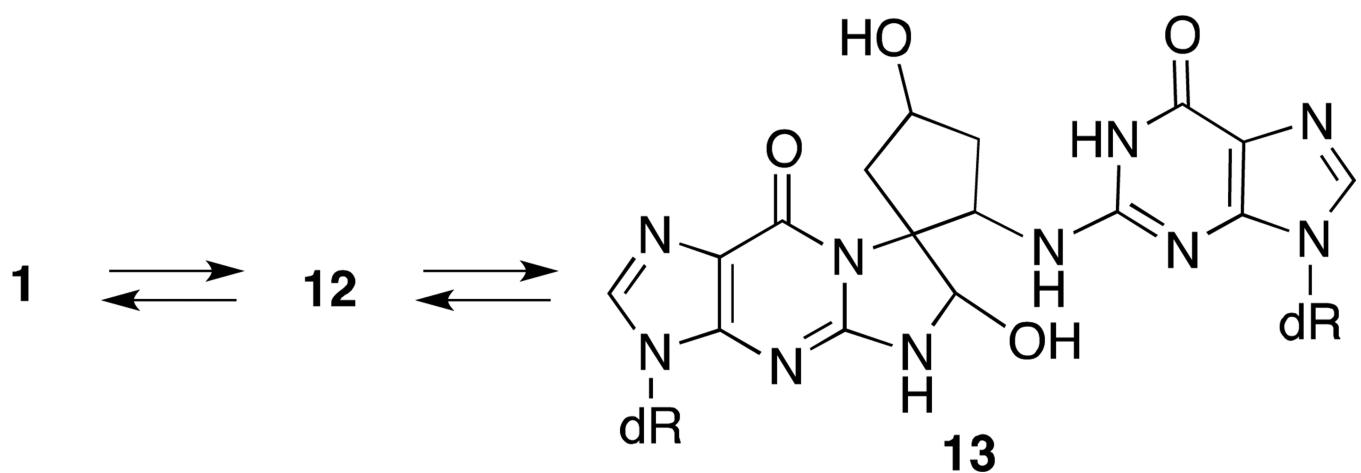
Equilibria of bis-nucleoside **1a** with **1d** and of **1b** with **1c**, reduction of **1a-d** to bis-nucleosides **4a-d** with NaBH₄, and configurational assignments of **1a-d** and **4a-d**.

Muconaldehyde



Scheme 5.

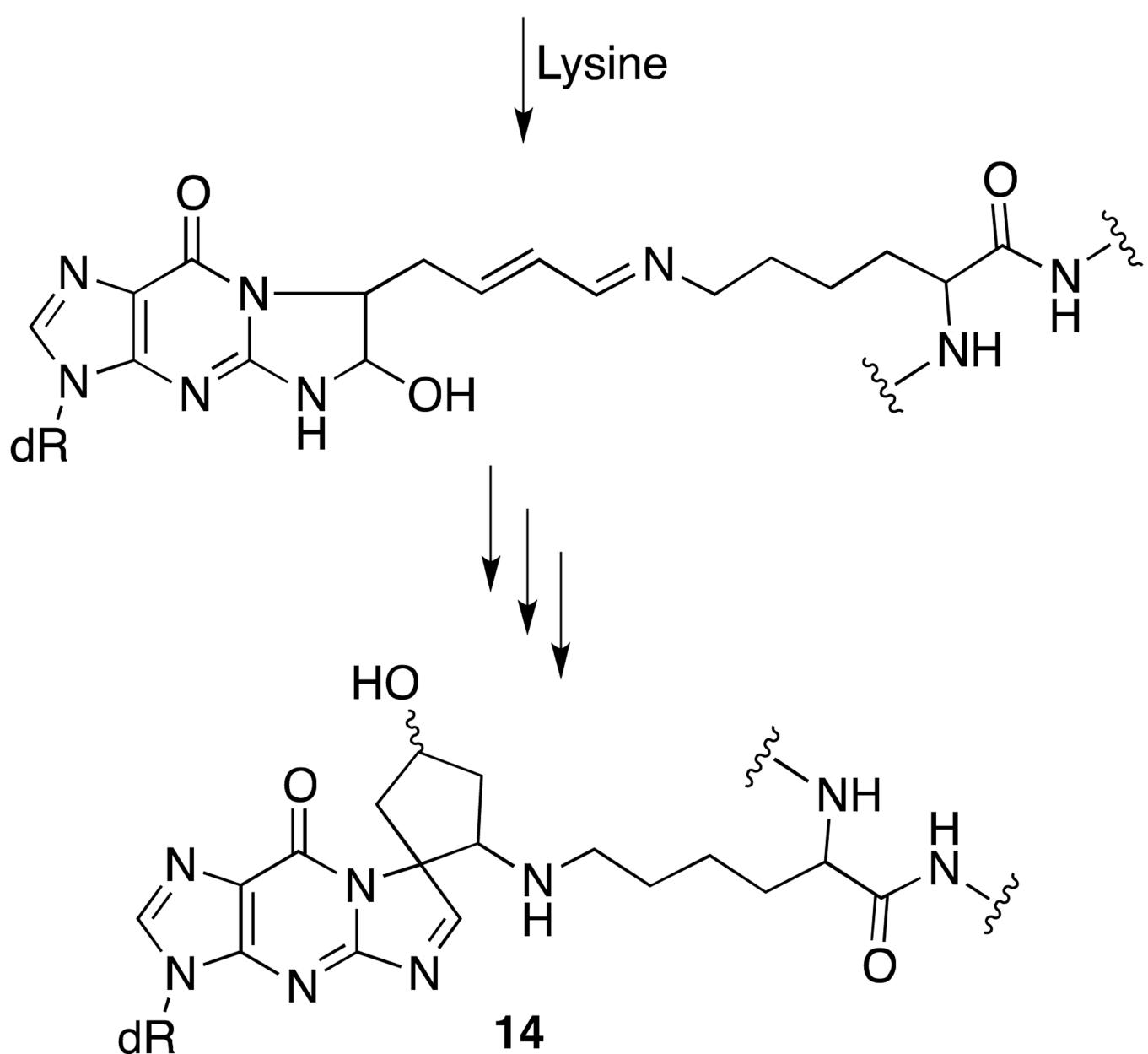
Proposed mechanism for formation of bis-nucleosides **1a-d**.



Scheme 6.

Reversible opening of ring C of bis-nucleosides **1** to form imines **12** and carbinolamines **13**.

5/6

**Scheme 7.**

Proposed reaction of the dGuo-muconaldehyde monoadduct with the ε -amino group of lysine to form DNA-protein cross-links.