Synthesis and Properties of N^2,3-Ethenoguanosine and N^2,3-Ethenoguanosine 5'-Diphosphate

Jaroslaw T. Kuśmierek,† David E. Jensen,‡ Sylvia J. Spengler, Ryszard Stolarski,§ and B. Singer*

Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

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N^2,3-Ethenoguanosine has not been reported as a product of guanosine reaction with haloacetaldehydes. This is in contrast to the reaction of the ribosides yielding the well-known etheno compounds 1,N^6-ϵAdo, 3,N^4-ϵCyd, and 1,N^2-ϵGuo. The base, N^2,3-ethenoguanine was, however, synthesized, but not by direct reaction. We now report the synthesis of the nucleoside via reaction of O^6-benzylguanosine with bromoacetaldehyde followed by hydrogenolytic debenzylation in the presence of palladium on charcoal. The structure of N^2,3-ethenoguanosine was confirmed by NMR, UV, and FAB-MS data. The conformation of this nucleoside resembles that of guanosine, being predominantly in the anti form. The nucleoside was stable at neutrality, 37 °C, but depurination was rapid at pH 1, 24 °C, with a $t_{1/2}$ = 16 min. In contrast to 1,N^2-ϵGuo, the angular N^2,3-etheno derivatives are fluorescent, with the base having considerably higher fluorescence than the nucleoside. N^2,3-Ethenoguanosine was converted to the 5'-diphosphate by conventional methods. This compound was a substrate for polynucleotide phosphorylase and could be copolymerized with CDP or ADP. There is little quench of the nucleotide fluorescence in copolymers. The recent report that N^2,3-ethenoguanine is detected in chloroacetaldehyde-treated DNA after depurination makes N^2,3-etheno modification of the guanine moiety of potential importance in understanding vinyl chloride induced carcinogenesis.

The reaction of haloacetaldehydes with nucleic acid components yields etheno derivatives of guanosine, adenosine, and cytidine; the latter two have been used as probes in enzymatic systems, and the high fluorescence of 1,N^6-ϵA is a tool for structural studies. (For a review see Leonard.[1]) Chloroacetaldehyde and bromoacetaldehyde are stable metabolites of vinyl chloride or vinyl bromide, which are carcinogens and mutagens. Etheno derivatives of both cytosine (ϵC) and adenine (ϵA) are formed in liver RNA of animals given vinyl chloride in vivo[2,3] or in single-stranded polynucleotides treated with chloroacetaldehyde in vitro.[4] However, there is contradictory opinion whether ϵC or ϵA is formed in vivo in double-stranded DNA or only in single-stranded RNA[5] since their formation is not possible in Watson-Crick paired bases. The very labile metabolite of vinyl chloride, chloroethylene oxide, does react with DNA or deoxyguanosine, yielding *N*-7-(2-oxoethyl)guanosine[6,7] and thus is likely to react at the N-3 of A and other base sites characteristic of alkylating agents.[8] The replicational ambiguities resulting from the presence of ϵA and ϵC in synthetic deoxypolynucleotide templates were studied with various polymerases,[9-11] but only ϵC appeared to act significantly as a promutagen.

Oesch et al.[12] found traces of the fluorescent derivative N^2,3-ethenoguanine [8,9-dihydro-9-oxoimidazo[2,1-*b*]-purine] after depurination of DNA treated with chloroacetaldehyde in vitro. More recently these authors claim N^2,3-ethenodeoxyguanosine was detected, by fluorescence, in enzyme digests of DNA reacted with chloroacetaldehyde in vitro.[13] No details regarding the reference compound are given. Laib et al., in 1985,[14] presented preliminary evidence for N^2,3-ethenoguanine in liver DNA of rats given [^{14}C]vinyl chloride. The etheno base was previously synthesized and characterized by Sattsangi et al.[15] We now report the synthesis and characterization of the nucleoside N^2,3-ethenoguanosine [8,9-dihydro-9-oxo-3-β-D-ribofuranosylimidazo[2,1-*b*]purine (**6**)] and preparation of the corresponding nucleoside diphosphate.

Results and Discussion

The reaction of guanosine (**1**) with chloroacetaldehyde (Figure 1) led to formation of a 1,N^2-etheno bridge, while with O^6-substituted guanines an N^2,3-etheno bridge is formed and the resulting derivatives can be converted to N^2,3-ethenoguanine (**5**).[15] The analogous situation should occur in the reaction of DNA with chloroacetaldehyde, where the N-1 and O^6 of guanine are involved in hydrogen bonding, whereas the N^2 and N-3 sites are exposed in the minor groove of the DNA helix.[12] In our approach to the synthesis of N^2,3-ethenoguanosine we followed the general approach of Sattsangi et al. for the synthesis of the base N^2,3-ethenoguanine. However, as detailed below, synthesis of the modified nucleoside presented special problems.

The reaction of O^6-ethylguanosine (**2a**) with bromoacetaldehyde at pH 4.5 gives N^2,3-etheno-O^6-ethylguanosine (**4a**) as the major product. However, the attempted alkaline hydrolysis of the O^6-ethyl group[16] was

† Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw 02532, Poland.

‡ Fels Research Institute, Temple University School of Medicine, Philadelphia, PA 19140.

§ Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Warsaw 02089, Poland.

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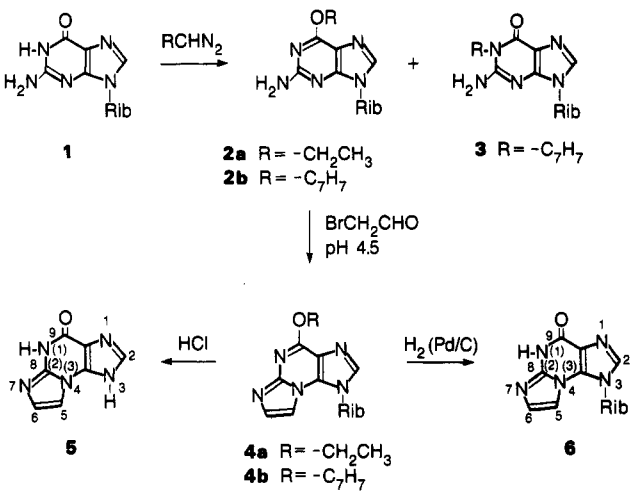

Figure 1. Reaction scheme.

unsuccessful due to the very rapid, but not unexpected, glycosyl bond breakage.[8] Heating of **4a** in 1 N KOH at 100 °C gave rapidly and quantitatively the new product, which had UV characteristics virtually identical with those of N^2,3-etheno-O^6-methylguanine[15] (data not shown). Acid hydrolysis of **4a** led to removal sequentially of ribose and then the O^6-ethyl group to give N^2,3-ethenoguanine (**5**).

The failure of the use of the protective ethyl group to allow synthesis of the desired derivative led us to employ a benzyl group, which has the advantage of being removed by hydrogenolysis under neutral conditions. The reaction of guanosine (**1**) with phenyldiazomethane gave O^6-benzylguanosine (**2b**) and the accompanying N-1 isomer **3** as the major products; these were identified on the basis of UV spectra.[17] The reaction of **2b** with bromoacetaldehyde under conditions similar to the reaction of **2a** gave N^2,3-etheno-O^6-benzylguanosine (**4b**). Isolation of **4b** from the reaction mixture (as well as the isolation of **4a**; see above) was by chromatography on an ion-exchange column. The strong bonding to Dowex 1 resin in the carbonate form would indicate hydrophobic interaction and allows facile desalting and isolation of these nonionic derivatives. The hydrogenolysis of **4b** in the presence of Pd/C under ambient conditions gave the desired N^2,3-ethenoguanosine (**6**) with a yield of 20%, based on O^6-benzylguanosine as the starting material. The UV spectrum of **6** is shown in Figure 2.

Fast atom bombardment–mass spectra (FAB–MS) of **4a**, **4b**, **5**, and **6** gave the expected fragmentation.[18] In each case the quasi-molecular ion ($M^+ - H$) was obtained. The fragmentation of nucleosides occurs by cleavage of the glycosyl bond to yield the corresponding base, and in the case of **4a** and **4b**, further fragmentation patterns confirm the structures in Figure 1. In addition, the structures of the compounds **4a**, **4b**, **5**, and **6** were confirmed by proton NMR spectra (see the Experimental Section). The differences of 0.2–0.4 ppm of proton chemical shifts of N^2,3-ϵ-guanine in comparison with those cited in literature[15] might be attributed to changes in stacking arising from minor differences in concentration.

From the vicinal proton–proton coupling constants it may be concluded that the three guanosine derivatives **4a**, **4b**, and **6** exhibit conformations close to those of guanosine, i.e., N ↔ S dynamic equilibrium of the sugar ring[19] with

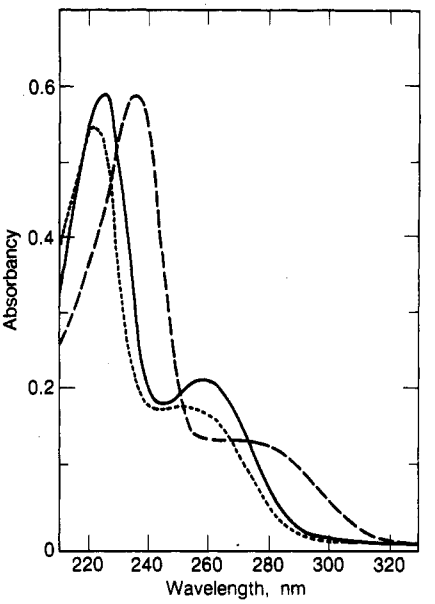

Figure 2. UV spectra of N^2,3-ethenoguanosine (**6**) at pH 7.2 (—), pH 2 (– –), and pH 12 (···). Extinction coefficients are in the Experimental Section.

about 50% populations each, and gauche–gauche ↔ gauche–trans ↔ trans–gauche dynamic equilibrium of the exocyclic 5′-CH_2OH[20] with 60% predominance of the gauche–gauche conformer. The substitution at N-3 constrains the conformation about the glycosyl bond to the anti form. The quantitative determination of the population of the anti form by comparison of sugar proton chemical shifts[21] in N^2,3-etheno derivatives and guanosine (60% anti) is not possible without additional studies, although qualitatively the dominance of the anti form is confirmed by the fact that only H-1′ chemical shifts show marked changes (0.3–0.5 ppm) upon etheno substitutions.

The first synthesis of an N-3-substituted purine ribonucleoside, 3-methylguanosine,[22] supported the observation from polynucleotide studies in that the glycosyl bond of such derivatives is extremely labile. The estimated stability of *N*-3-methyl purine nucleosides is at least 3 order of magnitude less than the parent nucleoside.[8] The stability ($t_{1/2}$) of N^2,3-ethenoguanosine (**6**) at pH 1, 24 °C, is 16 min. The glycosyl bond of the cyclic derivative is considerably more stable than that of *N*-3-methylguanosine, which is reported, under the same conditions, to be ≪6 min.[8] It appears that the etheno ring structure distributes the charge at N-3, thus increasing glycosyl bond stability. In addition no depurination was observed after 18 h, pH 7, 37 °C. Additional stabilization of the bond is conferred by O^6-alkylation, increasing with the size of the substituent, so that the benzyl group permits isolation of the modified nucleoside. This substituent not only directs the ring closure to the N-3 but also decreases depurination in the reaction steps.

The pK_a of **6** is pH 2 and 3, but an exact determination could not be made since below pH 2 instability of the glycosyl bond prevents accurate measurement. The second pK_{a_2} is at pH 8.85, which is lower than that of guanosine (pH 9.2).

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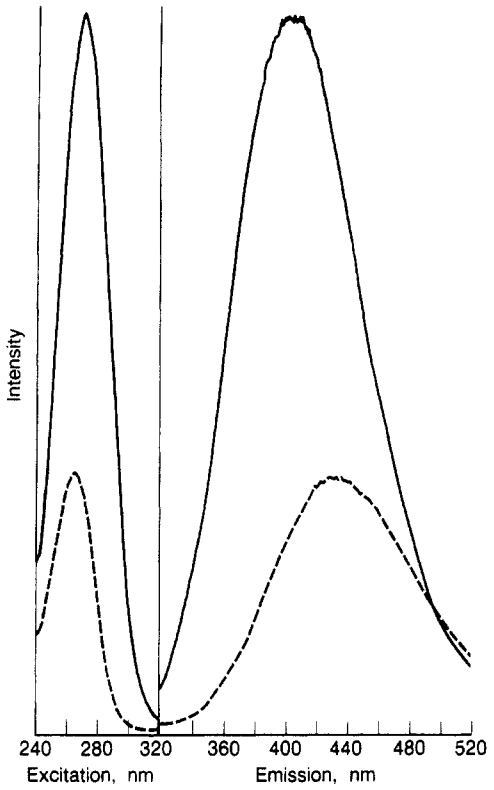

Figure 3. Uncorrected fluorescence spectra of N^2,3-ethenoguanine (**5**) (—) and N^2,3-ethenoguanosine (**6**) (– –) in 10 mM K_2HPO_4, pH 7.0. Part of the riboside **6** solution was depurinated at pH 1 (30 min, 80 °C) to form base **5** and then neutralized. Concentrations were 4.86×10^{-6} M for **6** and 4.43×10^{-6} M for **5**. After acid depurination of polynucleotides containing ϵG, followed by HPLC separation of ϵG, the amount of fluorescence detected indicates that 1 $\epsilon G/10^7$ bases (using 1 mg of DNA) could be detected and quantitated at maximum sensitivity.

The 5′-nucleotide is prepared enzymatically,[23] and under the reaction conditions (16 h, pH 4.5, 37 °C) depurination amounted to <10%. The second 5′-phosphate was added to the N^2,3-ethenoguanosine 5′-monophosphate by a chemical reaction under anhydrous neutral conditions.[24]

N^2,3-Ethenoguanosine 5′-diphosphate can be copolymerized with CDP or ADP using *Micrococcus luteus* polynucleotide phosphorylase. This permits study of its properties in different polymer structures. Polymers containing 6–32% of N^2,3-ethenoguanosine were prepared and used for fluorescence studies. Replication and transcription of such copolymers are the subjects of a separate study.

Of the two possible etheno derivatives of guanine, 1,N^2-ethenoguanine is not fluorescent while N^2,3-ethenoguanine exhibits fluorescence with an excitation maximum at neutrality of 264 nm and emission maximum at 400 nm.[15] The excitation maximum of **6** is also 264 nm, but the emission maximum is 430 nm (Figure 3). The intensity of fluorescence of the base **5** is 2.8 times that of the nucleoside **6** at pH 7. These data on the relative intensity of **5** and **6** are inverse to the relative quench observed when 1,N^6-ethenoadenine is compared to the nucleoside[25] (0.21, ϵAde; 0.56, ϵAdo).

A considerable number of laboratories have studied the fluorescence of ϵA in polynucleotides.[1] In all cases the fluorescence of ϵA is markedly quenched, even in dinucleoside diphosphates.[26] The implication has been that base interactions have absorbed the energy. In contrast, N^2,3-ethenoguanosine containing poly(C) or poly(A) exhibits fluorescence proportional to the content of N^2,3-ethenoguanosine and of similar magnitude to that of the free nucleoside. This is in sharp contrast to 1,N^6-ethenoadenosine and would indicate that the base interactions in polymers believed responsible for ϵA quenching are not significant for N^2,3-ethenoguanosine.

In conclusion, we have prepared a new etheno nucleoside, N^2,3-ethenoguanosine, and its 5′-diphosphate in order to study the possible role of this compound in vinyl chloride induced cancer. While the corresponding base has been synthesized[15] and detected[14] in chloroacetaldehyde-treated DNA, no biological or biochemical studies can be performed using the base. Thus, the present synthesis of a labile compound is an important step in initiation of studies on the mechanism of mutagenesis/carcinogenesis. Of all the etheno derivatives (1,N^6-ϵA, N^2,3-ϵG, 1,N^2-ϵG, 3,N^4-ϵC), only N^2,3-ϵG has the potential to form canonical Watson–Crick hydrogen bonds with other bases.

Experimental Section

The structural assignments were supported by FAB–mass spectrometry performed on a Kratos MS-50 mass spectrometer equipped with a Kratos FAB ion source operating at 7-kV accelerating voltage.

Proton NMR spectra were run on a Varian 500 spectrometer at ambient temperature and 0.02 M concentration in $(CD_3)_2SO$. Chemical shifts are according to the strongest $(CD_3)_2SO$ peak, taken as 2.50 ppm.

Fluorescence spectra were determined with a Perkin-Elmer 650-105, 1-cm path, 3-mm slits with a sensitivity of 10. Spectra are uncorrected. Quench of N^2,3-ethenoguanine and the riboside was determined by observing the riboside spectra at pH 7, followed by acid depurination (pH 1) and then neutralization to pH 7. Free-base spectra (4.4×10^{-6} M) were the control.

Resolution and sequential detection of the free base **5** and nucleoside **6** by UV and fluorescence were achieved on a Bondapak C_{18} column (300 × 3.9 mm, 10 μm) coupled with a Waters Model 440 UV detector and a Perkin-Elmer LS-5 fluorescence spectrophotometer connected in tandem.

Nucleoside **6** elution was in 10 mM K_2HPO_4, pH 6.5, at a flow rate of 2 mL/min. UV detection was at 254 nm; fluorescence excitation at 265 nm; emission at 430 nm.

Elution of free base **5** from the column was in 10 mM NH_4COOH, pH 3.8, at a flow rate of 2 mL/min. UV absorption was measured at 254 nm; fluorescence excitation at 265 nm; emission at 400 nm.

For spectral measurements of the protected substrates and final products, UV measurements and pK_a determinations were done in Britton–Robinson buffers at room temperature, using a Varian–Cary 219 spectrophotometer.

Solvents for thin-layer chromatography (TLC): system I, chloroform–methanol (8:2), silica gel plates; system II, 2-propanol–H_2O–concentrated NH_4OH (7:1:1), silica gel plates; system III $(NH_4)_2SO_4$(saturated)–H_2O–2-propanol (8:2:0.2), cellulose plates.

Bromoacetaldehyde. A mixture of 5 mL of bromoacetaldehyde diethyl acetal (Merck), 15 mL of 1 N HCl, and 5 mL of ethanol was stirred 3 days at 37 °C. No phase separation occurs when the mixture is brought to room temperature. This solution, containing approximately 1.3 mmol of bromoacetaldehyde in 1 mL, was stored at −20 °C and used for reactions without further treatment.

***N*²,3-Etheno-*O*⁶-ethylguanosine (4a).** Bromoacetaldehyde (10 mL of about 1.3 M solution) was added to the solution of **2a**·$2H_2O$[15] (1.5 g, 4.3 mmol) in the mixture of 30 mL of 1 M sodium acetate buffer (pH 4.5) and 15 mL of ethanol, and the reaction mixture was kept at 37 °C. After 48 h, TLC in system I showed

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the lack of substrate **2a** (R_f 0.50) and the presence of the major product **4a** (R_f 0.35) as well as several minor products. The mixture was evaporated to about half-volume to remove ethanol and 50 mL of Dowex 1X4 200/400 in HCO_3^- form was added in order to neutralize the mixture. The resulting slurry was degassed under vacuum and applied on the 2.6 × 45 cm Dowex 1X4 200/400 column in HCO_3^- form. The column was eluted with a 0–50% linear gradient of 2-propanol in water, and 20-mL fractions were collected at 9-min intervals. The main peak (fractions 61–80, 27–36% 2-propanol) containing 14 000 A_{270}^{pH7} units was evaporated, and the residue was crystallized from the mixture of boiling 95% aqueous ethanol (20 mL) and methanol (5 mL) to give 400 mg (27%) of **4a** in hydrated form, mp 154–157 °C. The mother liquor gave, after recrystallization, an additional 140 mg of chromatographically pure **4a**. The total yield was 36%.

4a: 8.34 (H8), 7.94 and 7.45 ppm (H5 and H6) [2.0 Hz (J_{56})], 4.51 (CH_2), 1.39 (CH_3), 6.17 (H1′), 4.43 (H2′), 4.14 (H3′), 4.06 (H4′), 3.65 (H5′), 3.56 ppm (H5″) [5.0 ($J_{1'2'}$), 5.0 ($J_{2'3'}$), 4.5 ($J_{3'4'}$), 3.2 ($J_{4'5'}$), 3.6 ($J_{4'5''}$), 12.4 Hz ($J_{5'5''}$)]; (pH 7.2) λ_{max} 229 nm (26 000), λ_{min} 250 (4800), λ_{max} 266 (br, 264–272 nm) (5900), (pH 12) λ_{max} 229 nm (26 100), λ_{min} 250 (5900), λ_{max} 266 (br, 264–274 nm) (5700), (pH 2) λ_{max} 222 nm (25 800), λ_{min} 237 (4900), λ_{max} 266 (9500); FAB–MS, 336 (M + 1), 204 (B + 1), 176 (B + 1 − ethyl). Anal. Calcd for $C_{14}H_{17}O_5N_5 \cdot 0.75H_2O$: C, 48.20; H, 5.35; N, 20.08. Found: C, 48.34; H, 5.12; N, 19.95.

N^2,3-Ethenoguanine[15] [8,9-Dihydro-9-oximidazo[2,1-*b*]purine (5)]. N^2,3-Etheno-O^6-ethylguanosine (**4a**; 200 mg, 0.6 mmol) was treated with 10 mL of 6 M HCl on a steam bath for 6 h. The resulting dark solution was evaporated to dryness, and the residue was evaporated three times with water and once with concentrated NH_4OH. The residue was treated with charcoal in 20 mL of boiling 50% aqueous methanol, and the hot solution was filtered. The filtrate after standing overnight at room temperature deposited crystals in the form of small needles that were collected by filtration to give 30 mg (29%) of **5**. The crystals changed their shape at about 290 °C but did not melt up to 360 °C.

5: 8.12 (H8), 7.63 and 7.15 ppm (H5 and H6) [2.0 Hz (J_{56})]; FAB–MS, 176 (M + 1). Anal. Calcd for $C_7H_5N_5O \cdot 0.25H_2O$: C, 46.8; H, 3.09; N, 38.99. Found: C, 46.50; H, 2.97; N, 38.51.

Preparation of Phenyldiazomethane. Sodium ethoxide (40 mL, about 1.5 M in 99.8% ethanol) was added stepwise to a stirred suspension of 10 g of *N*-benzyl-*N*-nitrosourea[27] in 200 mL of 99.8% ethanol until the reaction mixture became alkaline, then a minute amount of *N*-benzyl-*N*-nitrosourea was added to bring the reaction to neutrality. The precipitate was filtered off, and the dark red solution of phenyldiazomethane was used for alkylation of guanosine.

***O*^{6}-Benzyl- and *N*-1-Benzylguanosine (2b and 3).** A solution of 200 mL of phenyldiazomethane in ethanol was added dropwise to a suspension of guanosine (**3**; 3 g, 10.6 mmol) being refluxed in methanol over a 3-h period. Refluxing was then continued for another 4 h until the reaction mixture became essentially homogeneous and colorless. The precipitate that appeared after standing overnight at room temperature was filtered off. TLC in system I showed that the precipitate consisted mostly of unreacted **1** (R_f 0.0) and other other polar products. The filtrate contained **2b** (R_f 0.55) and **3** (R_f 0.30) as the major components, which were identified on the basis of UV spectra.[17] O^6-Benzylguanosine (**2b**) (λ_{max} 249 nm and 282, 25 200 A_{282}^{pH7} units, 2.4 mmol, 23%) and *N*-1-benzylguanosine (**3**) (λ_{max} = 258 nm, 27 200 A_{258}^{pH7} units) were separated by chromatography on preparative silica gel plates in system I and eluted with methanol. Several attempts to crystallize either derivative were unsuccessful due to gel formation. Therefore, O^6-benzylguanosine was used in succeeding steps without further purification.

A sample of **2b** was quantitatively hydrogenated to the parent guanosine (**1**) within 3 h in the presence of 5% Pd/C at atmospheric pressure and at room temperature. The *N*-1-benzyl isomer **3** was completely resistant to hydrogenolysis under these conditions during several hours.

N^2,3-Etheno-O^6-benzylguanosine (4b). Bromoacetaldehyde (10 mL of about 1.3 M solution) was added to a solution of O^6-benzylguanosine (**2b**) (23 500 A_{282nm}^{pH7} units, 2.2 mmol) in a mixture of 60 mL of 1 M sodium acetate buffer, pH 4.5, and 30 mL of ethanol. After 24 h at 37 °C, TLC in system I showed some substrate (**2b**, R_f 0.55), the presence of the major product **4b** (R_f 0.40), and several minor products. The mixture was evaporated at 35 °C to about half-volume to remove ethanol, and 80 mL of Dowex 1X8 200/400 in HCO_3^- form was added in order to neutralize the mixture. The resulting slurry was degassed under vacuum and applied to a 3.5 × 16 cm Dowex 1X8 200/400 column in HCO_3^- form. The column was washed with 400 mL of water and then eluted with a linear gradient of 0–75% 2-propanol in water, and 20-mL fractions were collected at 5-min intervals. The main peak (fractions 66–120, 35–75% 2-propanol) containing 9700 A_{270nm}^{pH7} units of **4b** was evaporated and found by TLC to be >95% pure. The residue was crystallized from 100 mL of boiling ethanol to give 217 mg (25%) of **4b** in the form of microneedles, mp 179–181 °C.

4b: 8.35 (H8), 7.95 and 7.47 ppm (H5 and H6) [2.0 Hz (J_{56})], 5.56 (CH_2), 7.52 and 7.38 (phenyl ring), 6.18 (H1′), 4.43 (H2′), 4.14 (H3′), 4.07 (H4′), 3.67 (H5′), 3.56 ppm (H5″) [5.0 Hz ($J_{1'2'}$), 5.0 ($J_{2'3'}$), 4.5 ($J_{3'4'}$) 3.2 ($J_{4'5'}$), 3.6 ($J_{4'5''}$), 12.4 Hz ($J_{5'5''}$)]; (pH 7.2) λ_{max} 229 nm (32 900), λ_{min} 250 (5500), λ_{max} 266 (br, 264–274) (6800), (pH 12) λ_{max} 231 nm (32 500), λ_{min} 251 (5300), λ_{max} 268 (br, 264–274 nm) (6700), (pH 2) λ_{max} 221 nm (37 000), λ_{min} 238 (5700), λ_{max} 262 (12 000); FAB–MS, 398 (M + 1), 266 (B + 1), 176 (B + 1 − benzyl). Anal. Calcd for $C_{19}H_{19}O_5N_5$: C, 57.34; H, 4.82; N, 17.62. Found: C, 57.52; H, 4.62; N, 17.50.

N^2,3-Ethenoguanosine [8,9-Dihydro-9-oxo-3-β-D-ribofuranosylimidazo[2,1-*b*]purine (6)]. N^2,3-Etheno-O^6-benzylguanosine (**4b**; 155 mg, 0.38 mmole) was hydrogenated at room temperature (1 atm) in the presence of 5% Pd/C (80 mg) in 350 mL of 85% aqueous ethanol for 20 h. TLC in system II showed over 95% transformation of the substrate (R_f 0.60) to **6** (R_f 0.23). The mixture was filtered and the filtrate evaporated to dryness. The residue was dissolved in a small amount of methanol with addition of concentrated aqueous NH_4OH, and this solution was applied on four TLC 20 × 20 silica gel 60 F_{254} plates with concentration zone (Merck, Art. 11798). The plates were run in system II, and the product was eluted with methanol. The eluate was concentrated to a small volume and filtered through a fine sintered glass funnel and the filtrate evaporated to dryness. The residue contained 3200 A_{258nm}^{pH7} units (0.3 mmol, 80%) of chromatographically pure **6**, which was used for further reactions.

A sample for analytical purposes was obtained by dissolving on a steam bath 1600 A_{258nm}^{pH7} units of **4b** in 1.5 mL of 50% aqueous ethanol, with addition of a few drops of concentrated NH_4OH. The solution was kept in an open flask at 37 °C for 2 days. The amorphous precipitate, which was formed during slow evaporation (final volume about 0.5 mL) was collected by filtration and dried under vacuum at 37 °C in the presence of P_2O_5. The yield of **6** in hydrated form was 20 mg. This material softens at about 170 °C without melting and changes into colorless needles at about 250 °C, which melt at 320 °C.

6: 8.20 (H8), 7.65 and 7.13 ppm (H5 and H6) [2.0 Hz (J_{56})], 6.03 (H1′), 4.41 (H2′), 4.11 (H3′), 4.04 (H4′), 3.65 (H5′), 3.55 ppm (H5″) [5.0 ($J_{1'2'}$), 5.0 ($J_{2'3'}$), 4.5 ($J_{3'4'}$), 3.2 ($J_{4'5'}$), 3.6 ($J_{4'5''}$), 12.4 Hz ($J_{5'5''}$)]; (pH 7.2) λ_{max} 225 nm (29 700), λ_{min} 244 (9000), λ_{max} 258 (10 700), (pH 12) λ_{max} 235 nm (29 700), λ_{min} 262 (6700), λ_{max} 270 (6800), (pH 2) λ_{max} 221 nm (27 700), λ_{min} 244 (8800), λ_{max} 252 (9100)° pK_a = 8.85 and pK_a = 2.5 (compound unstable at low pH); UV, see Figure 2; FAB–MS, 308 (M + 1), 176 (B + 1). Anal. Calcd for $C_{12}H_{13}O_5N_5 \cdot 1.5H_2O$: C, 43.12; H, 4.82; N, 20.95. Found: C, 42.99; H, 4.80; N, 20.75.

N^2,3-Ethenoguanosine 5′-Phosphate. The general conditions for the enzymatic phosphorylation of **6** were as described by Singer et al.[28] A 2 × 16 cm column packed with Dowex 1X4 200/400 HCO_3^- form was used with a linear gradient from 600 mL of water and 600 mL of 1 M triethylammonium carbonate (TEAB) at a flow rate of 4 mL/min for separation of the products from the phosphorylation of 930 A_{258nm}^{pH7} units (0.09 mmol) of **6**. The middle of the peak of inorganic phosphate appeared at 0.35 M TEAB, nucleoside at 0.60 M TEAB, 5′-nucleotide at 0.75 M TEAB, and

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p-nitrophenyl phosphate at 0.85 M TEAB. The yield of N^2,3-ethenoguanosine 5'-phosphate was 20%, but the nucleotide was contaminated by some nucleoside. The nucleoside was recovered, and phosphorylation was repeated twice. The total yield of pure N^2,3-ethenoguanosine 5'-monophosphate after three runs and rechromatography of pooled nucleotide fractions on the same column was 370 A^{pH7}_{258nm} units (40%).

N^2,3-Ethenoguanosine 5'-Diphosphate. The general synthetic procedure was according to Hoard and Ott,[24] using triethylammonium phosphate instead of tributylammonium pyrophosphate. The products from the reaction of 370 A^{pH7}_{258nm} units (0.035 mmol) of N^2,3-ethenoguanosine 5'-phosphate were separated on a 2 × 16 cm column packed with DEAE Sephadex A-25 (HCO_3^- form) with a linear gradient from 600 mL of H_2O to 600 mL of 0.5 M TEAB and flow rate 4 mL/min. N^2,3-Ethenoguanosine 5'-phosphate, 90 A^{pH7}_{258nm} units (24%), appeared as a sharp peak at 0.40–0.42 M TEAB. Fractions containing ethenoguanosine 5'-diphosphate were evaporated three times with water. Without further treatment the aqueous solution of triethylammonium salt of N^2,3-ethenoguanosine 5'-diphosphate was used for the preparation of polynucleotides. The structure of N^2,3-ethenoguanosine 5'-diphosphate was confirmed by limited digestion by bacterial alkaline phosphatase, which hydrolyzed stepwise yielding the nucleoside 5'-diphosphate, 5'-monophosphate, and finally the nucleoside (R_f 0.43, 0.36, and 0.24, respectively, in system III).

Preparation and Analysis of Polynucleotides. N^2,3-Ethenoguanosine 5'-diphosphate was copolymerized with varying proportions of CDP or ADP as described by Singer et al.[28,29] Analyses, after enzymatic digestion to nucleosides,[30] were performed on a BioRad HPLC system, which will be described in detail in a paper on the properties of such polynucleotides (Singer et al., *Carcinogenesis*, in press).

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Synthetic Studies on Cembranolides. Stereoselective Total Synthesis of Isolobophytolide†

James A. Marshall,* Robert C. Andrews, and Lukasz Lebioda‡

Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208

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The synthesis of (±)-isolobophytolide starting from diol **1**, the coupling product of the geraniol-derived sulfone IX and the epoxy alkoxide X, has been achieved. Macrocyclization was accomplished via the π-allyl palladium complex of the allylic pivalate **8**. The derived carboxylic acid **11** underwent carboxy inversion to the cis lactone **32**. Subsequent conversion to the trans lactone **42** followed by internal epoxidation to **44** and lactone α-methylenation completed the synthesis. Alternative methods were developed for carboxy inversion and α-methylenation.

Since their initial recognition some 25 years ago,[1a-c] the cembrane diterpenes have emerged as a major class of natural products with widespread origins in both the plant and animal kingdoms.[1d] In the latter category, Caribbean gorgonians and Pacific soft corals account for the greatest diversity of cembrane structures. These "cembranolides", with their characteristic fused and bridged lactone groupings, represent the most structurally complex and biologically interesting members of the entire family. To date, synthetic work has focused on the relatively simple cembranes.[2] Cembranolides have received only scant attention.[3]

One of our first efforts in the cembranolide area was directed toward isolobophytolide, the major terpenoid component of the soft coral *Lobophytum crassum*.[4] Bowden et al. initially proposed a cis-fused γ-lactone structure for isolobophytolide.[4] We succeeded in synthesizing this cis-fused lactone from the abundant natural cembranolide crassin acetate (Figure 1).[5] However, the spectral properties of our synthetic lactone clearly differed from those reported for natural isolobophytolide.[4] A major discrepancy was the chemical shift of the lactone carbinyl proton H14, which appeared at 4.7 ppm in our synthetic material vs. 4.1 ppm for natural isolobophytolide. It was subsequently found through single-crystal X-ray structure analysis that isolobophytolide is a trans-fused lactone with the relative stereochemistry depicted in I (Figure 2).[6]

† Dedicated to Prof. William G. Dauben in commemoration of the 25th anniversary of his structure elucidation of cembrene.

‡ To whom inquiries regarding X-ray structure analysis should be directed.

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