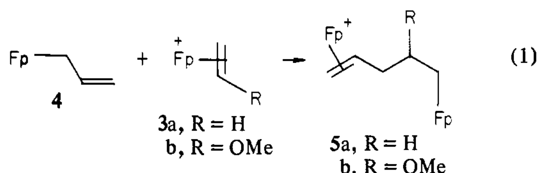
bond and the p oribital on C_β. Furthermore the C_α–C_β rotational barrier, determined from the coalescence temperature (–60 °C) of the AB set of vinyl proton resonances, is 10.5 kcal·mol^{-1}, well above the value expected for a relatively uncrowded σ-bonded complex.[8]

The vinyl ether complex is far less distorted. The complex cation is disordered about a crystallographic mirror plane which relates the α-carbon atom of the ligand to (approximately) a carbonyl carbon atom; the location of the β-carbon atom is largely unaffected by this disorder. The disorder is not completely resolved and thus has the effect of *lengthening* the Fe–CO distance and *shortening* the Fe–C_α distance. A realistic value for the latter distance is ~2.20 Å, based on structure determinations on Fp(tetramethylallene)[10] and Fp(propene).[11] Hence a lower limit for the value of the difference between the Fe–C_α and Fe–C_β distances is 0.12 Å. The greater olefinic character of the C_α–C_β bond is also evidenced by the NMR spectrum of the cation, which does not show line broadening of its AB set of proton resonances up to 80 °C. The C_α–C_β rotational barrier is estimated to be greater than 18 kcal·mol^{-1} in this cation.[12]

A measure of the chemical effect of these distortions is provided by a comparison of the reactivity of complexes **3a**,**3b**, and **3c** toward nucleophiles. In the absence of other electronic effects substitution of a vinyl hydrogen atom in **3a** by a group 5 or 6 heteroatom would be expected to result in decreased reactivity, since the interaction of the filled heteroatomic orbital with both vacant (a_1–$\lambda'\pi$) and (π^*–λb_2)[1] orbitals must raise their energies and increase their delocalization. Furthermore, such interactions should be more important for **3c** than for **3b**, since the energy levels of interacting orbitals are closer for the amine complex. The effects of molecular distortions such as those postulated by Eisenstein and Hoffmann[1] may be expected to countervail this reactivity sequence, although it is not clear that the theoretical treatment is applicable to the vinylamine complex since the iron atom is more nearly σ bonded to the ligand in this substance.[13]

Decreased reactivity of **3c** compared with **3b** is observed in reactions with water. The amine complex is inert to hydrolysis at room temperature and can be recovered from its aqueous solutions unchanged even after warming to 40 °C, while **3b** is instantaneously hydrolyzed at room temperature to give $FpCH_2CHO$. The reactivities of **3a**, **3b**, and **3c** with a relatively soft nucleophile such as **4** (eq 1), for which frontier orbital in-



teractions would be expected to be more important, is of greater interest. Both **3a** and **3b** condense rapidly with **4** at room temperature to give dinuclear complexes **5a** and **5b**, but **3c** fails to react on prolonged standing or even on warming to 40 °C. Interactions of the heteroatom lone-pair orbital with the ligand LUMO appear to play a dominant role in the reactivity of the amine complex. Significantly, this does not appear to be so for **3b**. The half-life for the reaction of **3a** with **4** at 10 °C is 82 min, while that for **3b** for the same concentration of reactants, but at –3 °C, is 3 min. A comparison of rate constants, obtained for the reaction of **4** with **3a** from 10 to 30 °C and extrapolated to –3 °C, with that for **3b** at this temperature shows that the vinyl ether complex is 530 times more reactive than the ethylene complex.[14]

The results provide the first experimental evidence that molecular distortions in metal–olefin complexes, of the form defined by Eisenstein and Hoffmann,[1] may play a significant role in determining their reactivity with nucleophiles.

Acknowledgment. This research was supported by a grant from the National Science Foundation (CHE-7816863) which is gratefully acknowledged.

Supplementary Material Available: Tables of atomic coordinates for complexes **3b** and **3c** (2 pages). Ordering information is given on any current masthead page.

(7) Where there are favorable steric effects,[5] the Fe–C–C–X torsion angle is quite close to 90°, while when no possible interaction exists, e.g., in similar complexes with saturated ligands, the torsion angle is 180°: Pope, L.; Sommerville, P.; Laing, M.; Hindson, K. J.; Moss, J. R. *J. Organomet. Chem.* **1976**, *112*, 309.

(8) Coalescence temperatures were determined at 90 MHz in CD_2Cl_2 solution over a temperature range of –103 to +10 °C. The rotational barrier was calculated by using the approximate equation $k_c = \pi\Delta\nu/2^{1/2}$, which has been shown to give reliable values of free energies of activation for coalescence of uncoupled pairs of doublets.[9] We are indebted to Dr. W. Priester for carrying out the measurement.

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(12) The C_α–C_β rotational barrier in Fp(isopropenyl methyl ether)BF_4 is estimated from NMR coalescence experiments to be 14.8 kcal·mol^{-1}.

(13) For example, the M–C_α–C_β bond angle in **3c** (104.2°) is close to that expected for a σ complex, while the theoretical treatment of Eisentstein and Hoffmann retains a maximum angle of 90° for the most distorted model system.

(14) Reactions were carried out on BF_4^- salts and were monitored by NMR spectrometry, employing a Bruker W-90 spectrometer. All reactions were carried out in CD_3NO_2 solutions and followed over 2–3 half-lives. Rate plots exhibited good second-order behavior throughout. The activation energy calculated from these measurements for the reaction of **3a** with **4** is 19.4 kcal·mol^{-1} (log A = 11.8).

Identification of Three Alkylated Nucleotide Adducts from the Reaction of Guanosine 5′-Monophosphate with Phosphoramide Mustard

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The chemical basis of the induction of the cytotoxic, mutagenic, and carcinogenic effects of alkylating agents is generally believed to be the covalent reaction with cellular DNA. Thus elucidation of molecular modification involved in the interactions of these agents with nucleic acids is of primary importance. The advent of a new ionization technique, fast atom bombardment (FAB) mass spectrometry,[1] has permitted us to identify successfully a series of alkylated nucleotide adducts from the reaction of guanosine 5′-monophosphate with phosphoramide mustard.

The bifunctional alkylating agent phosphoramide mustard was first synthesized in 1959 and has been shown to be active against a wide variety of animal tumors.[2] It was later isolated as an in vitro[3] and an in vivo[4] metabolite of the anticancer drug cyclophosphamide. It is now believed that the interaction of phosphoramide mustard with nucleic acids is responsible for the cytotoxic effect of the parent drug, cyclophosphamide. While cross-linking of DNA by phosphoramide mustard has been re-

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Table I. Capacity Factors (k') of Guanosine 5'-Monophosphate and Derivatives[a]

compound	k'
guanosine 5'-monophosphate	1.6
7-methyl-guanosine 5'-monophosphate	3.9
adduct I	9.1
adduct II	2.6
adduct III	1.0

[a] High-performance liquid chromatography analyses were carried out on an Altex Model 322MP. Capacity factors were determined isocratically using an Ultrasphere (5 μm) ODS column (15 cm × 4.6 mm) from Altex and a precolumn of CO:Pell ODS resin (Whatman, Inc.) eluted with 0.001 M formic acid (pH 3.5) at a flow rate of 1.0 mL/min.

ported,[5] no attempts were made to identify the sites of alkylation or the nature of the substituents. Recently, guanosine and deoxyguanosine phosphoramide mustard adducts bonded at the 7-position were isolated and identified by field desorption mass spectrometry.[6] These findings prompted us to study the alkylation reaction at the nucleotide level. We chose to react phosphoramide mustard with guanosine 5'-monophosphate since the guanine bases of DNA and RNA have been shown to be alkylated by most alkylating agents and have been postulated to be the sites of DNA cross-linking by bifunctional nitrogen mustards.[7] We wish to report the isolation of a cross-linked dimer, *N*,*N*-bis[2-(5'-phospho-7-guanosinyl)-ethyl]phosphorodiamidic acid (adduct III), and two simple adducts, *N*-(2-chloroethyl)-*N*-[2-(5'-phospho-7-guanosinyl)-ethyl]phosphorodiamidic acid (adduct I) and *N*-(2-chloroethyl)-*N*-[2-(5'-phospho-7-guanosinyl)-ethyl]amine (adduct II).

Phosphoramide mustard reacted readily with guanosine 5'-monophosphate in aqueous solution in a pH range 4.0–7.4 to yield two intact guanosine 5'-monophosphate–phosphoramide mustard adducts (I, III) and a guanosine 5'-monophosphate–nornitrogen mustard adduct (II). However, the best yields were achieved at pH 4.6. These adducts are extremely unstable in either acidic or basic media. Acidic conditions lead to deribosylation (cleavage of the *N*-glycosyl bond), and scission of the imidazole ring occurs under basic conditions. Successful stabilization of these adducts was achieved in the pH range 3.5–5.0. Therefore, 30 mg of guanosine 5'-monophosphate was reacted with 100 mg of phosphoramide mustard in 1.0 mL of 0.1 M sodium acetate buffer (pH 4.5) at 37 °C for 1 h. The reaction mixture was immediately separated by reverse-phase high-performance liquid chromatography using an Altex Ultrasphere (5 μm) ODS column (15 cm × 4.6 mm). This column was eluted with a linear gradient of methanol (0–15% in 15 min) in 0.005 M KH_2PO_4 (pH 4.5) at a flow rate of 1.0 mL/min. Major (I) and minor (II) adducts which amounted to approximately 10% of unreacted guanosine 5'-monophosphate coeluted much later than guanosine 5'-monophosphate. Adduct III, formed only in small quantities, eluted earlier than guanosine 5'-monophosphate. Fractions containing the adducts were collected and lyophilized overnight. Adducts I and II were then separated and purified by chromatography on the same column equilibrated with 0.001 M formic acid (pH 3.5). Under this chromatographic condition, adduct II eluted much earlier than adduct I. The order of elution as shown in Table I suggests that the order of polarity is adduct III > guanosine 5'-monophosphate > adduct II > adduct I, which indeed correlates with the chemical structures as assigned below.

The site of alkylation of the nucleotide was determined by ultraviolet spectrophotometry (Table II). The ultraviolet spectra of I, II, and III were obtained at pH 1.0, 7.0, and 13.0 and are typical of 7-substituted-guanosine 5'-monophosphate, shown by the irreversible spectral change in alkali, a consequence of opening

Table II. Ultraviolet Maxima (nm) of Guanosine 5'-Monophosphate and Derivatives[a]

compound	pH 1.0[b]	pH 7.0[c]	pH 13.0[d]
guanosine 5'-monophosphate	256 (276)	253 (270)	258–268
7-methyl-guanosine 5'-monophosphate	258, 280	256, 279	265
adduct I	258, 281	256, 280	265
adduct II	257, 282	256, 281	265
adduct III	257, 282	256, 279	265

[a] Ultraviolet spectra were recorded with a Varian Cary 15 spectrophotometer. [b] 0.1 N HCl. [c] 0.1 M potassium phosphate. [d] 0.1 N NaOH.

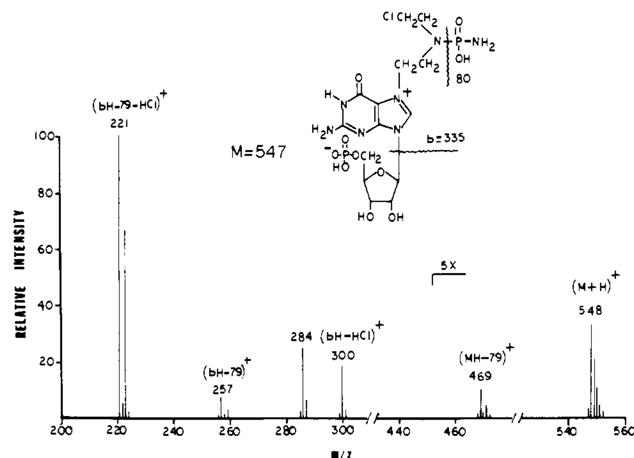

Figure 1. Positive-ion FAB mass spectrum of adduct I.

of the imidazole ring. The presence of the phosphorodiamidic group in adduct I was confirmed by ^{31}P NMR. The ^{31}P NMR spectra were recorded on a 100-MHz JEOL FX-100 instrument equipped with a fast Fourier transform unit. The spectrum of adduct I contains two signals which occur at 1.23 and 12.91 ppm with respect to the reference compound phosphoric acid, corresponding to the nucleotide phosphate and the phosphorodiamidic acid, respectively. The structures of these adducts were finally established by FAB mass spectrometry. Mass spectral analysis was performed on a Kratos MS-50 mass spectrometer equipped with a Kratos FAB ion source operating at 8-kV accelerating voltage. Samples were deposited in glycerol pastes onto the probe tip and irradiated by a beam of argon atoms derived by neutralizing ions which had been accelerated through 4 kV in a beam current of 0.4 mA. In our hands, field desorption mass spectra of these samples were not readily measurable.

The spectrum of adduct I (Figure 1) contains a protonated molecular ion peak $(M + H)^+$ at m/z 548 and an isotopic peak at m/z 550, evidence for the presence of chlorine. This molecular weight is consistent with a structure in which guanosine 5'-monophosphate has reacted with one arm of phosphoramide mustard, leaving the other reactive group intact. Additional information was obtained from fragment ions at m/z 469 and 471, which arise from the cleavage of the N–P bond in the molecular ions. The peaks at m/z 300, 257, and 221 correspond to ions whose formation involves cleavage of the *N*-glycosyl bond, representing $(bH - HCl)^+$, $(bH - 79)^+$, and $(bH - HCl - 79)^+$, respectively. Fragment ions of mass 284 appear to comprise the protonated guanosine residue. The facile loss of even-electron neutral residues observed here appears to be characteristic of fast atom bombardment spectra.

The mass spectrum of adduct II has a similar fragmentation pattern to that of adduct I. Here we observed molecular ion peaks at m/z 469 and 471, which correspond to the structure of a guanosine 5'-monophosphate–nornitrogen mustard adduct (Figure 2). Peaks at m/z 257 and 259 represent $(b + H)^+$ formed by loss of the sugar–phosphate moiety. Further elimination of HCl gives rise to the base peak at m/z 221. The peak at m/z 284 is characteristic of the protonated guanosine residue.

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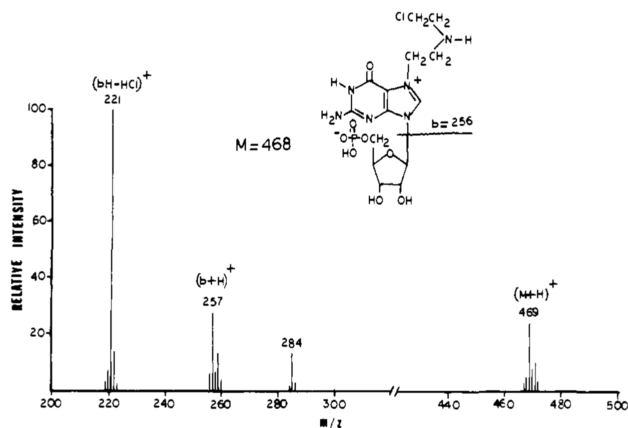

Figure 2. Positive-ion FAB mass spectrum of adduct II.

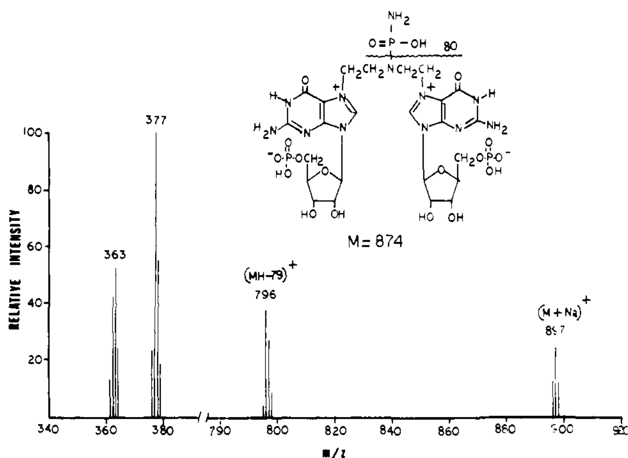

Figure 3. Positive-ion FAB mass spectrum of adduct III.

Adduct III produced an ion of mass 897 $(M + Na)^+$, which corresponds to the structure of a dimer adduct in which each arm of phosphoramide mustard has reacted with guanosine 5′-monophosphate at the 7-position (Figure 3). The fragment ion of mass 796 arises from the loss of the phosphorodiamidic group $(M + H - 79)^+$ as the result of N–P bond cleavage. Other peaks at m/z 363 and 377 probably represent the ions $(GMP + H)^+$ and $(7\text{-methyl-GMP})^+$.

We conclude that we have isolated three N-7-alkylated nucleotide adducts whose structures are shown in Figures 1–3. In agreement with a previous report,[6] we found guanosine nucleotide to be readily alkylated by phosphoramide mustard at the 7-position. Adduct II could arise either by scission of the N–P bond in phosphoramide mustard and subsequent alkylation of guanosine 5′-monophosphate by nornitrogen mustard or by alkylation of guanosine 5′-monophosphate by phosphoramide mustard and subsequent scission of the N–P bond.

Our study demonstrates that phosphoramide mustard, like other nitrogen mustards,[7,8] will alkylate N-7 of guanosine 5′-monophosphate. The cytotoxic effect of cyclophosphamide and phosphoramide mustard could be mediated by two major types of damage to DNA: depurination (deribosylation) of adducts I and II which could lead to chain scission and by interstrand and intrastrand cross-linking analogous to that of adduct III.

Acknowledgment. We are grateful to Dr. Ven L. Narayanan (NCI) for a generous gift of phosphoramide mustard. Mass spectral determinations were carried out at the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation Regional Instrumentation Facility. NMR analyses were made by Dr. Lou Kan in the Johns Hopkins School of Hygiene. This work was supported by NIH grants GM-21248 and CA-16783 and training grant T32-CA-09243.

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A Bacteriorhodopsin Analogue Containing the Retinal Nitroxide Free Radical

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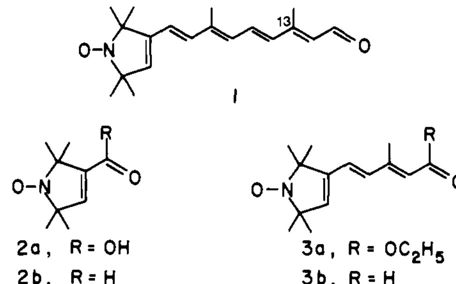
Synthetic analogues of retinal have been used to study the binding site and photochemistry of both the visual pigment rhodopsin[1] and the bacteriorhodopsin[2] membrane of *Halobacterium halobium.* Nitroxides are excellent "reporter" groups for studying protein environments by the use of electron spin resonance (ESR) spectroscopy. A retinal derivative which contains a nitroxide functional group as well as forms a pigment with the bleached purple membrane or with the visual pigment apoprotein, opsin, would indeed be a valuable probe of the binding site. We have reported the synthesis of one spin-labeled retinal and the pigment analogue formed between it and the bleached purple membrane.[3] The information which could be gained from this analogue was limited as the label was linked to the retinal molecule by an ester bond which hydrolyzed readily after pigment formation. We report here the synthesis of the nitroxide retinal **1**, which contains the *N*-oxide incorporated into the ring of the retinal. The properties of the stable pigment formed from the reaction of the 13-cis or all-trans isomers of **1** and the bleached purple membrane are discussed.



Esterification of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid (Eastman) (**2a**) with diazomethane followed by lithium aluminum hydride reduction and manganese dioxide oxidation, both in dry tetrahydrofuran, afforded the corresponding aldehyde **2b**[4] (44%). Condensation of **2b** with triethyl 4-phosphono-3-methylcrotonate[5] in the presence of sodium amide gave the ester **3a** (77%) which was reduced and oxidized as above to yield the aldehyde **3b** (39%) which was purified by preparative thin-layer chromatography (TLC). Anal. Calcd for $C_{14}H_{20}NO_2$: C, 71.75; H, 8.62; N, 5.98. Found: C, 71.59; H, 8.70; N, 6.01. The aldehyde **3b** was condensed, reduced, and oxidized as above to yield the spin-labeled retinal **1** in 41% yield. The product was purified by TLC and high-pressure liquid chromatography (HPLC) using 20% ethyl acetate/hexane on a μ-Porasil column. Two major isomers were obtained and identified as the 13-cis and trans isomers by elution profile on HPLC (13.5 and 15.2 min, respectively) and pigment formation capability. The mass spectrum of the two isomers each had a parent peak at m/e 300 (70 eV).

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