Covalent Modification of Proteins and Peptides by the Quinone Methide from 2-*tert*-Butyl-4,6-dimethylphenol: Selectivity and Reactivity with Respect to Competitive Hydration

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Covalent modification of cellular nucleophiles by electrophilic metabolites has been shown to be an important pathway in the toxicological activity of many xenobiotic compounds. The *p*-quinone methide (4-allylidene-2,5-cyclohexadien-1-one, QM) oxidative metabolites of the 4-alkylphenols are a chemical family whose toxicology has been well described. The reactivity of the QM from 2-*tert*-butyl-4,6-dimethylphenol (BDMP−QM) with respect to hydration has been studied in the 3 < pH < 11 range in order to examine selectivity for competitive reaction with biological nitrogen nucleophiles. The reactivity of BDMP−QM was examined with human hemoglobin A (HbA), angiotensin-III (AIII), amino acids, and amines. Nucleophilic selectivity (k_{Nu}/k_{H_2O}) and reactivity (k_{Nu}) in aqueous solution (7 < pH < 8) for addition of these biological nucleophiles to BDMP−QM demonstrate (i) that adduct formation is competitive with hydration at low nucleophile concentrations and (ii) that proteins, peptides, and tyrosine show enhanced reactivity toward QMs, relative to simple amino acids and related compounds. All adducts were characterized by electrospray mass spectrometry (ESMS). These results provide strong evidence supporting the toxicity of QM metabolites via facile alkylation of nucleophilic nitrogen sites in proteins and peptides, even when present in low concentration in aqueous solution under physiological conditions.

Introduction

The quinone methide (QM) oxidative metabolites of the 4-alkylphenols form a chemical family whose toxicology has been well reported.[1] Roles for QM metabolites have been demonstrated in several endogenous biochemical processes including melanization, sclerotization, and lignin formation.[1,2] In addition, QMs have been implicated as the potential ultimate cytotoxins responsible for the biological effects of antitumor drugs, antioxidants, and several naturally occurring hydroxylated aromatic compounds.[1] They are also of interest as synthetic intermediates in cyclization reactions,[3] as selective DNA alkylators,[4] and as side products of prodrug strategies.[5] In all cases, the mechanism of toxicological action is proposed to be via QM alkylation of biological nucleophiles. Indeed, convincing evidence has been presented to show that QM metabolites could become covalently attached to cellular proteins.[1]

The structural difference between a QM and a quinone is the replacement of one carbonyl by a methylene or substituted methylene group. This substitution results in a much more reactive electrophile and a diminished capacity for redox chemistry.[1] Consequently, reactions of QMs in biological systems are characterized by nonenzymic Michael additions at the exocyclic methylene carbon generating benzylic adducts with biological nucleophiles.[1a] The description of a QM as a resonance-stabilized carbocation emphasizes the importance of the cationic, aromatic resonance contributor and the role of inter- and intramolecular interactions in regulating electron deficiency at the methylene carbon and hence QM reactivity toward nucleophiles.[6,7]

Reaction of QMs with various nucleophiles, although often under nonaqueous, nonphysiological conditions, has been well studied.[8] While these findings demonstrate the wide-ranging reactivity of QMs toward O, S, and N nucleophiles, the issue of chemical selectivity is crucial. A QM metabolite may have one of three fates as an alkylating agent: (1) the QM may react with and not escape from the redox protein in which it was formed; (2) the QM may undergo hydration; and (3) the QM may escape to alkylate a biological nucleophile.[9] The efficacy of QM trapping by glutathione and the isolation of QM−SG conjugates from rat hepatocytes implies high selectivity toward S nucleophiles.[1] But what of nitrogen nucleo-

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(9) In addition, QM solutions made by chemical oxidation of phenols often contain a small amount of dimerized material. Dimerization as a result of oxidation of hindered phenols has been well described: (a) Cook, C. D.; Nash, N. G.; Flanagan, H. R. *J. Am. Chem. Soc.* **1955**, *77*, 1783. (b) Cook, C. D.; Norcross, B. E. *J. Am. Chem. Soc.* **1956**, *78*, 3797. (c) Bauer, R. H.; Coppinger, G. M. *Tetrahedron* **1963**, *19*, 1201.

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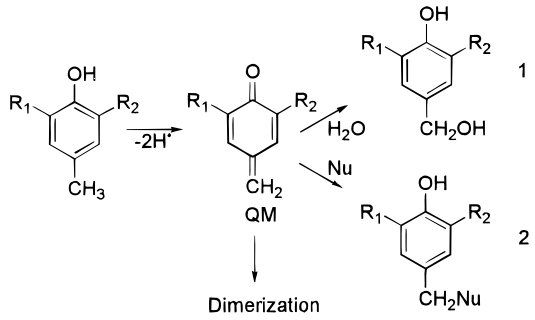
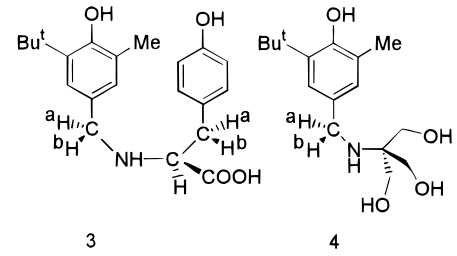
Scheme 1



Scheme 2



philes in aqueous solution? The observation of myoglobin (Mb) alkylation by 2-*tert*-butyl-6-methyl-4-methylene-2,5-cyclohexadienone (BDMP−QM, Scheme 1, R_1 = Bu^t, R_2 = Me), despite the absence of cysteine residues in this protein, demonstrates the ability of a QM to form adducts **2** by alkylation of nitrogen nucleophiles.[10] But, what is the relative reactivity and selectivity of a QM toward the amine nucleophiles of peptides and proteins, in competition with background hydration?

Selectivity is intimately linked with the toxicity of the alkylphenols, which has been correlated with the lifetimes of their corresponding QMs.[11−13] The lifetime of a QM in aqueous solution is governed by its rate of hydration to form benzyl alcohol **1**. BDMP−QM was reported as a highly hepatotoxic QM, possibly because the lifetime of this QM, intermediate relative to other QMs, allows escape from the parent redox protein, but then subsequent reaction with cellular nucleophiles to form adducts (**2**). A comparative study of the reactivity and selectivity of BDMP−QM toward hydration and toward alkylation of the nitrogen nucleophiles of proteins, peptides, amino acids, and related compounds is required. A pH−rate profile, 3.5 < pH < 10.2, has been obtained for BDMP−QM using various oxygen buffers. Nucleophilic selectivity and reactivity in aqueous solution (7 < pH < 8) for reaction of BDMP−QM with human hemoglobin A (HbA), angiotensin-III (AIII), trityrosine tripeptide (Tyr_3), amino acids, and amines have been examined using kinetic analysis and product identification by electrospray mass spectrometry (ESMS). The results provide strong evidence supporting a toxicity pathway for QM metabolites via facile alkylation of nucleophilic nitrogen sites in proteins and peptides.

Experimental Section

Materials. All chemicals were purchased from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) unless stated otherwise. BDMP−QM was synthesized as described previously by lead oxidation of the corresponding phenol.[12] HPLC solvents used were from BDH (Toronto, ON). Purified human Hb A in the CO form (HemAzero) was obtained from Hemosol Inc. (Etobicoke, Ont.). Distilled deionized water was purified using the Barnstead MegaPure MP-1 water purification system.

Alkylations for Nucleophilic Selectivity Study. Stock solutions of nucleophiles were adjusted to pH 7.4 and diluted in water to achieve final molar ratios Nu:QM of 20:1−1:5. Stock solution concentrations were determined spectrophotometrically where possible. The concentration of MeCN solutions of BDMP−QM was determined spectrophotometrically, using ϵ_{286} = 22 400 M^{-1} cm^{-1}.[12] BDMP−QM was added to the nucleophile solutions to achieve a final concentration of 0.2 mM QM and no more than 10% MeCN (v:v). Reactions were allowed to proceed overnight at room temperature.

Chromatography. Aliquots (400 μL) of the product mixtures were analyzed by reversed-phase HPLC at 280 nm using a 2.1 mm × 25 cm Ultrasphere C_{18} column (Beckman) using a linear gradient, from 3% B to 90% B in 45 min (A = 0.1% TFA, B = MeCN, 0.085% TFA) at 0.2 mL/min, on a Shimadzu LC-10A gradient HPLC with SPD-10AV UV detector. All experiments were run in triplicate, and standard errors ranged from 1 to 5%. Results were reproducible between batch preparations of BDMP−QM within 5%. The identities of the putative conjugates of BDMP−QM with nucleophile observed in HPLC chromatograms were confirmed by peak collection and subsequent ESMS analysis, as well as by chromatographic equivalence with synthetic standards in selected instances (Scheme 2). The presence of BDMP phenol as well as small (≤5% of total products by integration of peak areas) amounts of dimer[9] were observed consistently throughout all experiments.

Synthesis and Characterization of Products.

2-*tert*-Butyl-6-methyl-4-(hydroxymethyl)phenol (1). Five hundred mL of DDW was added to 500 mL of a 2.5 mM MeCN solution of BDMP−QM (1.25 mmol), and the mixture was stirred for 8 h. The MeCN was then removed under vacuum, and the remaining aqueous solution was extracted with two volumes of diethyl ether. The ether extracts were combined and washed with two volumes of brine and dried with sodium sulfate and then the ether removed under vacuum. The product was purified using flash chromatography (hexanes/EtOAc) and obtained as 200 mg of white powder (1 mmol, 82%); mp 93−95 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.34 (9H, s, Bu^t), 2.15 (3H, s, ArMe), 4.31 (2H, d, J_{CHOH} = 5.50 Hz, benzyl CH_2), 4.86 (1H, t, J_{OHCH} = 5.50 Hz, benzyl OH), 6.86 (1H, s, ArH), 6.96 (1H, s, ArH), 7.92 (1H, s, phenol OH) ppm; ^{1}H NMR (400 MHz; $CDCl_3$) δ 1.40 (9H, s, Bu^t), 2.24 (3H, s, ArMe), 4.55 (2H, s, benzyl CH_2), 4.78 (1H, s, OH), 7.01 (1H, d, J_{meta} = 1.59 Hz, ArH), 7.12 (1H, d, J_{meta} = 1.98 Hz, ArH) ppm; ^{13}C NMR (50 MHz, CD_3OD) *J*-mod δ 16.68 (ArMe), 30.31 (Bu^t Me), 35.62 (Bu^t C), 65.65 (benzyl CH_2), 124.98 (ArC), 126.08 (ArC), 128.82 (ArC), 133.13 (ArC), 138.14 (ArC-4), 154.32 (ArC-1) ppm; CI+ GC/MS (isobutane) *m*/*z* 195 (2) $(M + H)^+$, 194 (15) M^+, 193 (100) $(M - H)^+$ ($C_{12}H_{18}O_2$).

BDMP−N_α-Tyr (3). A 20 mM solution of tyrosine (Tyr) was prepared in 50 mL of water, pH = 7.4. A QM solution in MeCN (2.5 mM, 4 × 50 mL) was added in aliquots, and the MeCN was removed after each addition under vacuum. The product mixture was extracted with two volumes of ether and then purged of organic solvent by bubbling nitrogen through the solution. The remaining aqueous phase was passed through a C-18 extraction cartridge (Baker). The cartridge was washed with water, and the adducts were then eluted with MeOH. The MeOH was removed under vacuum, and the residue was redissolved in MeOH for chromatographic purification on a silica gel column with EtOAc/MeOH (1:4) as eluant. Final purification was carried out by HPLC using an Ultrasphere ODS column (10 × 250 mm, Beckman) with a flow rate of 3.5 mL/min and the same mobile-phase composition described above for analytical work: ^{1}H NMR 200 MHz (DMSO-d_6) δ 1.31 (9H, s, Bu^t), 2.11 (3H, s, ArMe), 2.79 (1H, dd, J_{ab} = 21 Hz, J_α = 5.91 Hz, Tyr benzyl CH^a), 2.82 (1H, dd, J_{ba} = 21 Hz, J_α = 5.91 Hz, Tyr benzyl CH^b), 3.14 (1H, t, J_α = 5.91 Hz, Tyr CH$^\alpha$), 3.54 (1H, dd, J_{ab} = 28 Hz, BDMP benzyl CH^a), 3.59 (1H, dd, J_{ba} = 28 Hz, BDMP benzyl CH^b), 6.64 (2H, d, J_{ortho} = 8.39 Hz, Tyr ArH), 6.77 (1H, s, BDMP ArH), 6.91 (1H, s, BDMP ArH), 7.0 (2H, d, J_{ortho} = 8.39 Hz, Tyr ArH),

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8.08 (s, 1H, BDMP phenol OH), 9.24 (s, 1H, Tyr phenol OH) ppm; ^{1}H NMR 200 MHz (D_2O) δ 1.18 (9H, s, Bu^t), 2.00 (3H, s, $ArCH_3$), 2.57 (2H, d, J_α = 6.6 Hz, Tyr benzyl CH_2), 3.08 (1H, t, J_α = 6.8 Hz, Tyr CH^a), 3.26 (1H, d, J_{ab} = 12.5 Hz, benzyl CH^a), 3.45 (1H, d, J_{ba} = 12.5 Hz, benzyl CH^b), 6.39 (2H, d, J_{ortho} = 8.4 Hz, Tyr ArH), 6.75, (1H, d, J_{meta} = 1.9 Hz, BDMP ArH), 6.77 (2H, d, J_{ortho} = 8.4 Hz, Tyr ArH), 6.90 (1H, d, J_{meta} = 2 Hz, BDMP ArH) ppm; ES$^+$ MS *m*/*z* 358 (100), MH$^+$ ($C_{21}H_{28}O_4N$).

BDMP−TRIS (4). The QM adduct of tris(hydroxymethyl)-aminomethane (TRIS) was synthesized and purified as described above for **3**: ^{1}H NMR (200 MHz, D_2O) δ 1.35 (9H, s, Bu^t), 2.21 (3H, s, ArMe), 3.81 (6H, s, TRIS CH_2), 4.16 (2H, s, benzyl CH_2), 7.15 (1H, d, J_{meta} = 1.96 Hz, ArH), 7.26 (1H, d, J_{meta} = 1.96 Hz, ArH) ppm; ^{13}C NMR (50 MHz, CD_3OD) *J*-mod δ 16.48 (ArMe), 30.11 (Bu^t Me), 35.75 (Bu^t C), 59.23 (benzyl CH_2), 61.03 (Tris C), 67.57 (Tris CH_2), 123.53 (ArC), 126.67 (ArC), 127.97 (ArC), 131.59 (ArC), 138.78 (ArC-4), 156.17 (ArC-1) ppm; UV−vis λ_{max} (H_2O) 272 nm; ES$^+$ MS *m*/*z* 298 (100) MH$^+$ ($C_{16}H_{28}O_4N$).

Rechromatography of **1**, **3**, and **4** using the analytical (2.1 mm × 25 cm) C_{18} HPLC column confirmed the identities of the corresponding putative adducts from the kinetic experiments based on identical retention times.

ESMS. Peaks collected from HPLC experiments were analyzed directly. All samples were delivered to the Fisons VG Quattro ESMS probe using a Harvard syringe pump at 8.3 μL/min and were scanned at 84 *m*/*z* per s. Alkylated protein product mixtures were acidified and diluted to 20 μM in protein, 5% (v:v) MeCN, 2% (v:v) HOAc. Ion evaporation produced an "envelope" series of ions for each protein species. The identity of the individual charge state of each ion in each series as well as the molecular mass of each protein species was computed using the algorithm of Mann et al.[14] The addition of BDMP−QM to a potential nucleophile results in a net increase in molecular weight of 177 amu.

Nucleophilic Selectivity. Nucleophilic selectivities k_{Nu}/k_{H_2O} were determined for each nucleophile using eq 1[6] as the slopes in the plots shown in Figure 4. The ratio A_2/A_1 is the

$$A_2\epsilon_1/A_1\epsilon_2 = k_{Nu}[Nu]/k_{H_2O}[H_2O] \quad (1)$$

peak area ratio of nucleophilic adduct **2** to benzyl alcohol **1** from C_{18} HPLC analysis of product mixtures at 280nm, and ϵ_1/ϵ_2 is the ratio of extinction coefficients at 280 nm. The extinction coefficient ϵ_1 for the BDMP−QM hydration product in water was determined to be ϵ_1 = 0.68 mM^{-1} cm^{-1} using a pure synthetic sample (*vide supra*). Extinction coefficients for nucleophilic adducts ϵ_2 were determined additively. For instance, for the AIII−QM adduct, ϵ_{AIII} = 0.91 mM^{-1} cm^{-1} from a pure synthetic sample and $\epsilon_{AIII-QM}$ was determined as $\epsilon_{AIII-QM}$ = 0.91 + 0.68 = 1.6 mM^{-1} cm^{-1}. For Tyr, ϵ_{Tyr} = 1.4 mM^{-1} cm^{-1}.

Hydration Kinetics. All buffers were prepared with distilled, deionized water. The ionic strength of diluted buffers was maintained at I = 0.1 M with NaCl for monobasic anions and with Na_2SO_4 for dibasic anions. Buffers of formic acid (pH 3.5−4.4), acetic acid (pH 4−5.3), phosphoric acid (pH 5.6−7.6), boric acid (pH 8.4−9.1), and carbonic acid (pH 9.3−10.2) were used to determine the pH−rate profile. A freshly prepared solution of QM in MeCN (30 μL) was added to 970 μL of buffer in a quartz cuvette, producing a QM concentration of approximately 45 μM. The disappearance of BDMP−QM was followed, under pseudo-first-order conditions, by monitoring the decrease in absorbance at 286 nm[12] using a Hewlett-Packard 8452A diode array spectrophotometer. Rate constants were determined for triplicate reactions run for at least 6 half-lives. The temperature was maintained at 22 °C with a thermostated cell holder. The pH of each buffer was measured using a Beckman Φ32 pH meter with a Beckman 39848 electrode. Experiments were carried out at six different buffer concentrations in order to extrapolate to zero buffer and obtain the buffer-independent rate constant k_o (s^{-1}) at each pH value. Plots of the experimental pseudo-first-order rate constants,

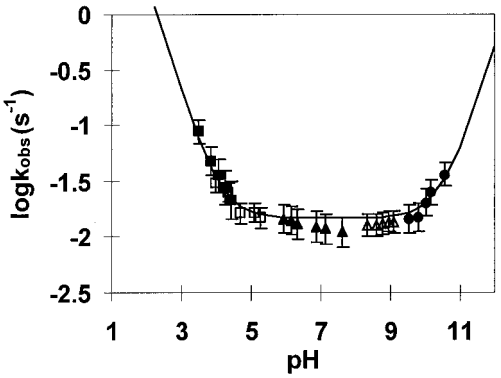

Figure 1. pH−rate profile for BDMP−QM hydrolytic decomposition from data in Table 1, at 22 °C, I = 0.1, substrate concentration 45 μM, formate (■), acetate (□), phosphate (▲), borate (△), carbonate (●). The line drawn is fit to the equation, $k_o = k_{H_3O^+}[H_3O^+] + k_{H_2O}[H_2O] + k_{OH^-}[OH^-]$, where $k_{H_3O^+}$ = 200 M^{-1} s^{-1}, k_{OH^-} = 50 M^{-1} s^{-1}, for $K_w = 10^{-14}$ M, $k_{H_2O} = 2.7 \times 10^{-4}$ M^{-1} s^{-1}. The latter value was used to correspond to previous estimates.[11]

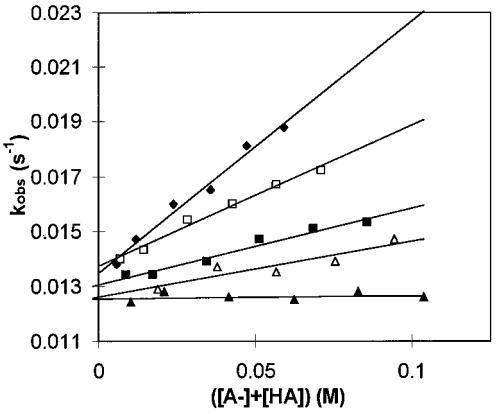

Figure 2. Buffer dilution plot of pseudo-first-order rate constant, k_{obs}, vs [borate], at pH 8.36 (▲), 8.63 (△), 8.79 (■), 8.98 (□), 9.12 (♦), I = 0.1, substrate concentration 45 μM, at 22 °C. This plot is representative of those obtained for the other buffers employed.

k_{obs}, vs total buffer concentration were found to be linear for all reactions of BDMP−QM studied, yielding rate constants $k_{cat.}$ (M^{-1} s^{-1}) from the slope of the correlation (Figure 2). Plots of $k_{cat.}$ vs the fraction of buffer as free base, f_B, were used to estimate the general base, k_{A^-}, and general acid, k_{AH}, contributions of the buffer to catalysis (eqs 2 and 3, Figure 3). The pH−rate profile (log k_o vs pH) was fitted to the standard relationship (eq 4).

$$k_{obs} = k_o + k_{cat.} \quad (2)$$

$$k_{cat.} = k_{A^-}[A^-] + k_{AH}[AH] \quad (3)$$

$$k_o = k_{H_3O^+}[H_3O^+] + k_{H_2O}[H_2O] + k_{OH^-}[OH^-] \quad (4)$$

Results and Discussion

Hydration. The pH−rate profile for the addition of water to BDMP−QM was obtained by monitoring disappearance of the QM by UV in various oxygen buffers. The profile obtained is similar in appearance to that previously described for a QM[15] (Figure 1). There are three distinctive regions corresponding to dominant acid-catalyzed (pH < 5), pH-independent (5 < pH < 9), and base-catalyzed reaction pathways (pH > 9) (Figure 1,

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Table 1. Rate Constants for BDMP−QM Breakdown Extrapolated to Zero Buffer

buffer	formate	acetate	phosphate	borate	carbonate
pK_a[a]	3.75	4.76	7.20	9.24	10.33
k_A^-/q[b] (M^{-1} s^{-1})	0.41 × 0.03	0.34 × 0.04	0.26 × 0.01	0.22 × 0.02	3.91 × 0.4

formate		acetate		phosphate		borate		carbonate	
pH	10^2k_o (s^{-1})	pH	10^2k_o (s^{-1})	pH	10^2k_o (s^{-1})	pH	10^2k_o (s^{-1})	pH	10^2k_o (s^{-1})
3.50	8.60 ± 0.38	4.00	3.03 ± 0.14	5.56	1.51 ± 0.02	8.36	1.26 ± 0.02	9.57	1.46 ± 0.70
3.84	4.84 ± 0.09	4.34	2.49 ± 0.20	5.95	1.46 ± 0.02	8.63	1.26 ± 0.03	9.81	1.51 ± 0.25
4.06	3.58 ± 0.19	4.69	1.64 ± 0.10	6.16	1.39 ± 0.03	8.79	1.31 ± 0.02	9.98	1.36 ± 0.38
4.27	2.77 ± 0.19	5.10	1.64 ± 0.06	6.36	1.30 ± 0.02	8.98	1.37 ± 0.02	10.03	2.03 ± 0.50
		5.29	1.49 ± 0.50	6.86	1.21 ± 0.30	9.12	1.35 ± 0.03	10.19	2.54 ± 0.50
				7.63	1.10 ± 0.10				

[a] pK_a values taken from: Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M.; *Data for Biochemical Research*, 2nd ed.; Oxford University Press: London, 1969. [b] q = the number of basic sites on the buffer anion.

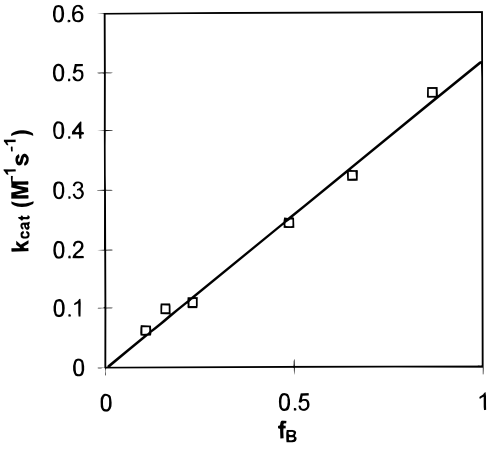

Figure 3. Plot of $k_{cat.}$ versus fraction of buffer in base form (f_B) for phosphate buffer, as representative example of plots for all buffers employed.

Table 1). Plots of k_{obs} vs total buffer concentration yielded rate constants k_{cat} for each pH studied (Figure 2). Plots of $k_{cat.}$ versus fraction of buffer in base form clearly indicate participation of the base form in breakdown of BDMP−QM for each oxygen buffer employed (Figure 3). It is probable that the rate constant for buffer base catalysis, k_A-, obtained from these plots describes mixed general base and nucleophilic catalysis, since a Bronsted plot of log (k_A-/q) versus [pK_a + log (p/q)] (p = the number of acidic protons on the buffer acid, q = the number of basic sites on the buffer anion) for these buffers shows considerable scatter (not shown), compatible with a mechanism other than pure general base catalysis (Table 1). It is not unreasonable, at the relatively high concentrations of buffer used, that buffer anions compete with water for nucleophilic addition to BDMP−QM. Indeed, comparison of k_A- values for buffer catalysis with second-order rate constants obtained for nucleophilic addition of nitrogen nucleophiles (Table 2) to BDMP−QM supports this hypothesis. For example, the second-order rate constant, k_{Nu}, for nucleophilic addition by Tris is lower than the buffer rate, k_A-, for some buffers (Tables 1 and 2). However, a stable buffer adduct has not been identified by HPLC analysis of the hydration products.

Nitrogen Nucleophile Selectivity. In order to examine reaction of BDMP−QM in amino acid, peptide, and protein solutions, unbuffered solutions were employed with pH measured before and after reaction at 7 < pH < 8. Since this is in the pH-independent region for hydration, small variations in pH are not of concern (Figure 1).

The major side products were isolated by HPLC and monitored in each reaction mixture by HPLC with UV-detection and assessed to be present in similar quantities independent of added nucleophile. All adduct peaks observed in the HPLC chromatograms and used for quantification of k_{Nu} were isolated and characterized as the 1:1 QM−nucleophile adducts by ESMS. The hydration product (**1**) was identified in the HPLC chromatogram by comparison to an authentic sample.

Benzylation of nucleophiles by QMs is well-documented.[16] Contrary to QMs derived from catechols, there is no competitive *o*-quinone reaction for QMs derived from simple phenols. Amino acids possessing nucleophilic side chains may be alkylated at the Nα and/or side chain N, O, or S. The QM conjugates of Tyr (**3**) and Tris (**4**) were characterized by NMR, since competitive *O*-alkylation must be considered. Comparison of the aromatic regions of the ^{1}H NMR spectra of **1**, **3**, **4**, and BDMP confirms addition to the methylene C of BDMP−QM (i.e., benzylation). That QM alkylation occurs at the N of Tris rather than one of the oxygens is evidenced by the equivalence of the six methylene protons of Tris in the isolated adduct and by the observed methylene chemical shifts. The ^{1}H NMR of **3** is incompatible with alkylation at a Tyr ring-C. Moreover, the relative upfield shift of the benzylic protons of the adduct (3.3, 3.5 ppm) is definitive for the *N*-adduct (cf: **1** 4.55, 4.31 ppm; **4** 4.2 ppm; lysine-N_α-adduct 3.88, 4.01 ppm;[17] histidine-N_α-adduct 3.88, 4.02 ppm[17]). The spectroscopic data are compatible with the absence of any adduct formation with N_α-acetyl-L-Tyr ethyl ester (*vide infra*).

Reactions with the "nitrogen nucleophiles," peptides, amino acids, and related species, were allowed to proceed to completion, and product ratios for adduct formation versus hydration (Scheme 1) were measured by HPLC with UV-detection to yield selectivity ratios (k_{Nu}/k_{H_2O}). The value of k_{H_2O} was determined spectrophotometrically,[11] and absolute values for k_{Nu} were thus derived. The more rigorous derivation of k_{Nu} was applied using varying nucleophile concentrations (40 μM to 4 mM), as employed by Richard *et al.* (Table 2, Figure 4).[6] The first point of interest is the high selectivity for all nitrogen nucleophiles studied over reaction with water, which ranges from a factor of 10^4 to a factor of 10^6 (Table 2). The second-order rate constant, for example, for reaction of BDMP−QM with His was measured as 17 M^{-1} s^{-1}, as compared to 2.7 × 10^{-4} M^{-1} s^{-1} for hydration.

The heptapeptide angiotensin III (AIII, Arg-Val-Tyr-Ile-His-Pro-Phe) was chosen as a readily available, representative oligopeptide containing various potential nucleophilic nitrogen sites. The reactivity of AIII toward

(16) (a) Lewis, M. A.; Graff, D. A.; Bolton, J. L.; Thompson, J. A. *Chem. Res. Toxicol.* **1996**, *9*, 1368. (b) Bolton, J. L.; Comeau, E.; Vukomanovich, V. *Chem.−Biol. Interact.* **1995**, *95*, 279.

(17) Bolton, J. L.; Thompson, J. A. Unpublished results.

Table 2. Second-Order Rate Constants for Adduct Formation of BDMP−QM with Nucleophiles

nucleophile	H_2O	Tris	His	His_{NAc}	Lys_{NAc}	Arg	Agm	Cys
pK_a[a]	15	8.3[b]	6.04, 9.33	ND[c]	ND	8.99, 12.84	ND	8.35, 10.46
k_{Nu}[d] (M^{-1} s^{-1})	2.7×10^{-4} [e]	3.73 ± 0.09	17.3 ± 0.9	10.1 ± 0.5	18.6 ± 2.8	17.2 ± 1.0	23.7 ± 3.8	3320 ± 66

nucleophile	Ile	Trp	Trpa	Tyr	Tyra	Tyr_3	AIII
pK_a[a]	9.76	9.44	10.2	9.11, 10.13	9.3, 10.4	ND	ND
k_{Nu}[d] (M^{-1} s^{-1})	7.9 ± 4.7	14.1 ± 2.8	3.48 ± 0.56	45.0 ± 3.0	4.62 ± 0.97	274.6 ± 10.7	123.9 ± 7.8

[a] pK_a values taken from Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M.; *Data for Biochemical Research*, 2nd ed.; Oxford University Press: London, 1969. [b] Good, N. E.; Winget, G. D.; Winter, W.; Connolly, T. N.; Izawa, S.; Singh, R. M.M. *Biochemistry* **1966**, *5*, 467. [c] Not determined. [d] Derived from k_{Nu}/k_{H_2O} values from product studies. [e] Reference 11.

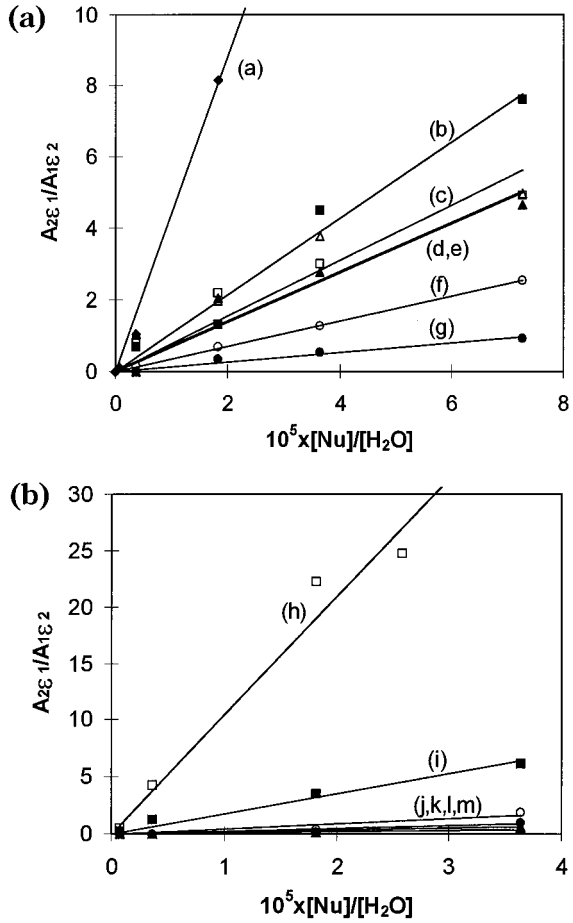

Figure 4. (a) Plot of extent of adduct formation with respect to nucleophile concentration: $A_2\epsilon_1/A_1\epsilon_2$ versus [nucleophile]. Nucleophiles used: (a) AIII (♦), (b) Agm (■), (c) His (□), (d) Lys_{NAc} (▲), (e) Arg (△), (f) His_{NAc} (○), (g) tris (●). Best fit lines are shown for each data series. (b) Plot of extent of adduct formation with respect to nucleophile concentration: $A_2\epsilon_1/A_1\epsilon_2$ versus [nucleophile]. Nucleophiles used: (h) Tyr_3 (□), (i) Tyr (■), (j) Trp (○), (k) Ile (●), (l) Tyra (△), (m) Trpa (▲). Best fit lines are shown for each data series.

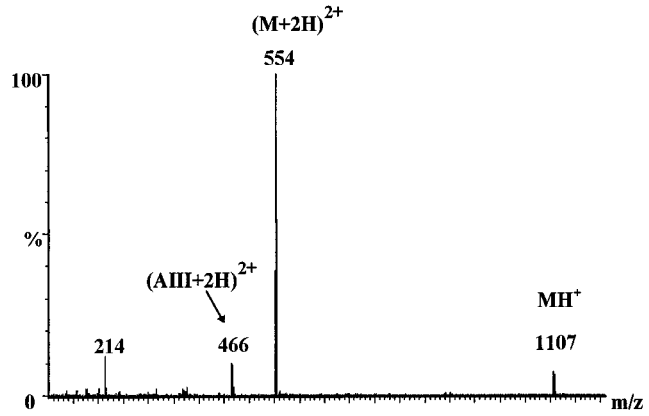

Figure 5. ESMS mass spectrum of AIII−QM adduct. The adduct was collected from HPLC separation of the product mixture and analyzed directly as described in the Experimental Section. The adduct is predicted to have an empirical formula $C_{58}H_{82}N_{12}O_{10}$, FW = 1106 (M). The unmodified peptide has FW = 931.

BDMP−QM is substantial. In comparison to its constituent nitrogenous-nucleophilic amino acid side chains, modeled by N_α-acetyl-L-lysine (Lys_{NAc}), N_α-acetyl-L-histidine (His_{NAc}), and arginine (Arg), AIII is more reactive by factors of 7, 10, and 7, respectively (Table 2, Figure 4). Moreover, agmatine (Agm), which is a good analogue of the nucleophilic *N*-terminus of AIII, is only one-fifth as reactive as AIII toward BDMP−QM. Addition of BDMP−QM to AIII leads to a small amount of precipitation (<8% w/w), as is observed to varying degrees on addition of QMs to many peptides and proteins. The ESMS chromatogram of the recovered, redissolved precipitate shows a mixture of AIII and 1:1, 1:2, and 1:3 adducts with BDMP−QM in an ion current intensity ratio of 18:8:3:1. However, the major adduct peak isolated from HPLC separation of the reaction solution is identified as the 1:1 AIII−QM adduct (Figure 5). Consideration of the chromatogram of the reaction products shows that this adduct represents >95% of AIII−QM adducts present in solution. Furthermore, reference to the reactivity trend, Agm > Arg > Lys_{NAc} > His_{NAc} > His_{NAc} > Tyr_{NAc} (Table 2), strongly suggests that the N-terminus is the primary site of modification. Further characterization is in progress using a combination of proteolytic digests and LC/ESMS/MS.

Tryptophan (Trp) was studied as an amino acid with side chain nitrogen and Tyr because of its presence in AIII. Trp is poorly reactive, whereas surprisingly, free Tyr has the highest reactivity toward BDMP−QM of all the free amino acids and derivatives studied, with the exception of cysteine (Cys) (Table 2, Figure 4). The unlikely possibility that the reactivity of AIII was due to the side chain phenolic nucleophile was ruled out by the absence of any adduct formation with N_α-acetyl-L-Tyr ethyl ester. Alkylation of Tyr itself occurs at N as confirmed by the synthesis, purification and characterization of the Tyr−QM adduct (**3**). Tyramine (Tyra), the decarboxylated analog of Tyr, has an activity that is reduced 10-fold compared to the parent amino acid. Reduced reactivity is also seen with tryptamine (Trpa), relative to Trp. However, the tripeptide Tyr_3 was examined and found to have remarkably high reactivity toward BDMP−QM: 6-fold greater than Tyr and twice that of AIII.

BDMP−QM is poorly hydrophilic, suggesting the possibility of a local concentration effect in which the local concentration of BDMP−QM with hydrophobic amino acids or with hydrophobic interiors of peptides and proteins would be greatly increased by aggregation. Thus, aggregation would lead to increased reaction rates.

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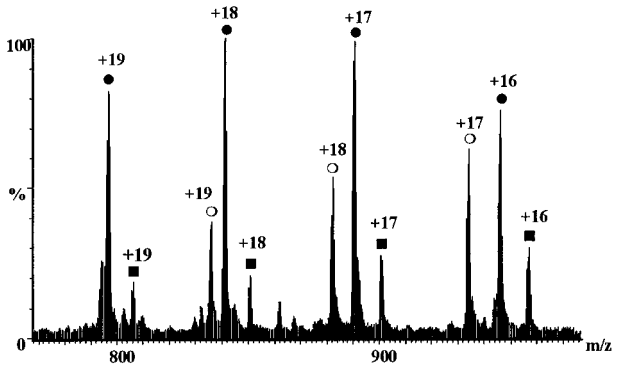

Figure 6. ESMS spectrum of HbA after alkylation by equimolar BDMP−QM at pH 7.4. Spectrum shows the formation of an adduct of 177 Da, corresponding to the addition of one QM, to HbA_α in preference to HbA_β. ● = HbA_α, M_r = 15122; ○ = (HbA_α+ BDMP−QM), M_r = 15 299; ■ = HbA_β, M_r = 15 861.

This possibility was examined using isoleucine (Ile), the amino acid with the most hydrophobic side chain. However, in this case, relatively low reactivity was observed.

The rate of addition of alcohols to QMs has been shown to depend to a substantial extent on the pK_a of the nucleophile.[18] However, no such simple correlation is seen with the amine nucleophiles studied herein. The selectivity of BDMP−QM for the primary amine Tris is 10^4-fold that for water, and selectivities increase up to 2×10^5 for Tyr, 5×10^5 for AIII, and 1×10^6 for Tyr_3. Peptide glycation is a biologically highly important protein modification in which reaction is site-specific. In this case, high rates of *N*-glycation have been observed when His is proximal to the *N*-terminus residue, possibly from intramolecular catalysis.[19,20] Similar site-specific catalysis is possible for peptide reaction with QMs.

Hydration studies demonstrated the importance of specific acid and specific and general base catalysis for BDMP−QM and indicate a possible role for general acid/base catalysis by certain amino acid side chains. However, catalytic motifs are not obvious in the reactive peptides studied (AIII and Tyr_3). Alternatively, in the case of poorly hydrophilic BDMP−QM, local concentration effects from hydrophobic binding of amine with BDMP−QM may influence observed reactivity. The hydrophobic aliphatic amino acid, Ile, was found to be poorly reactive toward BDMP−QM (*vide supra*); thus, the very high reactivity of AIII, Tyr, and Tyr_3 may suggest special hydrophobic binding with phenolic side chains via a favored arene−arene interaction.[21]

The addition of up to three units of BDMP−QM to Mb has been previously demonstrated using ESMS on unbuffered solutions.[10] Further experiments were conducted at equimolar concentrations of BDMP−QM for both Mb and HbA at pH 7.4 to compare the reactivities and selectivities for adduct formation over hydration. C_{18} HPLC analysis of product mixtures indicated that **1** was formed in the presence of Mb. Conversely, **1** was not observed in the reaction of BDMP−QM with HbA. This qualitative result demonstrates that Hb is more reactive toward BDMP−QM than Mb. ESMS analysis of HbA: QM mixtures at molar ratios from 1:1 to 1:8 gave spectra similar to that reported for Mb.[10] At a 1:1 ratio, it was found that one unit of QM is added selectively to the α chain of Hb A (Figure 6). At higher concentrations of BDMP−QM, a second unit is added to the β chain. This result demonstrates selectivity for the HbA α chain by BDMP−QM and an apparent reactivity trend: HbA α chain > HbA β chain > Mb. The simplest explanations for this selectivity are again based upon either (a) reactivity of protein side chains and catalytic motifs or (b) hydrophobic binding provided by the protein tertiary structure. The α and β chains of human HbA possess one and two cysteine residues, respectively, whereas Mb possesses no cysteine. In separate experiments, cysteine was determined to be more reactive toward BDMP−QM adduct formation than any of the nitrogen nucleophiles assayed by at least a factor of 10 (Table 2). The high reactivity of protein−thiol toward BDMP−QM may explain the high reactivity demonstrated by HbA compared to Mb. However, the intrinsic reactivity of cysteine does not explain the selectivity of BDMP−QM for the α chain, which was observed in the ESMS studies. There are, of course, several examples of proteins binding small hydrophobic arenes in the vicinity of reactive lysine residues, for example: (i) HbA, which catalyzes *p*-nitrophenyl acetate (PNPA) hydrolysis,[22] and (ii) serum albumin, which catalyzes PNPA hydrolysis and the Kemp elimination reaction of a benzisoxazole.[23]

Conclusions

Previous studies have shown wide-ranging reactivity of QM electrophiles with biological nucleophiles under varying reaction conditions. This study demonstrates, for the first time, the quantitative nucleophilic reactivity and selectivity of biological nitrogen nucleophiles toward adduct formation with a QM, in neutral aqueous solution. A full analysis of BDMP−QM hydration in the range $3 < pH < 11$ provides an essential background to this study. The observed selectivity for nitrogen nucleophiles is sufficiently high for adduct formation to compete with hydration, even at low concentrations of nucleophile. Furthermore, the observed reactivity of proteins (HbA, Mb), peptides (AIII, Tyr_3), and certain amino acids (e.g., Tyr) is anomalously high, suggesting that specific binding and/or catalytic motifs may be important in directing QM reactivity. Such specific motifs in peptides and proteins would be significant in dictating the cellular targets for the toxicological action of QMs.

BDMP−QM, a representative 2,4,6-trialkylphenol oxidative metabolite, forms 1:1 covalent adducts with amino acids, peptides, and proteins via reaction with nitrogen nucleophiles. The reactivity and selectivity for these nucleophiles is very high relative to background hydration and consistent with protein alkylation as an important mode of toxicological action of these phenols and their QM metabolites.

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