Formation of 4,4-Dialkoxycyclohexa-2,5-dienone *N*-(Thiol-*S*-yl)imine during Reaction of 4-Alkoxynitrosobenzenes with Thiols in Alcoholic Solvents

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During the interaction of nitrosoarenes with glutathione in aqueous media, intermediate generation of a highly resonance-stabilized sulfenamide cation has been repeatedly suggested. Most intermediates and end products could be explained by reactions of this sulfenamide cation with different nucleophiles such as excess thiol, solvent water, and metabolically produced arylamine. The present paper presents evidence for adduct formation of the sulfenamide cation with solvent alcohol at neutral pH. Sulfenamide cations generated from 4-nitrosophenetole and 4-nitrosoanisole, respectively, are strongly suggested to form the metastable ketals 4-ethoxy-4-methoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine and 4,4-dimethoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine, respectively, during reaction with solvent methanol. Reaction of the two sulfenamide cations in ethanol yielded 4,4-diethoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine and 4-ethoxy-4-methoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)-imine, respectively. Although the metastability of the ketals did not allow isolation of pure solid material, chromatographic and chemical behavior as well as tandem MS fragmentation substantiate a ketal structure of these intermediates. To confirm the proposed structure, new compounds, 2,6-dimethyl-4-nitrosophenetole, 2,6-dimethyl-4-nitrophenetole, 2,6-dimethyl-4-phenetidine, and *N*-(glutathion-*S*-yl)-*N*-hydroxy-4-aminoacetophenone, were synthesized and included in supportive experiments. In summary, the detection of ketals corroborates once more the occurrence of a sulfenamide cation which obviously not only reacts with soft nucleophiles such as GSH but, to a limited extent, also reacts with hard nucleophiles. The toxicological significance of this result is discussed.

Introduction

Reactions of nitrosoarenes with biological thiols have gained increasing interest among toxicologists when it became clear that nitrosoarenes and their redox couples *N*-hydroxyarylamines are involved in toxic, allergic, mutagenic, and carcinogenic effects (*1*). The rapid reactions with cellular thiols, leading mainly to unreactive metabolites, have been considered to be important for detoxication of nitrosoarenes. During the investigations of the underlying reaction mechanisms, a variety of observations pointed to the intermediate generation of an *N*-sulfenylnitrenium ion (also termed "sulfenamide cation"). Major hints at the occurrence of this reactive species came from kinetic investigations of nitrosoarene/GSH interactions (*2*), and from detailed studies of the metabolic pattern observed during the reaction of the phenacetin metabolite 4-nitrosophenetole (NOPt[1]) with GSH (*3*−*8*). The multiplicity of products has been most plausibly explained as resulting from interactions of the resonance-stabilized sulfenamide cation with various nucleophiles. Some of these products still await structural elucidation (*8*), one of them being characterized in this paper.

During the inceptive studies on NOPt/GSH reactions, Klehr tried to isolate the remarkably unstable semimercaptal [for structure, see Figure 1 in the preceding paper (*8*)] and finally performed his experiments at −40 °C in

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[1] Abbreviations: API, atmospheric pressure ionization; CID, collision-induced dissociation; EI-MS, electron impact mass spectrometry; Et/Me-ketal, 4-ethoxy-4-methoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine; Et/d_3Me-ketal, 4-ethoxy-4-trideuteriomethoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine; Et_2-ketal, 4,4-diethoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine; FAB-MS, fast atom bombardment mass spectrometry; GS-EPQDI, *N*-(4-ethoxyphenyl)-*N*′-(glutathion-*S*-yl)-1,4-benzoquinone diimine; GS-MPQDI, *N*-(4-methoxyphenyl)-*N*′-(glutathion-*S*-yl)-1,4-benzoquinone diimine; GS-NHAn, *N*-(glutathion-*S*-yl)-4-anisidine; GS-NHPt, *N*-(glutathion-*S*-yl)-4-phenetidine; GSO-NHAn, *N*-(glutathion-*S*-yl)-4-anisidine *S*-oxide; GSO-NHPt, *N*-(glutathion-*S*-yl)-4-phenetidine *S*-oxide; GS-NAn^+, *N*-(glutathion-*S*-yl)-4-anisidine cation; GS-Nd_3An^+, *N*-(glutathion-*S*-yl)-4-trideuteriomethoxybenzene cation; GS-NPt^+, *N*-(glutathion-*S*-yl)-4-phenetidine cation; GS-QI, *N*-(glutathion-*S*-yl)-1,4-benzoquinonimine; LC-MS/MS, liquid chromatography−tandem mass spectrometry; Me_2-ketal, 4,4-dimethoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine; NH_2An, 4-anisidine; NH_2Pt, 4-phenetidine; NOAn, 4-nitrosoanisole; NOPt, 4-nitrosophenetole; t_R, retention time; V_R, retention volume.

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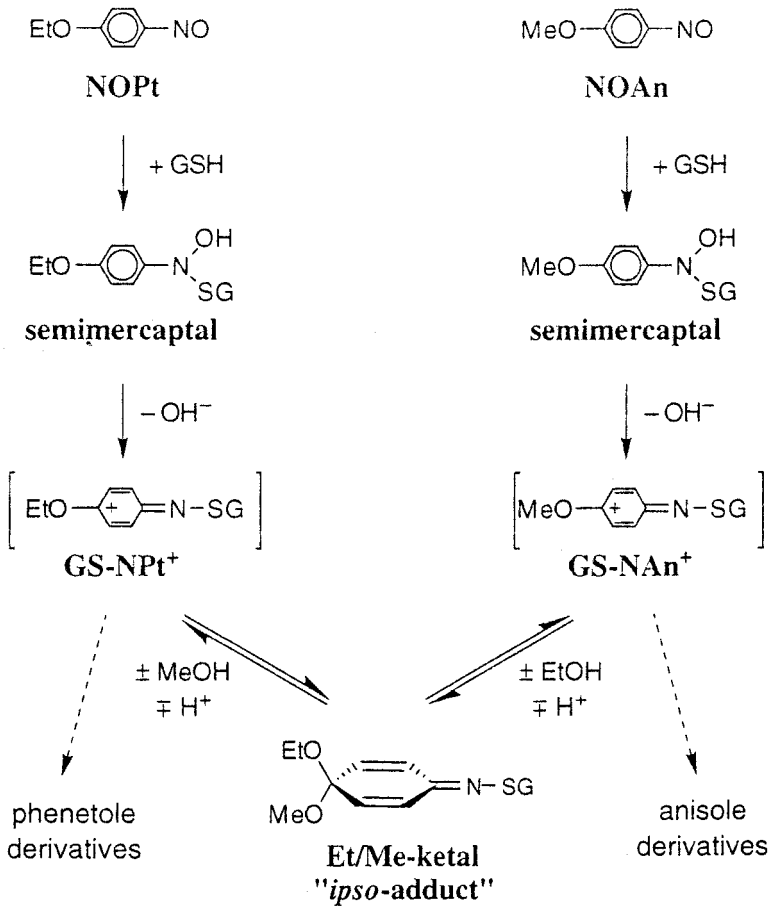


Figure 1. Supposed formation and decay reactions of alcohol adducts formed during reaction of 4-alkoxynitrosoarenes with GSH in alcoholic solvents.

the presence of 80% MeOH (*5*, *6*). However, instead of capturing the desired semimercaptal, he observed another metastable product in low yields (*6*). Tracer experiments using [u-ring-^{14}C]-NOPt and [Gly-2-^{3}H]-GSH indicated this compound to be a 1:1 adduct of nitrosoarene and thiol (*4*). Later experiments on the NOPt/GSH interaction showed that this metabolite was neither generated in pure aqueous reaction mixtures nor generated when MeOH was replaced by CH_3CN. These results fed the idea that MeOH may have added to the reactive sulfenamide cation, especially since analogous nitrenium ions are known to be captured by alcohols (*9*−*14*).

The scarce results reported hitherto on the reactivity of sulfenamide cations indicate the ring positions to react primarily with soft nucleophiles (*7*, *8*). The only reaction with a hard nucleophile, solvent water, is reported in the preceding paper (*8*). Therefore, proof of adduct formation with MeOH would be a further example of sulfenamide cation reactions with hard nucleophiles, thus also suggesting reactions with hard nucleophilic sites of biological macromolecules (e.g., DNA bases). To get more insight into the reactivity of sulfenamide cations, we tried to prove the presumed adduct formation with alcohols. Because of the metastability and low yields of the adducts, however, direct structural proof was not possible. However, different kinds of circumstantial evidence strongly supported the reversible formation of an alcohol adduct. The experimental strategy was based on trapping the sulfenamide cations of NOPt and 4-nitrosoanisole (NOAn) with MeOH and EtOH, respectively. Both educts should yield the same Et/Me-ketal that should be observable by HPLC methods and LC-MS/MS. Since the Et/Me-ketal may reversibly release one alcohol group, NOPt descendants should be observed from NOAn and vice versa. Figure 1 sketches the supposed reactions. For the sake of simplicity, the sulfenamide cations (shown in brackets) are presented only in the most important resonance structure as deduced from the product distribution {Table 1 of the preceding paper (*8*)].

Experimental Procedures

Chemicals. 4-Anisidine, calcium hydride, 4,4-dimethoxycyclohexa-2,5-dienone (96%), 2,6-dimethylphenol, ethyl iodide, methanol-d_4 (99.8 atom % D), nitrosonium tetrafluoroborate, NH_2Pt, and sodium methylate were purchased from Aldrich Chemie (D-89552 Steinheim). GSH was obtained from Boehringer (D-68305 Mannheim), 2-mercaptoethanol from Fluka Chemie (D-89231 Neu-Ulm), and ascorbic acid from Sigma Chemie (D-82041 Deisenhofen). All other reagents were purchased from Merck (D-64293 Darmstadt) at the purest grade available. NOPt (*15*) and *N*-(glutathion-*S*-yl)-4-benzoquinonimine (*8*) (GS-QI) were prepared as described earlier. 2,6-Dimethylphenetole was synthesized by reaction of 2,6-dimethylphenol with ethyl iodide according to standard methods (*16*). 4-Nitrosoacetophenone and *N*-hydroxyacetophenone had been synthesized previously (*3*).

Caution: *Nitrosoarenes bearing π-donating substituents have tested positive in the Ames mutagenicity assay with S. typhimurium without activation* (*17*−*21*).

Instrumentation and Analytical Methods. For ^{1}H NMR, EI-MS, FAB-MS, and analytical/semipreparative HPLC, see the preceding paper (*8*). IR spectra were registered with a Perkin-Elmer IR spectrometer, UV/vis spectra by a Shimadzu UV-

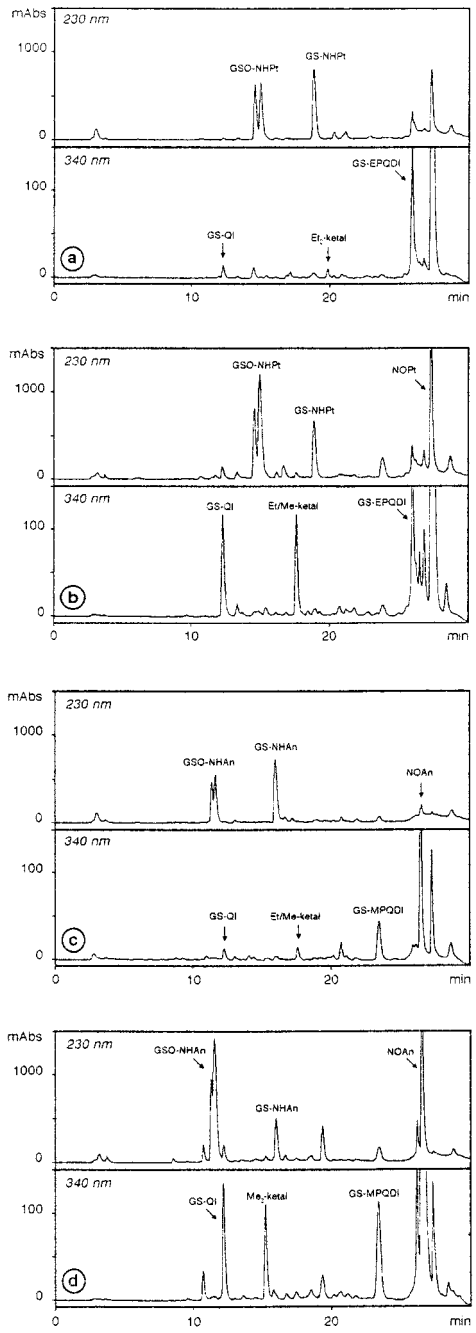


Figure 2. HPLC chromatograms of 4-alkoxynitrosobenzenes reacted with GSH in alcoholic solutions (90%); stoichiometry 5:1; reaction time 20 min; HPLC separation on Prep Nova-Pak (300 × 7.8 mm). (a) NOPt + GSH in EtOH; (b) NOPt + GSH in MeOH; (c) NOAn + GSH in EtOH; (d) NOAn + GSH in MeOH. For compound identification, see Figure 4 and Figure 1 of the preceding paper (*8*).

265 spectrophotometer. Analytical separation of the ketals was carried out on Lichrospher 100 RP 18 (4 × 125 mm; Merck) with MeOH/sodium phosphate buffer (10 mM, pH 6.5) gradients and detection at 230 and 340 nm (bandwidth 4 nm). Alternatively, separations were performed on Prep Nova-Pak C_{18} (7.8 × 300 mm, Waters, Milford, MA) using a MeOH/sodium phosphate buffer (10 mM, pH 6.5) gradient: 0−15 min, 5−50% MeOH; 15−19 min, 50% MeOH; 19−20 min, 50−90% MeOH; 20−23 min, 90% MeOH; 23−26 min, 90−5% MeOH; flow 3.0 mL/min. Peaks were identified by comparing the UV/vis spectra (220−500 nm) and retention times with authentic standards. In addition, glutathione sulfinamides were recognized by their characteristic double peaks (cf. Figure 2) and sulfenamides by their slow decay with formation of the corresponding arylamine (*1*). Peak purity of the ketal HPLC cuts was proved by diode array detection comparing the base-line-corrected UV/vis spectra at the beginning, top, and end of the respective HPLC peaks.

LC-MS and LC-MS/MS were performed on a Finnigan MAT triple quadrupole mass spectrometer TSQ 7000 (Finnigan MAT, San Jose, CA), equipped with an atmospheric pressure ionization (API) system. The API source was operated in the positive electrospray ionization mode. Measurements were accomplished using a capillary temperature of 200 °C, an electrospray ionization high voltage of 4.5 kV, nitrogen as auxiliary and sheath gas, and without sheath liquid. Quadrupoles were scanned with a scan time varying from 1 to 3 s over the mass range of interest. Collision-induced dissociation (CID) was performed using argon as the collision gas at a pressure of approximately 2 mTorr. To obtain product ion spectra, the collision energy was kept at −13 eV. Product ion abundances are expressed relative to the most abundant product rather than to the transmitted precursor. LC separation was performed on Lichrospher 100 RP 18 (125 × 4 mm, Merck) and gradient elution with MeOH/ammonium acetate buffer (0.01 M, pH 6.5): 0−10 min, 20−40% MeOH; 10−14 min, 40% MeOH; 14−16 min, 40−90% MeOH; 16−20 min, 90% MeOH; flow, 0.7 mL/min.

Syntheses. (A) 4-Nitrosoanisole (NOAn) was prepared and purified according to the procedure described for NOPt (*15*). Sky-blue crystals, mp 18.5−19.7 °C [lit. 21−23 °C (*22*, *23*)], yield 44% of theory, chromatographically pure. ^{1}H NMR in $CDCl_3$: δ 3.97 (3H, s, $-OCH_3$), 7.04 (2H), and 7.93 (2H) (ABCD system of the aromatic protons) [cf. (*23*)]. EI-MS [70 eV, cf. (*23*)]: *m*/*z* 137 (100%, $[M]^{+\bullet}$), 107 (36%, $[M-NO]^{+\bullet}$), 92 (33%, $[M-NO-CH_3]^{+\bullet}$). To determine ϵ (UV/vis) of NOAn, [ring-u-^{14}C]-NOPt (*4*) [specific activity 8.5 Bq/nmol, log ϵ_{342} = 4.26 (MeOH) (*24*)] was subjected to alkaline methanolysis (10 mM nitrosoarene, 50 mM sodium methylate in MeOH; complete reaction after 22 h at 37 °C under an argon atmosphere). After neutralization, NOAn was separated from its nitro derivative by RP-HPLC. UV/vis: λ_{max} = 340 nm, log ϵ_{340} = 4.22 (MeOH).

(B) *N*-(Glutathion-*S*-yl)-4-phenetidine *S*-Oxide (GSO-NHPt). One volume of GSH (50 mM; in ammonium bicarbonate buffer, 50 mM, pH 6.5) was added dropwise to a vigorously stirred solution of NOPt (50 mM; in ammonium bicarbonate buffer, 50 mM, pH 6.5/MeOH (1:1 v/v)]. After ether extraction of lipophilic metabolites, the batch was allowed to stand at ambient temperature overnight to allow decay of a hitherto unknown product which on HPLC separation coeluted with the desired sulfinamide. HPLC separation was performed the next day under the following conditions: Prep Nova-Pak C_{18} (7.8 × 300 mm, Waters); flow, 3 mL/min; elution with ammonium bicarbonate buffer (10 mM, pH 6.5)/MeOH; gradient: 0−14 min, 25−40% MeOH; 14−15 min, 40% MeOH. For ^{1}H NMR spectroscopy, the substance was used as obtained after lyophilization. For elemental analysis, the product was separated from buffer salts by gel chromatography on Superdex Peptide HR 10/30 (Pharmacia Biotech, D-79111 Freiburg) with water as eluent, following lyophilization to constant weight. Colorless powder (yield: 3% of theory). UV/vis (*6*): λ_{max} = 233/285 nm, log ϵ_{233} = 3.82 (MeOH/phosphate buffer, pH 6.5). ^{1}H NMR in D_2O: δ 1.38 (3H, t, J = 7.0 Hz, OCH_2CH_3), 2.1 (2H, m, Glu-β-CH_2), 2.5 (2H, m, Glu-γ-CH_2), 3.51 (1H, dd, J = 9 Hz/14 Hz, Cys-β_1), 3.59 (1H, dd, J = 5 Hz/14 Hz, Cys-β_2), 3.7 (1H, m, Glu-α), 3.80 (2H, s, Gly-α), 4.13 (2H, q, J = 7.0 Hz, OCH_2CH_3), 4.89 (1H, dd, J = 5 Hz/9 Hz, Cys-α), 7.03 (2H), and 7.15 (2H) (A_2B_2 system of the aromatic protons). FAB-MS (glycerol) (*6*): *m*/*z* 459 (43%, $[M+H]^+$), 322 (100%, $[GSO]^+$). Elemental analysis was unsuccessful as HPLC buffer salts could not completely be separated from the acid- and alkali-sensitive (*1*, *25*) tripeptide derivative. However, the above-mentioned physical properties and chemical reactivity of this derivative were in line with other well-characterized sulfinamides (*1*).

(C) *N*-(Glutathion-*S*-yl)-4-anisidine *S*-Oxide. GSO-NHAn was prepared as described above for GSO-NHPt. HPLC separa-

tion was performed with ammonium bicarbonate buffer (50 mM, pH 6.5)/MeOH; gradient: 0−10 min, 10−30% MeOH; 10−12 min, 30% MeOH. Colorless powder (yield: 16% of theory). UV/vis: λ_{max} = 233/285 nm (MeOH/phosphate buffer, pH 6.5). ^{1}H NMR in D_2O: δ 2.2 (2H, m, Glu-β-CH_2), 2.6 (2H, m, Glu-γ-CH_2), 3.52 (1H, dd, J = 9 Hz/13 Hz, Cys-β_1), 3.60 (1H, dd, J = 5 Hz/13 Hz, Cys-β_2), 3.79 (1H, t, J = 6.5 Hz, Glu-α), 3.88 (2H, Gly-α), 3.86 (3H, s, OCH_3), 4.91 (1H, dd, J = 5 Hz/9 Hz, Cys-α), 7.04 (2H), and 7.18 (2H) (A_2B_2 system of the aromatic protons). FAB-MS (glycerol): m/z 445 (85%, $[M+H]^+$), 322 (100%, $[GSO]^+$). As already discussed above, this derivative was not amenable to elemental analysis. Again, chemical reactivity corresponded to the sulfinamide structure.

(D) *N*-(Glutathion-*S*-yl)-*N*-hydroxy-4-aminoacetophenone was obtained by rapidly mixing 45 μL of 4-nitrosoacetophenone (22 mM in CH_3CN) with 5 μL of GSH (100 mM, in ammonium acetate buffer, 10 mM, pH 7 adjusted with ammonia). In view of the instability of this compound (almost complete decay within 0.5 h), no attempts at isolation were undertaken. Instead, 10 μL of the solution was applied to LC-MS/MS within 10 s and separated on Lichrospher 100 RP 18 (125 × 4 mm, Merck) with isocratic elution (70% ammonium acetate buffer, 10 mM, pH 6.5/30% MeOH). The first intense UV peak eluting after R_v = 1.6 mL exhibited an MS typical for semimercaptals (*1*): m/z 457 (100%, $[M+H]^+$), 439 (76%, $[M+H-H_2O]^+$), 152 (11%, $[M+H-GS]^+$). Chemical reactivity of the separated compound was in line with the presumed semimercaptal structure, too (*1*): Acidification (pH 2 for 0.5 h) liberated the corresponding arylamine as indicated by comparison with an authentic standard. Reaction with excess GSH yielded *N*-hydroxy-4-aminoacetophenone exclusively. During incubation at room temperature for 0.5 h, the compound degraded under formation of 4-nitrosoacetophenone, *N*-hydroxy-4-aminoacetophenone, and presumably *N*-(glutathion-*S*-yl)-4-aminoacetophenone *S*-oxide. The UV/vis spectrum of the semimercaptal, however, was atypical (*1*): λ_{max} = 309 nm (eluent); conceivably, this effect may result from the strong electron acceptor *para*-substituent.

(E) 2,6-Dimethyl-4-nitrosophenetole. To avoid any aqueous acidic conditions which could hydrolyze the 4-nitrosobenzene ether (*26*), 2,6-dimethylphenetole was nitrosated with nitrosonium tetrafluoroborate (3-fold excess) in dry acetonitrile as described recently (*23*). The reddish brown reaction mixture was poured into a vigorously stirred mixture (1:1 v/v) of *n*-pentane/sodium phosphate buffer (0.5 M, pH 8) in order to scavange the protons formed during hydrolysis of excess nitrosonium tetrafluoroborate. The organic layer was washed with sodium phosphate buffer and twice with water. After drying over sodium sulfate, the solvent was removed by vacuum and the viscous residue applied to flash chromatography on a short column of silica gel 60 (Merck) with hexane/dichloromethane (3:2 v/v) as eluent. The turquoise-green fraction containing 2,6-dimethyl-4-nitrosophenetole was collected. Removing the solvent by vacuum delivered a sky-blue, viscous liquid. RP-HPLC showed one single peak in the chromatogram, suggesting a pure product. However, on complete reduction of the nitroso compound with excess GSH or ascorbate, about 10% of the HPLC peak remained with a UV maximum shifted to shorter wavelength. Comparison with an authentic standard revealed the impurity to be 2,6-dimethyl-4-nitrophenetole (see below). In fact, formation of nitroaromatics is known during the reaction of activated arenes with surplus nitrosating agent (*16*). Besides, several nitroso- and nitroaromatics are known to be scarcely separated by any separation method (*27*, *28*).

To get the pure nitroso compound, the mixture was slowly fed in a solution of excess ascorbate (pH 7, H_2O/MeOH = v/v). After the insoluble azoxy compound had been filtered off, the *N*-hydroxy derivative was separated from the nitroarene by semipreparative RP-HPLC and collected at −70 °C under argon. The nitrosoarene was obtained by adding this fraction dropwise to an aqueous solution of excess potassium ferricyanide. The organic compound was extracted with pentane and applied again to RP-HPLC to remove impurities which coeluted with the *N*-hydroxy derivative and could not be separated by the final sublimation. After this treatment, the nitroso compound was chromatographically pure. Sublimation (p = 0.05 mbar, T_1 = 20 °C, T_2 = −35 °C) delivered sky-blue crystals. Yield: 10% of theory; mp 20.7−21.3 °C. IR: 2981 (m), 2930 (m), 1594 (w), 1489 (st), 1420 (m), 1388 (w), 1269 (st), 1220 (st), 1088 (st), 1029 (st), 962 (m), 899 (m), 745 (m) cm^{-1}. UV/vis [cf. 2,6-dimethyl-4-nitrosoanisole (*29*)]: λ_{max} = 230/327 nm, log ϵ_{327} = 4.05 (MeOH). ^{1}H NMR in $CDCl_3$ [cf. 2,6-dimethyl-4-nitrosoanisole (*23*, *29*)]: δ 1.47 (3H, t, J = 7.2 Hz, OCH_2CH_3), 2.41 (6H, s, Ar-CH_3), 3.97 (2H, q, J = 7.2 Hz, OCH_2CH_3), 7.61 (2H, s, ArH). ^{13}C NMR in $CDCl_3$: δ 16.4 (OCH_2CH_3), 17.2 (Ar-CH_3), 69.0 (OCH_2CH_3), 123.1 (C_3 and C_5), 132.8 (C_2 and C_6), 163.5 and 164.6 (C_1/C_4). EI-MS (70 eV): m/z 179 (100%, $[M]^{+\bullet}$), 151 (58%, $[M-C_2H_4]^{+\bullet}$), 121 (51%, $[M-C_2H_4-NO]^{+\bullet}$), 91 (62%), 77 (51%). Anal.: C, 66.99; H, 7.35; N, 7.86; O, 17.8. Calcd for $C_{10}H_{13}NO_2$: C, 67.02; H, 7.31; N, 7.82; O, 17.85.

(F) 2,6-Dimethyl-4-nitrophenetole was prepared by oxidation of unpurified 2,6-dimethyl-4-nitrosophenetole with Caro's acid (*30*) in MeOH/water (2:1 v/v; reaction at room temperature for 3 days). The product was extracted with dichloromethane and recrystallized from hexane. The compound was chromatographically pure and did not form any nitroso metabolites on mixing with GSH. Yellowish needles, mp 55.8−56.5 °C. UV/vis: λ_{max} = 289 nm, log ϵ_{289} = 3.90 (MeOH). ^{1}H NMR in $CDCl_3$: δ 1.45 (3H, t, J = 7.2 Hz, OCH_2CH_3), 2.36 (6H, s, ArCH_3), 3.92 (2H, q, J = 7.2 Hz, OCH_2CH_3), 7.93 (2H, s, ArH). EI-MS (70 eV): m/z 195 (100%, $[M]^{+\bullet}$), 167 (92%, $[M-C_2H_4]^{+\bullet}$), 137 (54%, $[M-C_2H_4-NO]^{+\bullet}$). High-resolution EI-MS: m/z 195.09029, $C_{10}H_{13}NO_3$ requires 195.08954.

(G) 2,6-Dimethyl-4-phenetidine was prepared by reduction of unpurified 2,6-dimethyl-4-nitrosophenetole with excess GSH (at pH 7) and subsequent hydrolysis of the major product sulfenamide (*1*) (pH 3, adjusted with phosphoric acid). Fifteen minutes later, the mixture was neutralized with sodium hydroxide and chromatographed on a Sep Pak C_{18} cartridge (Waters). Polar compounds such as GSH and GSSG were eluted with water before the desired arylamine was eluted with 0.1 N hydrochloric acid. After neutralization of the acidic fractions, the arylamine was extracted with peroxide-free ether. The reddish brown powder was chromatographically pure. UV/vis: λ_{max} = 233/287 nm (MeOH). ^{1}H NMR in $CDCl_3$: δ 1.39 (3H, t, J = 7.2 Hz, OCH_2CH_3), 2.21 (6H, s, Ar-CH_3), 3.77 (2H, q, J = 7.2 Hz, OCH_2CH_3), 4.6 (2H, broad, $-NH_2$), 6.46 (2H, s, ArH). EI-MS (70 eV): m/z 165 (35%, $[M]^{+\bullet}$), 136 (100%, $[M-C_2H_5]^{+\bullet}$). High-resolution EI-MS: m/z 165.11587, $C_{10}H_{15}NO$ requires 165.11536. Identical material was formed during the slow decay of the sulfenamide (*1*) and by reduction of 2,6-dimethyl-4-nitrosophenetole with sodium dithionite.

(H) 4-Ethoxy-4-methoxycyclohexa-2,5-dienone *N*-(Glutathion-*S*-yl)imine (Et/Me-ketal). GSH (20 μL, 20−50 mM in sodium phosphate buffer, 0.2 M, pH 7.4) was rapidly added to a vigorously stirred solution of NOPt (180 μL, 11 mM in MeOH). It took almost 20 min to withdraw the alcohol in a Speed Vac concentrator (p = 0.05 mbar, 20 °C) to ensure favorable HPLC separation. The solid residue (partly depleted from the volatile nitrosoarene and arylamine) was dissolved in 200 μL of water and applied to HPLC. (To chromatograph incubates of shorter reaction times, the reaction mixture was attenuated with 800 μL of water to obtain the composition of the HPLC eluent. Due to the high volume, this procedure caused significant loss of resolution.) Yield: about 0.02 μmol (2% of theory) at an estimated log $\epsilon_{340\ nm} \approx$ 4.2. [Due to the metastability of Et/Me-ketal, determination of ϵ was not possible. The order of magnitude indicated is estimated from that of *N*-(glutathion-*S*-yl)benzoquinonimines described in the preceding paper (*8*). Et/Me-ketal was obtained in distinctly lower yields when NOAn was reacted with GSH in EtOH. In this

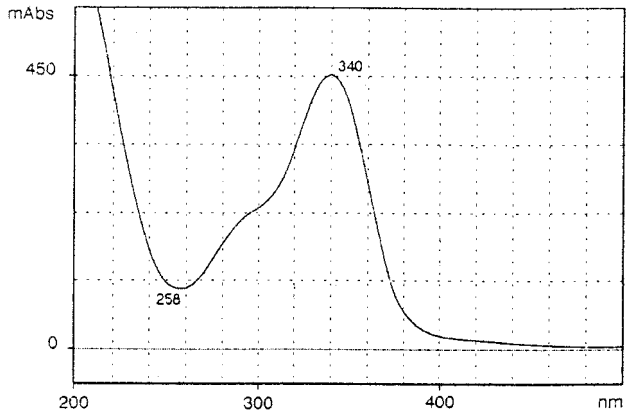


Figure 3. UV/vis spectrum of 4-ethoxy-4-methoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine (Et/Me-ketal) [MeOH/sodium phosphate buffer (10 mM, pH 6.5), 1:1 v/v] obtained by diode array detection.

experiment, the ketal could only be detected after 20 min reaction time using a 5-fold excess of NOAn.

(I) 4,4-Diethoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine (Et_2-ketal) was detected during interaction of a 5-fold excess of NOPt with GSH in EtOH after 20 min reaction time (for details, see synthesis of Et/Me-ketal).

(J) 4,4-Dimethoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)imine (Me_2-ketal) was prepared according to Et/Me-ketal using NOAn instead of NOPt.

Chemical reactivity of the ketals was investigated by adding 0.01 mL of reactant (GSH, ascorbate, and sodium dithionite, respectively; 0.1 M solutions in 0.2 M sodium phosphate buffer, pH 7 readjusted) to a freshly prepared HPLC cut of the ketal (approximately 0.5 mL). Acid hydrolysis was performed by adjusting to pH 1 with 1 M HCl in freshly prepared HPLC cuts. After 10 min reaction at ambient temperature (and neutralization of acid cuts), the whole incubate was reinjected into the HPLC system.

Results

During reaction of 10 mM NOPt with 2−5 mM GSH in solutions of 90% MeOH/10% sodium phosphate buffer (0.2 M, pH 7.4) at room temperature, a low-yield product was formed (Et/Me-ketal, Figure 2b) exhibiting a UV spectrum (λ_{max} = 340 nm; Figure 3) uncommon to the nitrosoarene metabolites known hitherto (*1*). Et/Me-ketal was already formed within 0.5 min and was detectable in the reaction mixture even 20 min later. On RP-HPLC separation, Et/Me-ketal was eluted similar to other monocyclic glutathione conjugates (Figure 2b), indicating incorporation of the polar GSH into the ketal. Et/Me-ketal was not observed in pure aqueous reaction mixtures. When MeOH was replaced by CH_3CN (90%) in order to mimic the solvent effect of MeOH, Et/Me-ketal was not formed either. However, when MeOH was replaced by EtOH, the HPLC chromatogram of the reaction mixture showed a very small peak immediately *after* the sulfenamide (Et_2-ketal; Figure 2a) exhibiting the same UV/vis spectrum as Et/Me-ketal (Figure 3). Thus, adduct formation between NOPt, GSH, and the solvent alcohol was presumed.

To isolate Et/Me-ketal in amounts sufficient for structural elucidation, factors affecting product formation were examined. Variation of the reaction temperature (−20 °C to +20 °C), pH shifts between 9 and 6, and increase of the MeOH portion in the reaction mixture did not essentially alter the yield of Et/Me-ketal. Increasing the educt ratio from 1:0.2 to 1:1 (NOPt:GSH) resulted in higher yields, but caused a more rapid product decay from 1:0.5 ratios upward. Unfortunately, increased educt concentrations did not elevate the amount of the desired product. Thus, the maximum yield from a single HPLC separation topped at about 0.02 μmol (for detailed reaction conditions, see Experimental Procedures).

Stability. Immediate rechromatography of the HPLC-isolated Et/Me-ketal revealed a distinct decay with formation of the known NOPt metabolites *N*-(glutathion-*S*-yl)-4-benzoquinonimine (GS-QI) (*8*) and GSO-NHPt (*5*) (Figure 4) and a few unknown minor products. Immediate freezing of the collected HPLC peak at −70 °C could not prevent decomposition. When the frozen HPLC cut was lyophilized overnight, the compound completely decomposed under formation of the quinonimine GS-QI, the sulfinamide GSO-NH*Pt*, and the sulfinamide GSO-NH*An*. Both sulfinamides were identified by their HPLC double peaks (*1*), by their UV/vis spectra, and by sample spiking with authentic standards. These findings appear to be in agreement with mechanistic considerations presented in Figure 4: The sulfenamide cation resulting from the interaction of NOPt with GSH (GS-NPt^+) adds to solvent MeOH to yield a metastable ketal (Et/Me-ketal). Thereupon, liberation of the *ipso*-methoxy and -ethoxy groups, respectively, generates two different sulfenamide cations (GS-NPt^+ and GS-NAn^+), giving rise to formation of the sulfinamides GSO-NHPt and GSO-NHAn.

Since the marked instability of Et/Me-ketal did not allow characterization by ^{1}H NMR or FAB-MS spectroscopy, more stable derivatives were intended to be synthesized. As previously reported by Fernando et al. (*31*), inclusion of two methyl groups *ortho* to the *ipso*-position should result in an increased stability of an *ipso*-compound, since steric interaction complicates the *ipso*-residue turning back into a coplanar position. To this end, a synthetic route to 2,6-dimethyl-4-nitrosophenetole was developed. When this NOPt derivative was reacted with GSH (90% MeOH, 20 min at 0 °C), the chromatogram of the mixture exhibited a small peak with the same UV/vis spectrum as Et/Me-ketal. As expected, this compound was distinctly more stable than its alkyl-deficient derivative, but not stable enough to yield pure solid samples for ^{1}H NMR or FAB-MS. Upon lyophilization, a significant decay was noticed, but this derivative could still be detected in the dry powder kept at −20 °C for 2 days.

Chemical Reactivity. Since synthesis of a stable ketal derivative failed, HPLC cuts of the Et/Me-ketal were used for investigation of chemical reactivity. When the HPLC cut was supplemented with excess GSH, slow formation of *N*-(glutathion-*S*-yl)-4-phenetidine (GS-NHPt) and subsequent NH_2Pt was observed (cf. Figure 4). The decay product GSO-NHPt was observed as well while GS-QI was not detected, possibly because of further reactions (*8*). Reaction of isolated Et/Me-ketal with excess ascorbate resulted in the same product pattern. The strong reductant sodium dithionite immediately formed NH_2Pt with traces of NH_2An (cf. Figure 4). Acid hydrolysis of Et/Me-ketal yielded the quinonimine GS-QI, NH_2Pt, and traces of NH_2An.

To gain additional evidence for the presumed structure of Et/Me-ketal, an alternative synthetic route was pursued: NOAn was reacted with GSH in EtOH (cf. Figure

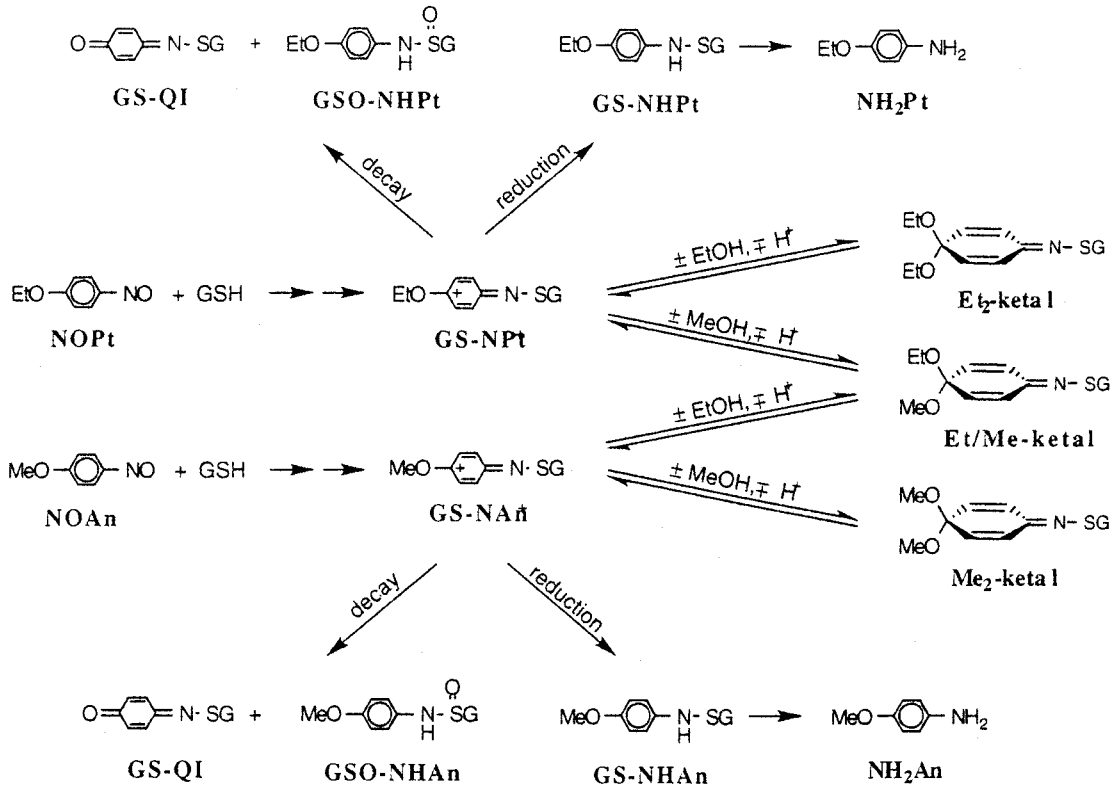


Figure 4. Formation of different ketals during reaction of 4-alkoxynitrosobenzenes with GSH in alcoholic solutions and their consequent reactions.

4). In fact, the HPLC chromatogram of this reaction mixture (Figure 2c) showed a small peak at the same retention time as Et/Me-ketal from reaction mixtures of NOPt and GSH in MeOH (Figure 2b). When both reaction mixtures were chromatographed on another C_{18} column (see Experimental Procedures), the respective peaks eluted again at the same retention time. The UV/vis spectra of both peaks were congruent. Because of the extremely small yields during syntheses in EtOH, the NOAn-derived Et/Me-ketal was only subjected to acid hydrolysis, resulting in formation of NH_2An and NH_2Pt with traces of quinonimine GS-QI.

NOAn was also reacted with GSH in a MeOH-containing solvent. Expectedly, a peak exhibiting the known UV/vis spectrum was detected at a shorter retention time (Me_2-ketal, Figure 3d). This ketal displayed the same instability as the above-described derivatives and yielded the analogous decay products (GS-QI and GSO-NHAn), the corresponding sulfenamide GS-NHAn, and the arylamine NH_2An. Mild reduction with excess GSH delivered the sulfenamide GS-NHAn. The only products found during acid hydrolysis were NH_2An and the quinonimine GS-QI. A variety of other 4,4-disubstituted 2,5-cyclohexadienone imines have been observed to split off the imino group upon acidification, yielding the respective cyclohexadienone derivative. However, formation of 4,4-dimethoxycyclohexa-2,5-dienone or its subsequent hydrolysis product 1,4-benzoquinone (*32*, *33*) could not be traced during acid hydrolysis of Me_2-ketal, as proved with authentic standards. Another ketal derivative, Et_2-ketal, resulting from reaction of NOPt with GSH in EtOH, liberated only NH_2Pt and the quinonimine GS-QI upon acidification. Attempts to obtain ketal derivatives of higher alcohols such as *i*-PrOH failed.

Investigation by LC-MS/MS. To unequivocally prove the structure of the presumed Et/Me-ketal, the reaction mixture of NOPt and GSH (in 90% MeOH, stoichiometry 1:0.5, 2 min reaction at ambient temperature) was investigated by LC-MS/MS. The quinonimine GS-QI, the sulfinamide GSO-NHPt, and the sulfenamide GS-NHPt emerging in the reaction mixture were identified by their retention times and relative peak height at the UV/vis detector which was included between the LC and the MS/MS unit. In addition, identity was confirmed by the respective mass spectra. GS-QI: *m/z* 435 (22%, $[M+Na]^+$), 413 (100%, $[M+H]^+$). GSO-NHPt: *m/z* 459 (100%, $[M+H]^+$), 322 (45%, $[GSO]^+$). GS-NHPt: *m/z* 443 (100%, $[M+H]^+$), 306 (37%, $[GS]^+$) (*1*, *8*). The presumed Et/Me-ketal eluting immediately in front of the sulfenamide GS-NHPt (Figure 2b) was detected by its UV absorption at 340 nm. The corresponding mass spectrum exhibited intense peaks at *m/z* 441 (100%) and 427 (36%), most probably reflecting the sulfenamide cations $GS\text{-}NPt^+$ and $GS\text{-}NAn^+$ generated by protonation of one ketal oxygen and immediate loss of MeOH and EtOH, respectively (Figure 5). To prove these presumed sulfenamide cation structures, the product ion spectra of *m/z* 441 and 427 were recorded (Figure 6a,b). Both spectra exhibited seven intense peaks with the same mass while five other peaks showed the characteristic difference between the ethoxy and the methoxy group of 14 units (Table 1).

An additional LC-MS/MS experiment was undertaken using CD_3OD instead of CH_3OH as reaction solvent. The MS spectrum recorded during elution of the deuterated derivative Et/d_3Me-ketal again exhibited the intense peak at *m/z* 441 (100%). The peak at *m/z* 427 (10%), however, was attended by a more intense peak at *m/z* 430 (41%), probably corresponding to a trideuteriomethoxy sulfenamide cation $GS\text{-}Nd_3An^+$ (Figure 5). Upon CID, *m/z* 441

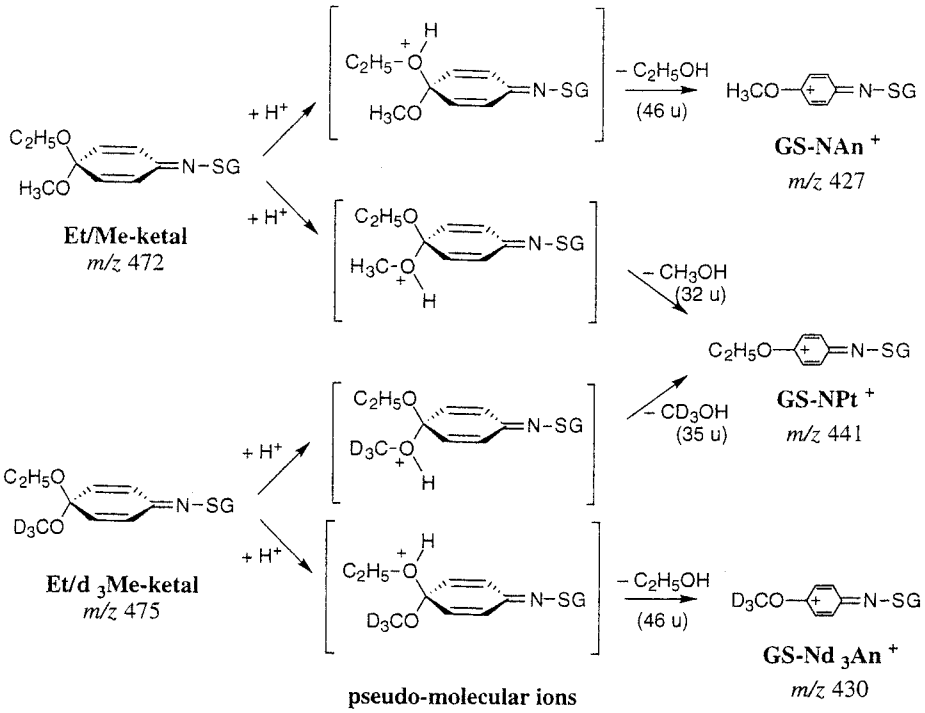


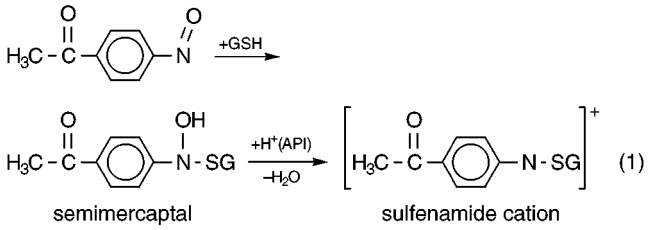
Figure 5. Presumed fragmentation of the ketal derivatives upon protonation.

Table 1. Comparison of the Product Ions of Substituted Sulfenamide Cations

		substituents			
fragments	proposed structure	C_2H_5O- (Figure 6a)	CH_3O- (Figure 6b)	CD_3O- (Figure 6c)	CH_3CO- (Figure 6d)
precursor ion	M^+	441	427	430	439
aryl substituent containing product ions	$[M-H_2O]^+$	423	409	412	421
	$[M-GluNH]^+$	295	281	284	293
		245	231	234	–
	$[ArNHS]^+$	168	154	157	–
	$[ArS]^+$	153	139	142	–
aryl substituent deficient product ions	$[GS-2H]^+$	304	304	304	304
	$[GS-2H-S]^+$	272	272	272	272
	$[GS-2H-S-H_2O]^+$	254	254	254	254
	$[GS-2H-CH_2CO_2H]^+$	245	245	245	245
	$[GS-2H-Glu]^+$	175	175	175	175
	$[GS-2H-S-(GlyCO)-OH]^+$	153	153	153	153
	$[GluNH]^+$	145	145	145	145

produced the same product ion spectrum as *m/z* 441 from the undeuterated ketal. The product ion scan of *m/z* 430 (Figure 6c) revealed a shift of 3 units of the methoxy-containing fragments, but not of the alkoxy-deficient fragments (Table 1).

Some effort was undertaken to prove the sulfenamide cation structure of the three fragments *m/z* 441, 427, and 430. To this end, we synthesized a glutathione semimercaptal since this family is known to yield the corresponding sulfenamide cation during FAB-MS fragmentation (*1*, *5*, *6*, *25*, *34*). Semimercaptals are unstable, especially in the case of donor aryl substituents (*1*), so that the 4-methoxy or 4-ethoxy derivative was not amenable to LC-MS/MS analysis. [The only stable *glutathione* semimercaptals described at all have been obtained from the acceptor substituted 3- and 4-nitronitrosobenzene, respectively (*34*).] Hence, we reacted the acceptor substituted 4-nitrosoacetophenone with glutathione to obtain the respective semimercaptal *N*-(glutathion-*S*-yl)-*N*-hydroxy-4-aminoacetophenone (eq 1) which proved to be stable enough for separation and detection by LC-MS/MS.



(1)

In fact, this semimercaptal—upon protonation inside the ion source—delivered an intense fragment at *m/z* 439 corresponding to the respective sulfenamide cation (eq 1). This sulfenamide cation was further fragmented by CID, delivering the product ion spectrum shown in Figure 6d. Again, seven substituent-deficient fragments were formed which had already been observed in the product spectra of the precursors *m/z* 441, 427, and 430 (Table 1). The acetyl-containing fragments $[M-H_2O]^+$ (*m/z* 421) and $[M-GluNH]^+$ (*m/z* 293) were observed likewise, while the corresponding product ions *m/z* 243, 166, and 151 were lacking (Table 1).

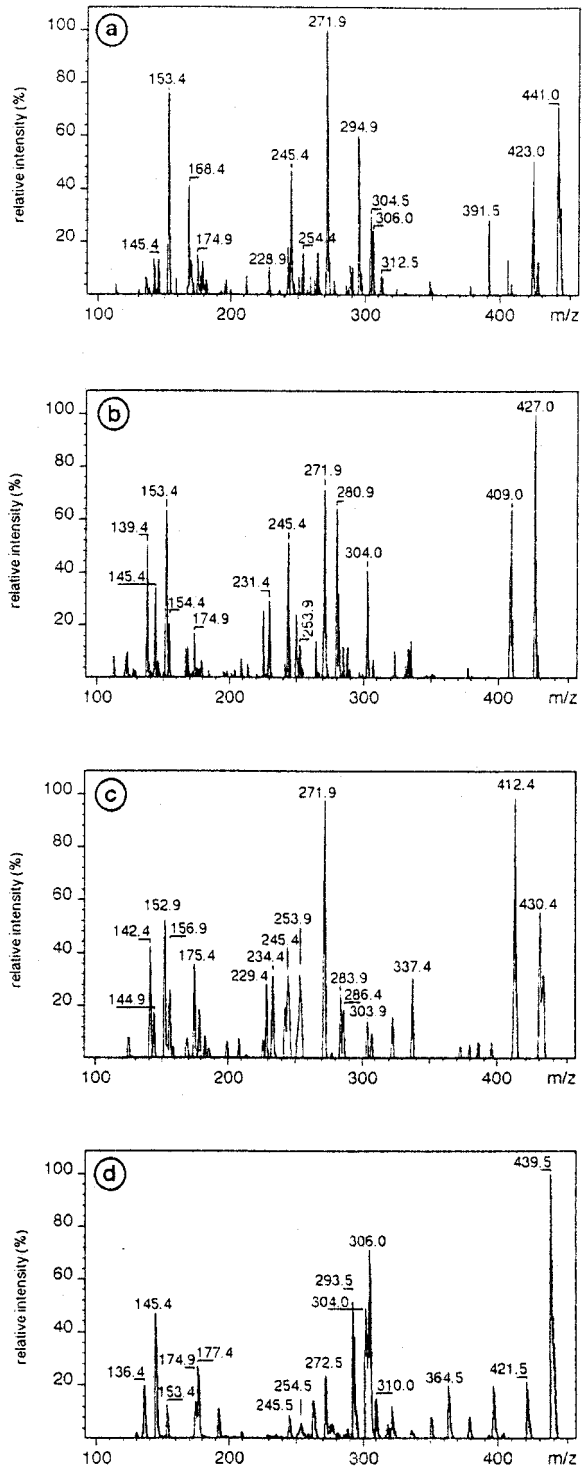


Figure 6. Product ion spectra (CID at −13 eV) of the sulfenamide cations (a) *m/z* 441 (R = OC_2H_5), (b) *m/z* 427 (R = OCH_3), (c) *m/z* 430 (R = OCD_3), and (d) *m/z* 439 (R = $COCH_3$) (for details, see Experimental Procedures).

Discussion

The results presented in this paper indicate formation of an alcohol adduct during reaction of 4-alkoxynitrosobenzenes with GSH in alcoholic solvents. Even if direct identification of the alcohol adducts by NMR and MS failed due to the high lability of the compounds, numerous circumstantial evidence supports their intermediate existence. First, NOPt in MeOH and NOAn in EtOH yielded the same adduct (HPLC, UV) upon reaction with GSH. Hence, alcohol addition in position 4 has to

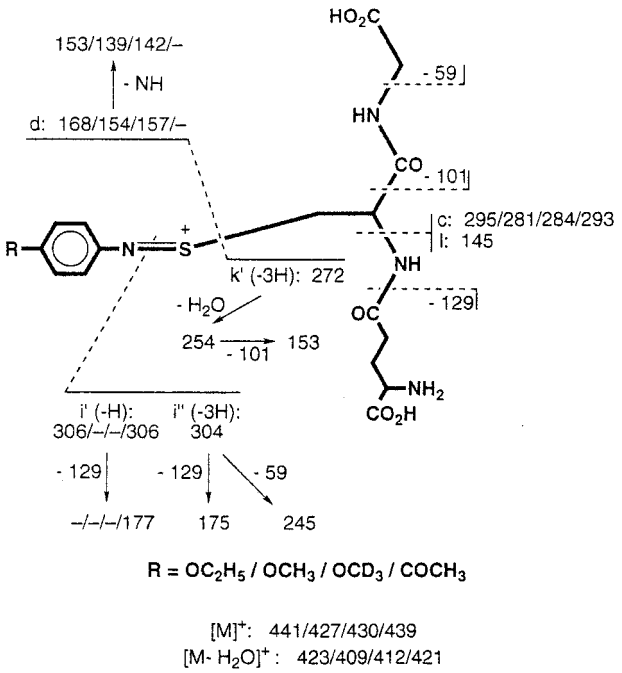


Figure 7. Tentative fragmentation pattern of the glutathione sulfenamide cations from Figure 6 and Table 1, respectively. The fragments indicated by lower case letters refer to (*37*, *38*, *58*, *59*). Neutral loss fragments are designated by the respective molecular masses.

be assumed. Second, incorporation of solvent alcohol was further proven when carrying out NOPt/GSH reactions in CD_3OD which resulted in fragments with masses 3 units higher than observed in MeOH. Third, typical end products such as the sulfinamides both of NH_2Pt and of NH_2An were observed, indicating the intactness of the N−S linkage of the glutathione moiety and the exchange of the alcohol group.

The LC-MS/MS experiments did not allow detection of the molecular ion of the *ipso*-adducts but showed fragments due to the loss of one alcohol group. As evidenced by molecular orbital calculations, the most probable sites of protonation are the two alkoxy oxygen atoms. In fact, the ab initio Mulliken net atomic charge distribution for the structural analogue 4-hydroxy-4-ethoxycyclohexa-2,5-dienone *N*-acetylimine revealed both ketal oxygen atoms as the most negatively charged reaction centers (*35*). Hence, the ketals once protonated at one of both sites will immediately lose the respective alcohol due to the extensive resonance stabilization of the resulting sulfenamide cations (Figure 5). To corroborate this mechanism, considerable effort was undertaken to actually prove the sulfenamide cation structure of the API fragments *m/z* 441, 430, and 427, particularly as their product ion spectra were recognized to be remarkably different from C-glutathione derivatives reported in the literature (*36*−*41*). Subsequent investigation of different C−S and N−S conjugated glutathione derivatives revealed the fragmentation patterns to be highly dependent on the nature of glutathione binding and the residue.[2] The glutathione sulfenamide cations exhibited particular interesting product ion spectra which may be rationalized by the fragmentations indicated in Figure 7. The hitherto unknown glutathione fragments *m/z* 304,

[2] D. Gallemann, J. Dasenbrock, and E. Wimmer (1996). unpublished result.

272, 254, 245, 175, and 153 suggest dehydrogenation during fragmentation corresponding to the reactive and oxidative character of the nitrenium ion moiety. Finally, the sulfenamide cation generated from a synthetic semimercaptal exhibited almost the same fragmentation as the presumed sulfenamide cations formed from the ketals[3] (cf. Figure 6 and Table 1). These findings substantiate not only the *ipso*-structure of the alcohol adducts, but also the arylamine nitrogen atom as the binding site of the glutathionyl residue as well. Hence, despite the lack of a molecular ion in the MS and other spectroscopic proof of the entire alcohol adducts, all the experimental results strongly suggest the ketal structure 4,4-dialkoxycyclohexa-2,5-dienone *N*-(glutathion-*S*-yl)-imine.

Formation of such ketals is clearly corroborated by mechanistic considerations of the NOPt/GSH interaction. As evidenced previously, an intermediate reactive sulfenamide cation originates during reactions of nitrosoarenes with alkylthiols (*1*, *2*, *6*−*8*). This sulfenamide cation was recently shown to undergo ring addition reactions not only with soft nucleophiles such as GSH and arylamines (*7*), but also to a limited extent with the hard nucleophile H_2O (*8*). Therefore, addition of alcohols to sulfenamide cations forming an *ipso*-adduct is reasonable, particularly as a substantial positive partial charge is located at the 4-position (*8*), i.e., the obvious site of alcohol attack.

The reported addition of solvent alcohol and water, respectively, are the only hitherto known examples of ring addition reactions of *hard* nucleophiles to sulfenamide cations (also termed *N*-sulfenylnitrenium ions[4]) (*1*). Other arylnitrenium ions are well-known to react with alcohols, and this interaction was frequently used for trapping these reactive cations (*9*−*14*, *26*, *42*−*48*). Similar to the results reported here, these arylnitrenium ions gave lower adduct yields with solvent EtOH compared to MeOH (*11*, *47*). During alcohol adduct formation, the occurrence of similar *ipso*-adducts has been proved by structural elucidation of some metastable derivatives (*10*−*12*, *46*−*48*). In contrast to the formation of *ipso*-adducts of 4-alkoxysulfenamide cations reported here, the *ipso*-adducts of arylnitrenium ions follow different decay pathways depending on their aryl substituents (Figure 8).

Basically, both types of *ipso*-adducts can be protonated at two different positions: the imino nitrogen atom and the alkoxy oxygen atom. *ipso*-Adducts derived from arylnitrenium ions apparently are protonated at the imino nitrogen atom, delivering the positive charge into the ring (*48*, *49*). If the *ipso*-substituent R^1 is a hardly migrating group, there is considerable chance for solvent methanol and water to add at the 3- or 1-position, respectively (Figure 8a,b). Subsequent leaving of methanol and amine, respectively, yields stable end products, i.e., 3-methoxyaniline (*13*, *14*, *46*, *48*) and cyclohexa-2,5-dienone derivatives (*12*, *46*, *48*, *49*).

In contrast, *ipso*-adducts derived from sulfenamide cations apparently are not protonated at the imino nitrogen atom, since formation of 4,4-dimethoxycyclohexa-2,5-dienone or 1,4-benzoquinone during acid hydrolysis of Me_2-ketal could not be established (cf. Figure 8b). Instead, all decay products identified are known to be sulfenamide cation descendants. Probably, the sulfenamide cation is restored back from the ketal by protonation of the alternative site, i.e., the alkoxy oxygen atom (Figure 5). Thus, sulfenamide cations appear to be much more stable than other arylnitrenium ions, which is explicable by a distinct mesomeric delocalization of the positive charge to the sulfur atom. This prominent resonance effect has been clearly demonstrated by kinetic investigations (*2*), and was corroborated by molecular orbital calculations (*8*).

The additional resonance contributor only possible in sulfenamide cations enables a unique detoxication reaction, i.e., addition of H_2O to the sulfur atom, yielding a sulfinamide [cf. discussion in (*8*)]. It might be argued, therefore, that at limited availability of soft nucleophiles such as GSH, this reaction with solvent H_2O may suffice for complete detoxication of sulfenamide cations inside cells, particularly as their pronounced resonance stabilization does not require an instant reaction with the nearest nucleophile. The reported formation of ring addition products with weak nucleophilic alcohols and H_2O (*8*), however, indicates striking similarity of the

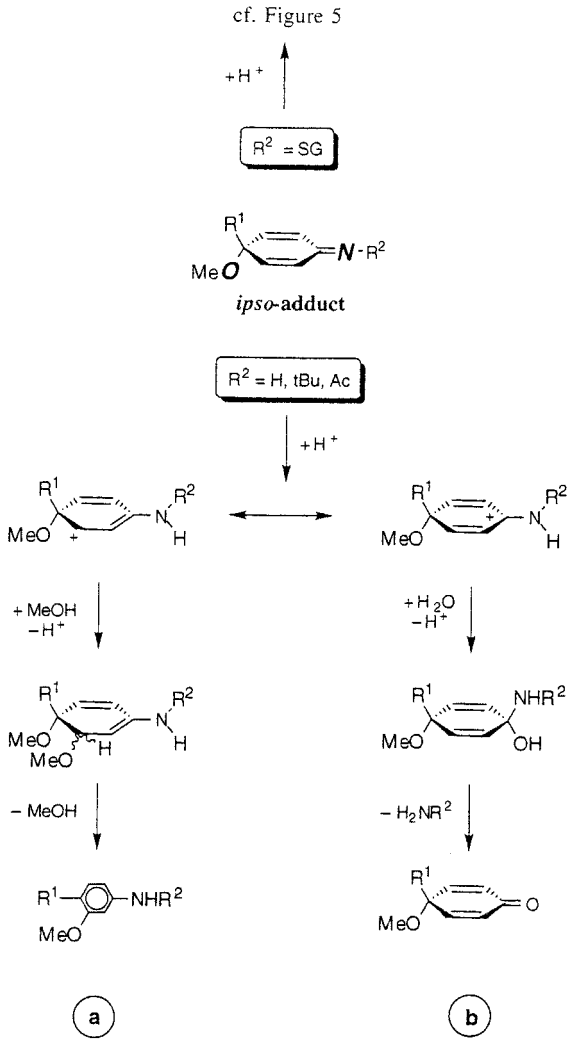


Figure 8. Decay possibilities of metastable/unstable *ipso*-adducts formed from various arylnitrenium ions in MeOH [R^1 = Me (*10*, *12*, *46*), Ph (*10*, *14*, *48*), fluorenyl (*13*); R^2 = H (*12*), tBu (*10*), Ac (*13*, *14*, *46*, *48*)]. For R^1 = OAlkyl and R^2 = SG, see Figure 5.

[3] The lack of some product ions (Table 1) may stem from electronic effects of the strong electronacceptor substituents on CID fragmentation.

[4] The term "sulfenamide cation" will be used throughout in order to distinguish *N*-sulfenylnitrenium ions from other arylnitrenium ions.

sulfenamide cations with other arylnitrenium ions. Since the latter are well-known to be highly mutagenic, reactions of sulfenamide cations with weak nucleophilic sites of DNA (and proteins) have to be assumed as well.

Going over to the conditions in mammalian cells, GSH-mediated activation of nitrosoarenes seems conceivable. Nuclei are known to contain distinct amounts of GSH (*50*−*53*), so that nitrosoarene molecules produced in the vicinity of nuclei, e.g., by cellular peroxidases (*54*, *55*), may reach the DNA compartment and be activated to the sulfenamide cation. Consequent detoxication reactions with excess GSH and H_2O should predominate, of course (*8*). But, as indicated by the ring addition of weak nucleophiles such as alcohol and water, reactions with weak nucleophilic DNA bases have to be considered, particularly in view of the high DNA concentrations caused by compartmentation and the electrostatically caused GSH-diminution around the DNA double strand (*56*). In fact, mutagenic action of NOPt was suggested to occur by an alternative activation pathway not using the *N*-hydroxyarylamine−nitrenium ion route (*19*−*21*). Among others, a GSH-mediated generation of the ultimately reactive species has been supposed (*21*, *57*), thus fostering a sulfenamide cation mechanism.

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