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Nucleophilic Addition of Thiols to Deoxynivalenol

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Supporting Information

ABSTRACT: Conjugation of deoxynivalenol (DON) with sulfur compounds is recognized as a significant reaction pathway, and putative DON−glutathione (DON−GSH) conjugates have been reported in planta. To understand and control the reaction of trichothecenes with biologically important thiols, we studied the reaction of DON, T-2 tetraol, and de-epoxy-DON with a range of model thiols. Reaction conditions were optimized for DON with 2-mercaptoethanol. Major reaction products were identified using HRMS and NMR spectroscopy. The results indicate that thiols react reversibly with the double bond (Michael addition) and irreversibly with the epoxide group in trichothecenes. These reactions occurred at different rates, and multiple isomers were produced including diconjugated forms. LC-MS analyses indicated that glutathione and cysteine reacted with DON in a similar manner to the model thiols. In contrast to DON, none of the tested mercaptoethanol adducts displayed toxicity in human monocytes or induced pro-inflammatory cytokines in human macrophages.

KEYWORDS: *deoxynivalenol, DON, thiol, mercaptoethanol, glutathione, cysteine, trichothecene, T-2 tetraol, de-epoxy-DON, NMR, MS/MS, HRMS, Michael reaction*

INTRODUCTION

Edible plants are often contaminated with toxigenic fungi, in the field or under storage conditions, leading to food and feed contamination. Among the most common genera of fungi is *Fusarium*, which includes many species that are pathogenic to agriculturally important crops. Among the major toxins produced by several *Fusarium* species are the trichothecenes.[1] Deoxynivalenol (DON) is a type B trichothecene mycotoxin characterized by the presence of a ketone group in the C-8 position (Figure 1). Although type B trichothecenes are generally less acutely toxic than type A-, C-, and D-trichothecenes, they are the most prevalent congeners in grain in Europe and North America.[2] DON possesses structural features that may give rise to chemical reactivity—the epoxy ring, hydroxyl groups, and an α,β-unsaturated carbonyl group. Consequently, DON may be biotransformed by metabolic processes in plants and animals. Although only a few biotransformation products have been identified so far, the potential presence of a wide range of biotransformation products has recently been shown in studies employing stable carbon isotope labels.[3] The chemical analysis of such biotransformation products is challenging, as they are usually not detected by routine analytical methods.

A significant part of the recent research on DON has been dedicated to pathways of conjugation or chemical derivatization of DON with sulfur-containing compounds. Several DON derivatives have been identified as DON-sulfonates,[4,5] DON-sulfates,[6] or DON−glutathione conjugates.[3,7] Glutathione (GSH) is a natural tripeptide containing cysteine and possesses a free thiol (−SH) group that behaves both as a nucleophile and as a reducing agent. For this reason, GSH is of biological

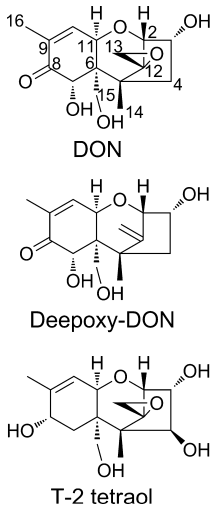

Figure 1. Structures of the trichothecenes used in the experiments.

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importance because it reacts with electrophilic or oxidizing species before they interact with critical cellular constituents such as nucleic acids or proteins.[8] Gardiner et al.[9] reported that RNA profiling of DON-treated barley spikes showed strong up-regulation of gene transcripts encoding glutathione-*S*-transferases and cysteine synthases. Suggested structures for DON−GSH conjugates include addition of GSH on the double bond at C-9[3,9] and nucleophilic attack on the hydroxylated C-7 atom or at the carbonyl carbon at C-8.[9] Frühmann et al.[10] proposed that DON−GSH is broken down in planta, resulting in methylthiodeoxynivalenol as the final product. They synthesized methylthiodeoxynivalenol and showed that their product was a mixture of the C-8 hemiketal and ketone, produced via addition of thiourea to the double bond of DON.

Here we report studies using mercaptoethanol as a model thiol for reaction with DON and other trichothecenes to identify the reaction pathways for DON with thiols. We also show that the same pathways are important for reaction of DON with a wide variety of thiols including cysteine and glutathione. In contrast to earlier reports, we identified the epoxide group as an important target for thiol addition to DON.

MATERIALS AND METHODS

Chemicals and Reagents. HPLC grade water and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Ammonium formate (Fluka, puriss. p.a. for HPLC), deoxynivalenol (DON) (≥98%), lipopolysaccharide (LPS) from *Escherichia coli*, CD_3CN (99.95 atom % D), 5.0 mm Wilmad NMR tubes, sodium carbonate (pro analysis), 2-mercaptoethanol (≥99.0%), 2-aminoethanethiol (≥98%), sodium methanethiolate (technical grade (>90%)), sodium 2-mercaptoethanesulfonate (≥98%), L-cysteine (≥98%), and reduced L-glutathione (≥98%) were from Sigma-Aldrich (Steinheim, Germany). Phosphate-buffered saline (pH 7.3, 0.172 M) was prepared from ready-to-use tablets (Oxoid, Hampshire, UK). Sodium bicarbonate (pro analysis, Merck, Darmstadt, Germany) and sodium carbonate were used to prepare 0.2 M buffers at pH 9.2 and 10.7. Buffer pH was measured at ambient temperature with a Mettler Delta 320 pH meter. Solutions of T-2 tetraol (50.3 μg/mL) and de-epoxy-DON 50.5 μg/mL) in acetonitrile were from Biopure (Romer Laboratories, Tulln, Austria). Standards of norDON B and norDON C were provided by Institute of Food Chemistry, Münster, Germany. Alamar Blue and human TNF-α Cytoset were from Invitrogen (Life Technologies, Carlsbad, CA, USA), and human IL-1β/IL-1F2 Duoset was from R&D Systems (Minneapolis, MN, USA). RPMI 1640, culture medium, penicillin/streptomycin, fetal bovine serum (EU standard, FBS), and PBS were from Lonza (Verviers, Belgium). Phorbol-12-myristate-13-acetate (PMA) was from Calbiochem (La Jolla, CA, USA). Hydrogen peroxide (30%) was from Merck.

Reaction of Trichothecenes with Thiols. All reactions were conducted in 2 mL HPLC vials and monitored by LC-MS or LC-HRMS.

Procedure 1. Aliquots (0.33 μmol) of the stock solutions of DON, de-epoxy-DON, and T-2 tetraol in acetonitrile were evaporated under a stream of N_2 at 60 °C and dissolved in carbonate buffer (pH 10.7; 1.0 mL), and mercaptoethanol 0.5 μL (7.13 μmol) was then added. The vials were flushed with argon, sealed, and placed in the autosampler set to 25 °C. Reactions (together with thiol-free controls) were followed by LC-MS (method A1), half-hourly for the first 8 h and then at 1−2 day intervals thereafter for 1 month. The reaction of DON with mercaptoethanol was also carried out in buffer at pH 9.2 and 7.3.

Procedure 2a. Mercaptoethanol (10 μL, 142 μmol) was added to DON (1.0 mg, 3.3 μmol) in buffer (1 mL; pH 10.7), and the reaction was followed by LC-MS for ca. 3 weeks (method A1), by which time DON was absent and the peak intensity of **1a**/**1b** was equal to that of **2b** (Figure S1).

Procedure 2b. Mercaptoethanol (15 μL, 210 μmol) was added to DON (1.5 mg, 5.1 μmol) in buffer (1.5 mL; pH 10.7), and the reaction was followed by LC-MS for 14 days, by which time the peak intensities of **2a** and **2b** were about equal and dominated the chromatogram (Figure S2a).

Procedure 3. Solutions of other thiols (71.3 mM, 1 mL) (i.e., other than mercaptoethanol) in buffer (pH 10.7) were added to solid DON (100 μg, 0.33 μmol), and reactions were monitored by one or more LC-MS methods (methods A1, A2, B1, or B2) for 3 weeks.

Oxidation of 1a/1b. Hydrogen peroxide (30%, 50 μL) was added to an aliquot (150 μL) of the reaction mixture obtained from the procedure 1 containing **1a**/**1b**. Reaction was followed for 1 h, when **1a**/**1b** were no longer detectable.

LC-MS. HPLC was performed using an Atlantis T3 column (150 × 2.1 mm, 3 μm; Waters). The flow rate was 0.3 mL/min, and the injection volume was 1.5 μL. Mobile phase A was 5 mM ammonium formate in MeCN/water (19:1), and mobile phase B was aqueous ammonium formate (5 mM). Gradient 1: Separation was performed by elution with a linear gradient of 5−15% A over 15 min and then to 100% A at 20 min, with a 3 min hold followed by a return to 5% A with a 3 min hold to equilibrate the column. Gradient 2: This was identical to gradient 1 except that the gradient started and ended with 0.5% A (instead of 5% A). Gradient 3 was identical to gradient 1, apart from the mobile phase containing 2.5 mM ammonium formate and 2.5 mM formic acid.

Method A1. A Finnigan Surveyor HPLC system was used with gradient 1 and interfaced to an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) operated in negative ionization full scan mode (m/z 180−600) and fitted with an electrospray ionization (ESI) interface. This method was used to monitor the reactions of DON, T-2 tetraol, and de-epoxy-DON with mercaptoethanol, as well as reactions of DON with sodium methanethiolate and sodium 2-mercaptoethanesulfonate. Capillary voltage and tube lens offset were tuned with continuous infusion of DON (10 μg/mL) in acetonitrile into a mobile phase composed of 10% A. The spray voltage was set to 3 kV, the sheath gas and auxiliary gas flow rates were 58 and 11 units, respectively, and the capillary temperature was 250 °C

The MS^n (n = 2−5) fragmentation of DON was studied using collision-induced dissociation in the ion trap by directly infusing 10 μg/mL DON in acetonitrile into a mobile phase composed of 10% A, via the instrument syringe pump (5 μL/min). The ESI settings were as described above. Individual precursor ions were selected with an isolation width of 2 m/z, the activation Q was set to 0.25, and the activation time was set to 30 ms. The collision energy was chosen such that the intensity of the precursor ion was <10% relative peak intensity. Product spectra were recorded for 30 s.

Method A2. Method A2 was performed as for method A1, except that gradient 2 was used for elution and the MS was run in positive mode for m/z 100−1000 in order to monitor the reaction of DON with 2-aminoethanethiol, glutathione, or cysteine.

Method B1. A Waters Acquity UPLC (Milford, MA, USA) used with gradient 1 was interfaced to a Q Exactive Fourier-transform high-resolution mass spectrometer (Thermo Fisher Scientific) and used for analysis of the products from the reactions of DON, T-2 tetraol, and de-epoxy-DON with mercaptoethanol and of DON with sodium methanethiolate and sodium 2-mercaptoethanesulfonate. A heated electrospray interface was used for ionization with a spray voltage of 3.8 kV and a temperature of 300 °C. The mass spectrometer was run in the negative full-scan mode in the mass range m/z 150−600. The mass resolution was set to 70000 at m/z 200. Other important interface parameters included a capillary temperature of 250 °C, a sheath gas flow rate of 55 units, and an auxiliary gas flow rate of 25 units.

For targeted MS/MS, $[M + HCOO]^-$ ions of mercaptoethanol derivatives were selected and subjected to higher energy collisional dissociation at a normalized collision energy of 35 eV. The resolution was set to 17500 at m/z 200, and the product ions were scanned in the mass range m/z 50−365, 50−445, or 50−525 (as appropriate).

Method B2. Method B2 was as described for method B1 except that gradient 2 was used for elution and the MS was run in positive mode

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for m/z 100−1000 in order to monitor the reaction of DON with 2-aminoethanethiol, glutathione, or cysteine.

Method C. Preparative LC-MS was performed by injecting portions (150 μL) onto an Atlantis T3 column (250 × 10 mm, 5 μm; Waters). The column was eluted with a Shimadzu LC-20AD pump (Shimadzu Corp., Kyoto, Japan) at 3 mL/min. A portion of the column effluent (0.1%) was continuously split into an LCQ Fleet ion trap mass spectrometer (Thermo Fisher), whereas individual fractions containing target compounds were collected manually.

Purification of DON−Mercaptoethanol Derivatives. Reaction mixtures from pocedures 2A and 2B were fractionated on Strata-X SPE columns (500 mg; Phenomenex, Torrance, CA, USA). The SPE column was conditioned with methanol (10 mL) and then water (10 mL), and the reaction mixture was applied. The columns were eluted with 5 mL each of 0, 5, 10, 15, 20, 25, 40, and 50% MeOH in water and then finally with MeOH. The 40 and 50% MeOH fractions from procedure 2A contained **1a**/**1b** and **2a**/**2b** and were combined and evaporated to ca. 1 mL under a stream of N_2. The components were purified by preparative LC-MS (method C) using a linear gradient of water (A) and acetonitrile (B) (from 5 to 21% B over 17 min, then 100% B for 3 min, and finally to 5% B for 5 min to equilibrate the column), with **1a**/**1b** eluting at 14.5 min and **2a**/**2b** (ca. 1:5 by NMR) at 16.8 min.

When the mixture obtained from procedure 2B was chromatographed by SPE (as above), the 40% MeOH fraction contained **2a**/**2b**. This material was concentrated to 1 mL as above and purified by preparative LC-MS (method C) using isocratic elution with 21% MeCN in water, with **2a**/**2b** (ca. 1:1 by NMR) coeluting at 6.6 min (Figure S2b).

NMR Spectroscopy. 1-D (^{1}H, SELTOCSY, SELROESY, ^{13}C, JMOD, DEPT135) and 2-D (COSY, TOCSY, HSQC, HMBC, NOESY, ROESY) NMR experiments were conducted with Bruker Avance AV 600 MHz and AVII 600 MHz NMR spectrometers equipped with 5 mm CP-TCI (^{1}H/^{13}C, ^{15}N−^{2}H) triple-resonance inverse cryoprobes with *Z*-gradient coils. Compounds were dissolved in CD_3CN in 5.0 mm Wilmad NMR tubes. Data were recorded and processed using Bruker TOPSPIN (version 2.1 and 3.0) software, and chemical shifts, determined at 25 °C, are reported relative to internal CHD_2CN (1.96 ppm) and CD_3CN (118.26 ppm).[11]

Cell Culture and Treatments. The human acute monocyte leukemia cell line (THP-1) was obtained from the European collection of cell cultures (ECACC) and grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were cultured at 37 °C under 5% CO_2 in a humidified incubator and kept in a logarithmic growth phase at (5−15) × 10^5 cells/mL through routine subculturing, according to standard ECACC protocol. The passage number was kept below 20. DON and its derivatives were dissolved in PBS and applied to the cells at a final concentration of 4 μM.

Proliferation. THP-1 cells (monocytes) were seeded at 150,000 cells/cm^2 and, following exposure to DON and the conjugates, the metabolic activity of the cells was measured using the Alamar Blue assay according to the manufacturer's protocol. The dark blue oxidized form of Alamar Blue is reduced to a highly fluorescent form in functional mitochondria,[12] and the measured fluorescence intensity is thus proportional to the number of viable cells. The fluorescence (585 nm) was quantified using a VICTOR^2 Multilabel Counter (PerkinElmer, Boston, MA, USA).

Cytokine Measurement, ELISA. Secreted cytokines were measured with an enzyme-linked immunosorbent assay (ELISA). THP-1 cells were seeded at 260,000/cm^2 and differentiated into macrophages by treatment with PMA (50 ng/mL) for 24 h. The medium was then replaced, and the cells were rested for 24 h before exposure. The cells were then treated with LPS (0.05 ng/mL) for 3 h, followed by toxin exposure for an additional 24 h. The medium was harvested and centrifuged (500*g*, 4 °C, 10 min) to remove cell debris. Levels of TNF-α and IL-1β in supernatants were measured by ELISA, using human TNF-α Cytoset or human IL-1β/IL-1F2 Duoset, respectively, according to the manufacturers' guidelines. The absorbances were measured using a plate reader (TECAN Sunrise, Phoenix Research Products, Hayward, CA, USA) equipped with analyzing software (Magellan VI).

Statistical Analysis. Data analyses were performed using Sigma Plot version 13.0. Statistical significance ($p < 0.05$) was assessed using one-way-ANOVA, followed by Dunnett's post-test.

RESULTS AND DISCUSSION

Reaction of DON, De-epoxy-DON, and T-2 tetraol with Mercaptoethanol. Initial experiments followed the reaction of DON using LC-MS (method A1, gradient 3) for up to 1 month, with mercaptoethanol as a model thiol under neutral and weakly basic conditions (procedure 1). Mercaptoethanol was used because it was expected to produce DON derivatives with retention times and ionization properties similar to those of DON itself, thereby facilitating monitoring of the reaction and its products by LC-MS. At pH 10.7 there was rapid formation of **1d** and **1e** (Figure 2) with a mass difference of 78 Da relative to the formate adduct of DON, corresponding to a single addition of mercaptoethanol. The broad peak observed for **1e** was suspected to be caused by the presence of formic acid in gradient 3; however, use of a neutral eluent (gradient 1) did not appreciably sharpen this peak. After 30 min, peaks corresponding to **1a**/**1b** (not resolved) and **1c** were also detected with the same m/z (Figure 2), along with **2a** and **2b** (Figure 2) exhibiting a mass difference of 156 Da

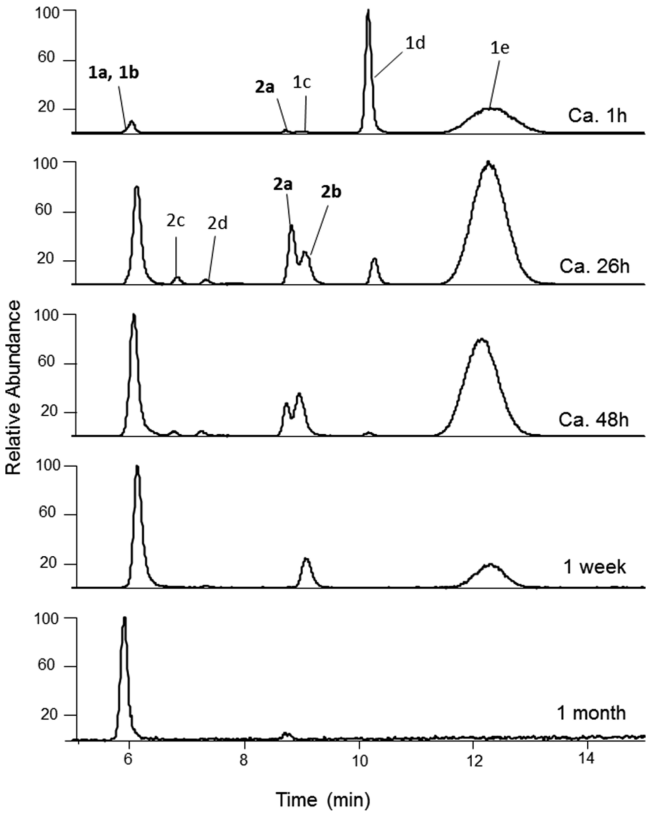

Figure 2. LC-MS extracted ion chromatograms (m/z 419 + m/z 497) from the reaction of DON with mercaptoethanol (method A1, pH 10.7, 25 °C). Adduct peaks are annotated 1 (m/z 419) or 2 (m/z 497) to indicate the number of mercaptoethanol units adducted to DON and with lower case letters then used to indicate separate peaks. Peaks labeled with bold text refer to adducts with structures determined by NMR spectroscopy (Figure 4). Note, a small drift in retention time occurred toward the end of the study.

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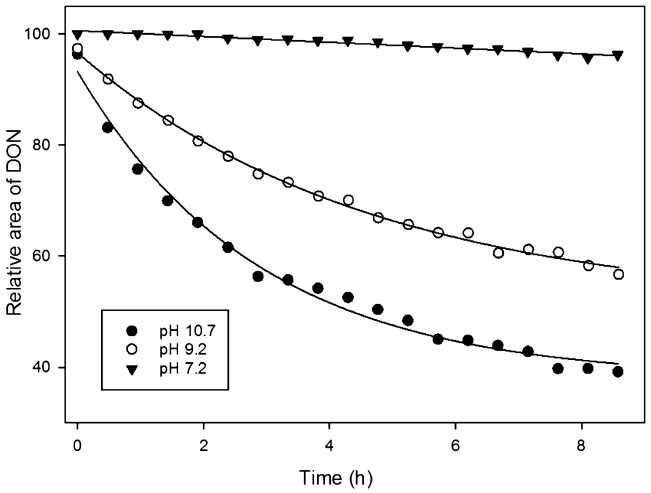

Figure 3. Change in the concentration of DON (measured by LC-MS, as a percentage of the total concentration of DON derivatives) with time upon treatment with mercaptoethanol at 30 °C at selected pH values. Lines show data fitted to exponential decay curves (pseudo-first-order kinetics).

relative to DON (corresponding to the addition of two molecules of mercaptoethanol). The ratios of these peaks continued to change over time, with **1a**/**1b** becoming more prevalent until they were the only compounds remaining in the mixture. Initially, four peaks corresponding to addition of two molecules of mercaptoethanol were detected, but **2c** and **2d** were minor and were no longer detectable after 1 week. Products **2a** and **2b** were the prominent double-addition conjugates, but **2a** disappeared in less than a week, leaving **2b** as the most prominent double-addition conjugate. However, **2b** also slowly disappeared with time, so that after 1 month the major peak was from **1a**/**1b**. The intensity of the LC-MS peak from DON gradually decreased as the reaction progressed (Figure 3) and was no longer detectable after 3 weeks.

We anticipated that thiol addition was most likely to occur reversibly at the C-10 carbon of the α,β-unsaturated ketone in DON and irreversibly at the epoxy group in DON. To test this hypothesis, we compared the reactions of DON, T-2 tetraol, and de-epoxy-DON with mercaptoethanol (Figure S17). In T-2 tetraol the C-8 carbon is hydroxylated, resulting in a 9,10-double bond that is not conjugated, whereas in de-epoxy-DON the epoxide ring has been reduced to an olefinic methylene (Figure 1). The reaction of T-2 tetraol was followed for 10 days by LC-MS (method A1), during which time just one new, later-eluting peak appeared, corresponding to addition of one mercaptoethanol molecule. LC-HRMS analysis (method B1) of this peak revealed it had m/z 421.1541, corresponding to $C_{18}H_{29}O_9S^-$ ([T-2 tetraol + HSEtOH + HCOO]$^-$, Δ 0.9 ppm) (Figures S3). Similarly, the reaction mixture containing de-epoxy-DON gave in total four peaks with m/z 403.1437−403.1443 corresponding to $C_{18}H_{27}O_8S^-$ ([de-epoxy-DON + HSEtOH + HCOO]$^-$, Δ 1.2−2.9 ppm). After 10 h of reaction, an earlier-eluting peak had almost the same intensity as de-epoxy-DON, whereas a later-eluting peak was higher in intensity (Figure S4a), and two other peaks had very low intensities. After 10 days, analysis revealed de-epoxy-DON was the most intense peak, with the later-eluting mercaptoethanol adduct now having an intensity 4 times lower than that of de-epoxy-DON (Figure S4b), indicating reversibility of the reaction. In contrast, reanalysis of the T-2 tetraol reaction after 10 days revealed the same two peaks as detected early in the reaction, but with the mercaptoethanol adduct having increased in intensity relative to T-2 tetraol. No double adducts of mercaptoethanol with de-epoxy-DON or T-2 tetraol were detectable at any stage of the reactions. These observations are consistent with reversible Michael addition of thiols to the conjugated double bond in type B trichothecenes and a slower irreversible addition of thiols to C-13 on the 12,13-epoxy group of trichothecenes (Figures 11 and S17).

These results suggested that **1c**, **1d**, and **1e** were products from Michael addition of mercaptoethanol to the 9,10-double bond of DON, whereas the relatively early eluting **1a**/**1b** was attributable to addition of mercaptoethanol to the epoxy group in DON.

During the reaction of DON with mercaptoethanol under basic conditions, several minor products with masses similar to that of DON were detected by LC-HRMS. The same products were also observed in thiol-free control samples, and these appeared to be identical to those reported earlier by Bretz et al.,[13] thought to be products from degradation of DON under basic conditions. Two of the degradation products, norDON B and norDON C,[13] were confirmed in the DON−mercaptoethanol reaction mixtures using authentic standards. Because these DON degradation reactions compete with the thiol addition, higher concentrations of thiols were used in the semipreparative reactions to reduce the reaction time and thereby minimize the effect of the degradation reactions.

Minor peaks affording ions with a 16 Da higher mass (m/z 435), relative to the DON−mercaptoethanol adducts, were also detected during the reaction, suggesting the presence of sulfoxide derivatives. Addition of H_2O_2 to an aliquot of a reaction mixture containing **1a**/**1b** resulted in rapid oxidation, affording products with the same masses and retention times as the putative sulfoxide derivatives (Figure S5). This indicates that m/z 435 peaks are due to the sulfoxides of thiol adducts **1a**/**1b**. Similar autoxidation was also seen with DON derivatives of the other thiols including cysteine or glutathione. Purified DON conjugates were therefore stored under argon to minimize the autoxidation.

Preparative Synthesis and Purification. DON and mercaptoethanol were reacted on a semipreparative scale to identify the major products. Two reactions were followed by LC-MS: one was stopped by SPE when almost equal ratios of **1a**/**1b** and **2b** were present (procedure 2a) and the other when the ratios of **2a** and **2b** were identical (procedure 2b). Fractionation of the reaction mixtures by SPE followed by semipreparative LC-MS afforded pure mixtures of **1a** together with **1b** and of **2a** together with **2b**.

Structural Elucidation. NMR structural elucidation of major products was done on mixtures of **1a** and **1b**, and of **2a** and **2b**, in CD_3CN, with DON used for spectral comparison. LC-HRMS showed a single peak for **1a**/**1b**, with m/z 419.1383 consistent with $C_{18}H_{27}O_9^-$ (DON + HSEtOH + HCOO]$^-$), indicating the addition of one molecule of mercaptoethanol to DON. However, NMR spectroscopy revealed the presence of two isomeric compounds (Table 1) in a ratio of 5:1. Three of the most diagnostic pairs of signals were methyl singlets at 1.27 and 1.24 ppm (H-14), methyl triplets at 1.74 and 1.81 ppm (H-16), and signals due to olefinic protons (both doublets of quartets) at 5.35 and 6.44 ppm (H-10). The presence of an olefinic proton in **1a** and **1b** indicated the presence of a double bond and thus showed that mercaptoethanol had not been added to C-10.

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Table 1. ^{1}H and ^{13}C NMR Assignments (δ) for DON, 1a, 1b, 2a, and 2b in CD_3CN

	DON		1a		1b		2a		2b	
atom	^{1}H[a]	^{13}C	^{1}H[a]	^{13}C	^{1}H[a]	^{13}C	^{1}H[a]	^{13}C	^{1}H[a]	^{13}C
2	3.51 (d, 4.5)	81.4	3.72 (d, 4.5)	81.6	3.76 (d, 4.5)	80.9	3.78 (d, 4.5)	80.9	3.81 (d, 4.5)	83.1
3	4.34 (dq, 11.1, 4.4)	69.4	4.55 (dq, 10.5, 4.3)	70.5	4.52 (dq, 10.6, 4.5)	70.3	4.50 (dq, 10.5, 4.3)	70.4	4.58 (dq, 10.6, 4.4)	70.8
4[b]	2.30 (dd, 14.6, 4.4)	44.3	1.42 (dd, 13.8, 3.7)	45.9	1.91 (dd, 14.0, 3.2)	44.5	1.50 (dd, 14.2, 3.5)	45.9	1.48 (dd, 13.8, 3.8)	46.9
	1.95 (dd, 14.6,11.1)		1.97 (dd, 13.8, 10.7)[c]		2.06 (dd,14.0, 10.6)		2.00 (dd,14.2, 10.4)		1.99 (dd,13.8, 10.6)	
5	–	46.7	–	50.8	–	50.9	–	50.4	–	51.0
6	–	53.1	–	51.8	–	53.4	–	54.1	–	55.4
7	4.80 (d, 2.9)	75.2	3.92 (d, 3.8)	77.7	4.71 (d, 2.7)	75.2	3.94 (d, 5.8)	72.9	3.95 (d, 5.4)	78.8
8	–	200.7	–	104.7	–	200.4		107.7	–	106.6
9	–	136.0	–	142.4	–	134.9	1.80 (dq, 9.2, 7.0)[d]	46.3	1.78 (dq, 12.0, 6.5)[d]	44.2
10	6.60 (dq, 5.9, 1.6)	139.6	5.35 (dq, 3.7, 1.6)	122.9	6.44 (dq, 5.3, 1.5)	139.7	2.41 (dd, 9.2, 6.6)	52.0	2.77 (dd, 12.0, 7.2)[d]	50.2
11	4.86 (d, 5.9)	70.9	4.81 (dq, 3.7, 1.6)	77.0	4.94 (d, 5.3)	71.8	4.48 (d, 6.6)	85.9	4.62 (d, 7.2)	76.5
12	–	66.7	–	81.7	–	81.4	–	81.6	–	81.3
13	3.03 (d, 4.4)	47.8	2.99 (d, 13.7)	36.3	2.98 (d, 13.2)	37.7	2.94 (d, 13.8)	36.2	3.09 (d, 13.7)	37.0
	3.05 (d, 4.4)		3.55 (d, 13.7)		3.54 (d, 13.2)		3.54 (d, 13.8)		3.63 (d, 13.7)	
14	1.05 (s)	14.6	1.27 (s)	15.8	1.24 (s)	15.7	1.23 (s)	16.3	1.22 (s)	15.6
15[b]	3.70 (dd, 11.9, 4.9)	61.7	3.31 (d, 8.7)	70.0	3.61 (dd, 11.7, 5.2)	64.8	3.52 (d, 8.3)	71.6	3.49 (d, 8.1)	70.3
	3.67 (dd, 11.9, 5.7)		4.09 (d, 8.7)		3.87 (dd, 11.7, 6.5)		4.11 (d, 8.3)		4.16 (d, 8.1)	
16	1.82 (dd, 1.6, 0.9)	15.3	1.74 (t, 1.7)	16.2	1.81 (t, 1.4)	15.5	1.13 (d, 6.9)	17.4	1.12 (d, 6.5)	13.3
1′	–		2.71 (dt, 3.1, 6.2)	37.0	2.69 (dt, 1.6, 6.2)	37.2	2.74 (m)	36.8	2.73 (m)	37.1
2′	–		3.68 (m)	62.0	3.64 (m)	61.9	3.72 (m)	62.3	3.71 (m)	62.03[e]
1″	–	–	–		–	–	2.69 (dt, 13.8, 6.3)[b]	35.6	2.66 (dt, 13.5, 6.4)	35.1
							3.00 (dt, 13.7, 6.2)		2.90 (dt, 13.5, 6.4)	
2″	–	–	–		–	–	3.66 (m)	62.00[e]	3.62 (m)	61.9
3-OH	3.25 (br d, 4.4)		3.13 (d, 4.4)		3.15 (d, 4.7)		3.14 (d, 4.5)		3.31 (d, 4.3)	
7-OH	3.79 (d, 2.9)		4.17 (bs)				3.50 (d, 5.8)[f]		4.10 (br s)	
12-OH	ND[g]		ND		ND		ND		ND	
2′−OH	ND		3.06 (t, 5.2)		ND		3.16 (t, 5.5)		3.11 (t, 4.8)[f]	
2″−OH	ND		ND		ND		3.12 (t, 6.6)[f]		3.08 (t, 6.4)	
15-OH	2.70 (br t, 5.5)		ND		2.66 (t, 6.2)		ND		ND	

[a]In the format: chemical shift in ppm (multiplicity, coupling constants in Hz). [b]Orientation of the protons was distinguishable (except for H-15 of DON and **1b**), and they are presented in the order α-H and β-H. [c]Resonance hidden under solvent signal. [d]Coupling constant measured from the HSQC spectra. [e]Shifts to two decimal places due to very close, but distinguishable resonances. [f]Coupling constants measured from TOCSY spectra. [g]ND, not detected.

Examination of the COSY and TOCSY spectra of the major isomer (**1a**) revealed a number of relatively short spin systems corresponding to H-16/H-10/H-11, H-2/H-3/3-OH/H-4α/H-4β/H-14, H-13a/H-13b, H-7/7-OH, H-15α/H-15β, and H-1′/H-2′/2-OH. The corresponding protonated carbon resonances were assigned from HSQC correlations, and connections between spin systems were established via correlations observed in HMBC spectra. Of particular note were HMBC correlations from the methyl triplet at 1.74 ppm (H-16) to 104.7 (C-8), 142.4 (C-9), and 122.9 (C-10) ppm and from H-15α/H-15β (3.31 and 4.09 ppm) to 104.7 (C-8), 77.7 (C-7), 50.8 (C-5), and 51.8 ppm (C-6). The latter established **1a** as a C-8 hemiketal derivative generated by intramolecular addition of the oxygen atom on C-15 to the C-8 carbonyl. Furthermore, correlations observed in the HMBC spectra from H-13a/H-13b (2.99/3.55 ppm) and C-1′ (37.0 ppm) and between H-1′ (2.71 ppm) and C-13 (36.3 ppm), showed attachment of the mercaptoethanol moiety at C-13 as a result of nucleophilic attack on the epoxy group of DON.

Examination of the corresponding spectra of the minor component (**1b**) revealed similar spin systems and correlations. Notable differences from **1a** included the presence in **1b** of HMBC correlations of the methyl protons at 1.81 ppm (H-16) to carbon signals at 200.7 (C-8), 134.9 (C-9), and 139.7 (C-10) ppm (Figure S6). Also, the COSY/TOCSY spectra of **1b** included a spin system corresponding to H-15α/H-15β/15-OH. The multiplicity of the signals from the H-15 resonances in **1b** (doublet of doublets) was consistent with ^{3}J coupling to the 15-OH proton, in contrast to **1a**, where H-15 resonances were observed as a pair of doublets due to the absence of a 15-OH. These results revealed the minor component (**1b**) to be the ketone analogue of **1a** (Figure 4). These observations are in accord with the known keto−hemiketal equilibration of type-B

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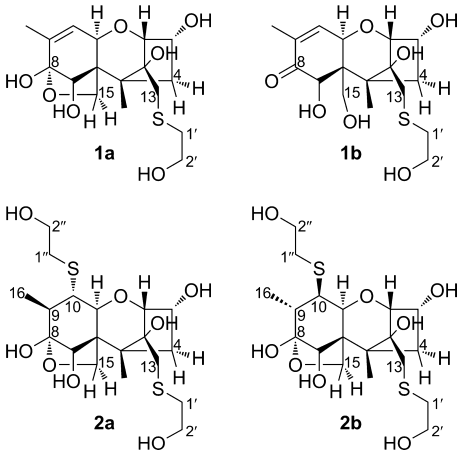

Figure 4. Structures of the major products purified from the reaction of DON with mercaptoethanol. **1a** and **1b** are the ketone and hemiketal pair resulting from addition of mercaptoethanol to the epoxide ring of DON, whereas **2a** and **2b** are a pair of diastereoisomers of the hemiketal form of adducts of mercaptoethanol to both the double bond and the epoxide ring of DON.

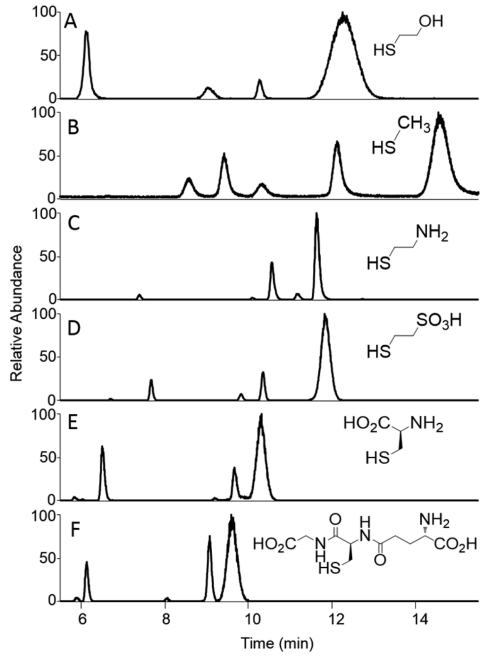

Figure 6. Extracted ion LC-MS chromatograms at the specified value of *m/z* for monoadducts of DON after reaction with various thiols at pH 10.7: (A) mercaptoethanol at 24 h, *m/z* 419.1381 ($[DON + HSCH_2CH_2OH + HCOO]^-$), negative ion mode; (B) sodium methanethiolate at 7 h, *m/z* 389 ($[DON + HSCH_3 + HCOO]^-$), negative ion mode; (C) 2-aminoethanethiol at 6 h, *m/z* 374.1632 ($[DON + HSCH_2CH_2NH_3]^+$), positive ion mode; (D) sodium 2-mercaptoethanesulfonate at 18 h, *m/z* 437.0945 ($[DON + HSCH_2CH_2SO_3]^-$), negative ion mode; (E) cysteine at 46 h, *m/z* 418.1530 ($[DON + CYS + H]^+$), positive ion mode; (F) glutathione at 48 h, *m/z* 604.2171 ($[DON + GSH + H]^+$), positive ion mode.

trichothecenes,[14] with the hemiketal form appearing to be favored by derivatization of the 9,10-olefinic group.[5,10,15]

Compounds **2a** and **2b** were isolated twice as a mixture, once in an approximate 1:1 ratio and once in a 1:5 ratio, which facilitated identification of NMR signals associated with the two components (Figure S7). LC-HRMS showed that **2a** and **2b** afforded ions with *m/z* 497.1524 and 497.1523, respectively, corresponding to $C_{20}H_{33}O_{10}S_2^-$ (Δ 0.7 and 0.6 ppm, respectively), consistent with the addition of two mercaptoethanol molecules to DON (i.e., $[DON + (HSEtOH)_2 + HCOO]^-$). The COSY, TOCSY, and SELTOCSY NMR spectra were used to identify spin systems corresponding to H-16/H-9/H-10/H-11, H-2/H-3/3-OH/H4α/H-4β/H-14, H-15α/H-15β, H-7/7-OH, H-13a/H-13b, H-1′/H-2′/2′-OH, and H-1a″/H-1b″/H-2″/2″-OH for both **2a** and **2b**. Protonated carbon resonances were assigned from correlations in HSQC spectra, whereas HMBC correlations were used to assign nonprotonated carbon resonances and to connect the spin systems. Correlations observed in the ROESY and SELROESY spectra established the relative stereochemistries in **2a** and **2b**. In contrast to **1a**/**1b** and DON, the NMR spectra of **2a** and **2b** lacked resonances attributable to olefinic protons or carbons, indicating thiol addition to C-10 in **2a** and **2b**. Consistent with this, the H-16 methyl resonances of **2a** and **2b** were shifted upfield relative to DON and **1a**/**1b** and appeared as doublets (J = 7 Hz), indicating the presence of a proton at C-9. The H-9 signal in **2a** and **2b** was further coupled (COSY) to H-10 as well as to the H-16 methyl, establishing that addition of a mercaptoethanol molecule had occurred at C-10 (Michael

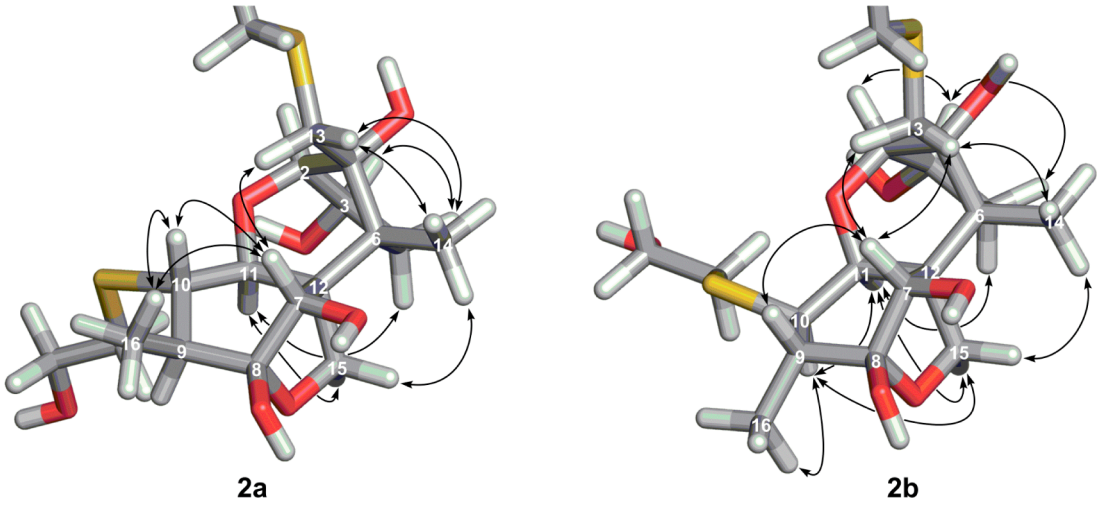

Figure 5. 3D models of **2a** and **2b**, showing selected structurally significant correlations (arrows) observed in ROESY and SELROESY NMR spectra.

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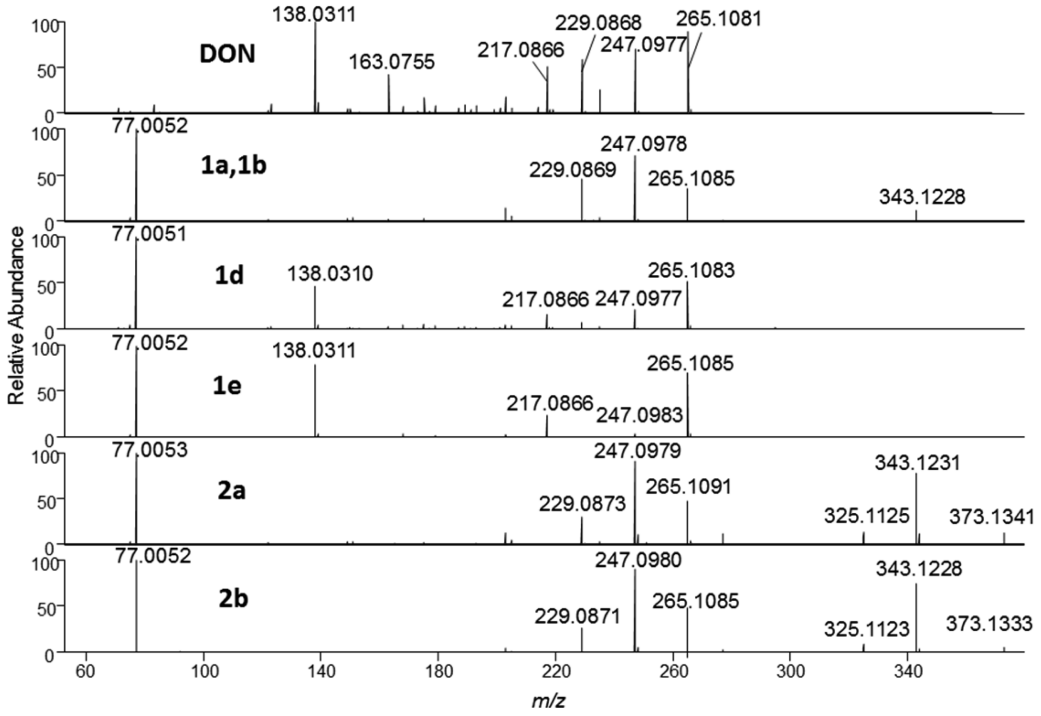

Figure 7. Product ion mass spectra from LC-HRMS^2 of the formate adduct ions of DON and its mercaptoethanol derivatives **1a**/**1b** (derivatized at the epoxide), **1d** and **1e** (Michael addition at C-10) and **2a** and **2b** (both epoxide and Michael addition). The prominent ion at *m*/*z* 77.0052 appears to correspond to $HOCH_2CH_2S^-$ (Δ 19 ppm) and is present in the spectra of all mercaptoethanol adducts regardless of the position of attachment of the thiol.

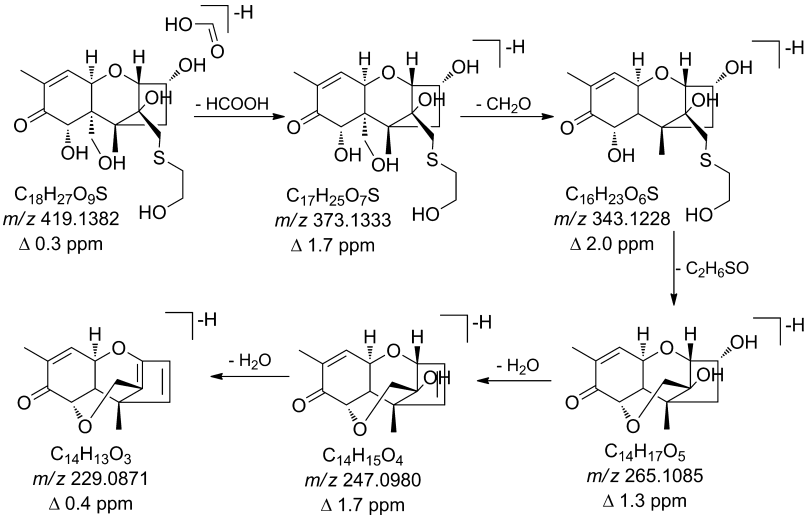

Figure 8. Possible negative ion mass spectral fragmentation pathway for mercaptoethanol adducts of the epoxide group of DON. The *m*/*z* values were measured by LC-HRMS.

addition) of DON. This conclusion was supported by HMBC correlations observed between H-10 (2.41/2.77 ppm) and both C-16 (17.4/13.3 ppm) and C-1″ (35.6/35.1 ppm) (Figure S8). No carbonyl resonances were observed in the ^{13}C, JMOD, or HMBC spectra of **2a** and **2b**. However, the HMBC spectra showed that both H-16 (1.13/1.12 ppm) and H-15 (3.51/4.11 and 3.49/4.16) correlated with a hemiketal carbon (C-8, 107.7/106.6 ppm) for both **2a** and **2b**. Furthermore, the H-15 doublets showed no TOCSY correlations to a 15-OH, indicating that **2a** and **2b** were both hemiketals (Figure 4). Correlations from H-13a (2.94/3.09 ppm) to C-2 (80.9/83.1 ppm), C-12 (81.6/81.3 ppm), C-5 (50.4/51.0 ppm), and C-1′ 36.8/37.1 ppm) confirmed the presence of a mercaptoethanol moiety at C-13 of **2a** and **2b**. Thus, **2a** and **2b** have the same skeleton and differ only in their stereochemistries at C-9 and C-10, which is reflected in the differing ^3J coupling constants for this spin system (Table 1). The stereochemistries at C-9 and C-10 were established from ROESY (Figure S9) and SELROESY NMR experiments (Figure 5). For **2a**, the ROESY spectra showed correlations between H-7 and both H-16 (not observed for **2b**) and H-10, locating H-10 and the 16-methyl together with H-7 on the β-face of the molecule. In the case of **2b**, however, H-7 showed a ROESY correlation to H-9, whereas H-10 showed ROESY correlations to H-11, H-15α (3.49 ppm),

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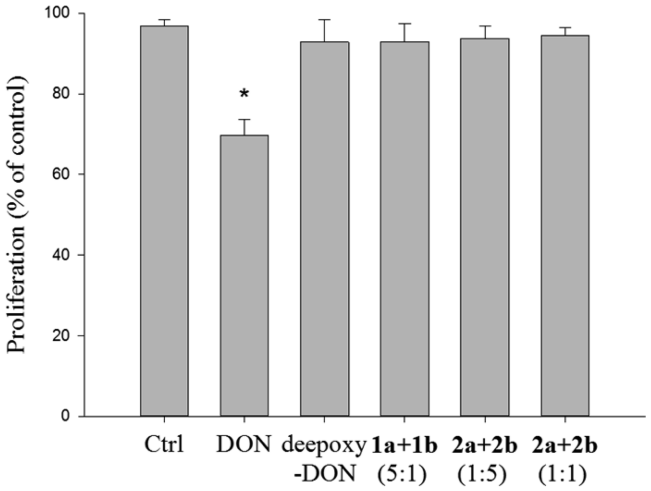

Figure 9. Proliferation of THP-1 cells after treatment with 4 μM DON or its derivatives for 24 h, measured with Alamar Blue. Data represent mean ± SD of three independent experiments. (∗) Significantly different from control (one-way-ANOVA with Dunnett's post-test, $p < 0.05$). Two samples with different ratios of **2a** and **2b** were tested.

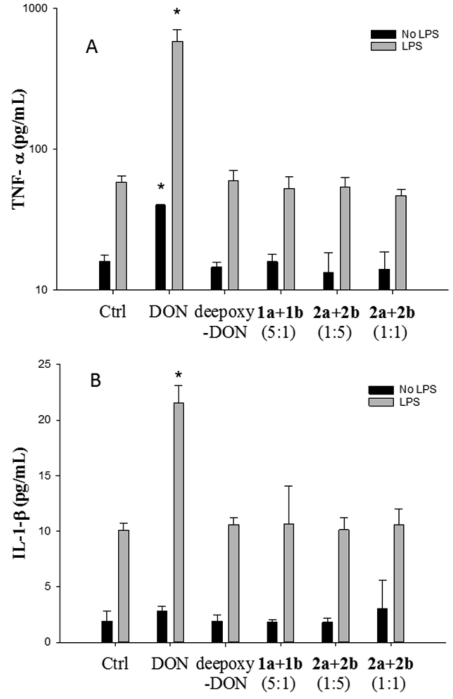

Figure 10. Cytokine secretion measured by ELISA in THP-1 cells treated for 3 h with LPS (0.05 ng/mL) followed by treatment with DON and its derivatives (4 μM) for 24 h. Data represent the mean ± SD of three independent incubations. (∗) Significantly different from control (one-way-ANOVA with Dunnett's post-test, $p < 0.05$). Two samples with different ratios of **2a** and **2b** were tested.

and H-16. This locates H-10 and the 16-methyl group on the α-face and H-9 on the β-face of **2b**. This situation places the 16-methyl and mercaptoethanol substituents attached at C-9 and C-10, respectively, equatorially on the ring in both **2a** and **2b** (Figures 5 and Figure S10) and would be expected to result in the least steric crowding in this region of these molecules. Thus, **2a** and **2b** have the structures shown in Figure 4.

Reaction of DON with Other Thiols. A set of experiments was carried out to see if DON reacted similarly with other thiols, including the biologically relevant amino acid cysteine and the tripeptide glutathione. Reactions were monitored for 1 week by LC-MS (Figure 6) and the chromatograms all showed peaks corresponding to the addition of a single thiol molecule to DON. The chromatograms displayed patterns of broad and sharp, later- and earlier-eluting peaks similar to those observed from the mercaptoethanol reaction (Figure 2). A wider array of thiol addition products formed in a shorter time for the reactions with smaller thiols, presumably as a consequence of reduced steric hindrance. Double conjugates were also present in all of the reaction mixtures (Figure S11). When mixtures were analyzed after aproximately 45 days at room temperature, there was only one early-eluting peak present having a mass that corresponded to addition of a single thiol (data not shown) believed to be analogous to the identified **1a**/**1b** epoxide adducts in the DON−mercaptoethanol reaction.

LC-HRMS and LC-MS^n. We studied the MS characteristics and fragmentation patterns of DON and its mercaptoethanol derivatives using LC-HRMS and LC-HRMS^2 as well as multiple-stage ion trap LC-MS^n. Upon electrospray ionization in the negative ion mode, **1a**−**1e** afforded formate adducts with m/z 419.1382−419.1384, corresponding to an elemental composition of $C_{18}H_{27}O_9S^-$ (Δ 0.3−0.5 ppm), whereas **2a**−**2d** afforded formate adducts with m/z 497.1523−497.1524 corresponding to an elemental composition of $C_{20}H_{33}O_{10}S_2^-$ (Δ 0.5−0.7 ppm). The formate adducts of **1a**/**b**, **1d**/**1e**, and **2a** and **2b** showed different fragmentation patterns during LC-HRMS^2 (Figure 7), attributable to different fragmentation pathways for the epoxide conjugates and Michael addition products, potentially allowing the two types of addition products to be distinguished by MS alone. To understand these differences, the fragmentation spectra of the mercaptoethanol adducts were compared to those of DON (Figure 7). The fragmentation pathways of DON were studied in more detail by multiple-stage ion trap MS^n (Figure S12). Fragmentation of the deprotonated molecular ions of DON (m/z 295), obtained from collision-induced MS^2 fragmentation of the corresponding formate adduct (m/z 341), primarily yielded m/z 265 (loss of CH_2O). Further fragmentation of m/z 265 (MS^4) yielded m/z 247 (loss of H_2O), m/z 217 (loss of H_2O and CH_2O), and m/z 138 as the most abundant product ions. The two losses of CH_2O during MS fragmentation of DON are attributable to cleavage of the CH_2OH side chain at C-6 and cleavage of the epoxide, as proposed previously.[16] The m/z 138 fragment appears to be a rare radical anion and has earlier been suggested to be the result of cleavage through the B-ring of the molecule.[16] An m/z 138.0311 product ion was present in the LC-HRMS^2 spectra of the DON, **1d** and **1e**, but not in compounds where mercaptoethanol was known to be added to the epoxy group (**1a**/**1b**, **2a**, and **2b**) (Figure 7). Additionally, HRMS^2 analysis of de-epoxy-DON did not reveal the presence of a m/z 138.0311 fragment (Figure S13). The foregoing information suggests that the formation of the m/z 138 fragment ion is associated with the presence of an intact 12,13-epoxy group, and **1d** and **1e** are thus suspected to be Michael adducts of mercaptoethanol at C-10 of DON. Another diagnostic feature that may assist in distinguishing between a Michael addition product and a reaction product from monoaddition of mercaptoethanol to the epoxy group is the presence of an m/z 343.1228 product ion ($C_{16}H_{23}O_6S^-$, Δ 2.9 ppm) in the LC-HRMS^2 spectra of the epoxide conjugates (Figure 7). These ions are formed by loss of formate and of CH_2O, presumably from the C-6 position of the molecular ion.

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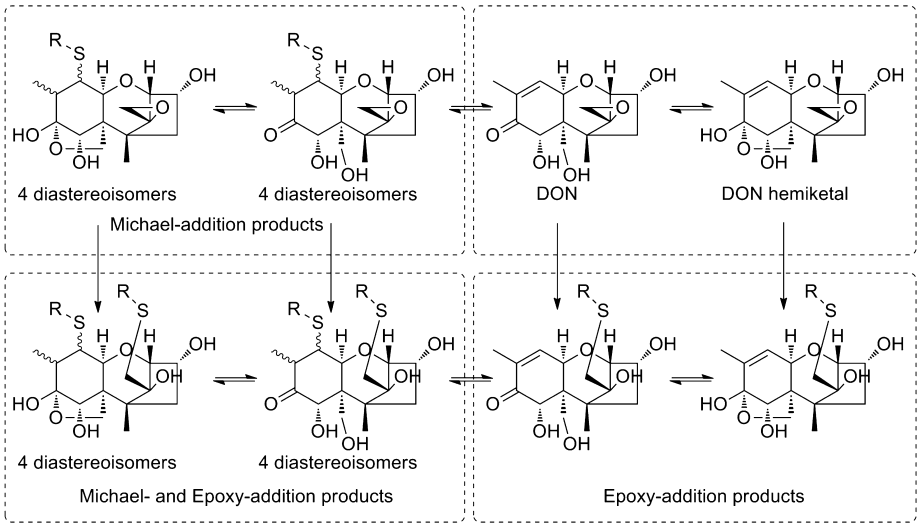

Figure 11. Generalized scheme showing proposed reaction of DON with a thiol (RSH) under basic conditions, as well as hemiketal−ketone equilibration at C-8.

In the LC-MS^n spectra, fragmentation of m/z 343 (MS^4) afforded m/z 265 (loss of 78 Da, corresponding to the mass of mercaptoethanol), m/z 247 (additional loss of H_2O), and m/z 229 (further loss of H_2O). Formation of the m/z 265 ion could occur via linkage of the 7-OH group and C-13 during the mercaptoethanol elimination to form a six-membered ring (Figure 8).

Cellular Effects. DON is able to cause cellular effects, such as inflammation and toxicity, through its ability to target ribosomes and to cause ribotoxic stress.[17] Because de-epoxy-DON does not affect ribosomes, it has been proposed that the 12,13-epoxy group is critical for DON's action on ribosomes.[17] To compare the effects of DON, de-epoxy-DON, and mercaptoethanol derivatives **1a**/**1b** and **2a**/**2b** on proliferation of THP-1 monocytes, we used the Alamar Blue assay, as previous studies[18] showed toxicity of DON with Alamar Blue in different cell lines. THP-1 cells were treated with DON, de-epoxy-DON, and purified mixtures of **1a**/**1b** and **2a**/**2b** for 24 h. DON decreased the proliferation of THP-1 monocytes, but de-epoxy-DON and DON−mercaptoethanol adducts **1a**/**1b** and **2a**/**2b** did not (Figure 9). These results support the epoxide moiety as an important structural feature for the toxicity of DON.

DON induces the expression of several pro-inflammatory cytokines, such as TNFα, IL-1b, IL-6, and IL-8,[19] and pretreatment (i.e., priming) with LPS potentiates this effect.[20,21] To examine pro-inflamatory responses induced by DON, de-epoxy-DON, and mercaptoethanol derivatives **1a**/**1b** and **2a**/**2b**, we analyzed the levels of TNF-α and IL-1β secretion by PMA-differentiated macrophages, with and without LPS priming. Regardless of the absence or presence of LPS, there was an increase in TNF-α in response to DON, but not to any of the derivatives (Figure 10A), although the sensitivity was higher with LPS present. Similarly, DON increased the release of IL-1β in LPS-primed cells, whereas de-epoxy-DON, **1a**/**1b**, and **2a**/**2b** did not (Figure 10B), although cells that were not treated with LPS did not show an IL-1β response to any of the compounds, including DON.

Thus, DON, but not de-epoxy-DON or mercaptoethanol derivatives **1a**/**1b** and **2a**/**2b**, reduced proliferation of THP-1 monocytes and induced pro-inflammatory responses in PMA-differentiated LPS-primed THP-1 macrophages. The lack of toxic responses to **1a**/**1b** and **2a**/**2b** (both of which involve derivatization of the 12,13-epoxy group of DON) supports the involvement of the epoxy group in the toxicity of trichothecenes—a hypothesis hitherto based solely on observations with de-epoxy-DON. Whereas all of the mecaptoethanol adducts isolated in the present study were derivatized at C-13 of the epoxy group of DON and were nontoxic, previous studies[22,23,10] have also demonstrated reduced toxicity for Michael addition products of DON.

Effect of pH on Reaction Rate. DON and mercaptoethanol were reacted at pH 7.3, 9.2, and 10.7, and the reaction was monitored by LC-MS. Neither DON nor mercaptoethanol contain acidic or basic groups, so DON and its mercaptoethanol derivatives can be expected to have similar responses in LC-MS. This makes it possible to estimate the proportions of DON and its reaction products by integrating areas under the peaks in the LC-MS chromatograms. We used a large excess of the thiol and observed pseudo-first-order reaction kinetics and followed the depletion of DON by plotting the relative peak area over time (Figure 3). However, the reaction kinetics are expected to be more complex, with several reversible reaction equilibria including Michael addition of the thiol to the double bond and hemiketal−ketone exchange (Figure 11). Furthermore, autoxidation of the thiol to form the disulfide can be expected to reduce the thiol concentration over time. Therefore, the reaction of DON with mercaptoethanol displayed pseudo-first-order kinetics only for the first few hours. Nevertheless, a dramatic increase in reaction rate was observed when the pH was increased from 7.2 to 9.2, with a further increase when the pH was increased to 10.7. These observations support nucleophilic attack by the thiolate ion as the dominant reaction mechanism, as the pK_a of mercaptoethanol has been reported to be approximately 9.7.[24] Regardless of the pH, there was always the same progression in the formation of the reaction products, as shown in Figure 2, and even at physiological pH adducts **1d** and **1e** appeared first. Attack of thiolate on the 12,13-epoxy group is expected to be essentially irreversible, whereas thiolate attack on C-10 of DON (Michael addition) should be reversible. In contrast, attack of thiolate on the isolated double bond in DON hemiketal is not expected, and

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isomerization of the thia-Michael addition products of DON to their hemiketal forms should hinder the elimination of thiol from C-10. The reaction scheme shown in Figure 11 thus accounts for the products identified in the reaction and, together with the well-known autoxidation of thiols to disulfides,[25] for the presence of only epoxy adducts **1a** and **1b** at extended reaction times. The late-eluting broad peak in the LC-MS analyses (Figures 2 and 6) may be due to on-column ketone−hemiketal isomerization, as this peak partially resolved when the mercaptoethanol addition products of DON were analyzed without the presence of formic acid in the mobile phase (data not shown).

Thus, DON and other trichothecenes have been shown to react with several natural and synthetic thiolates via Michael addition to C-10 of the α,β-unsaturated ketone and addition to C-13 of the 12,13-epoxy group. These reactions occurred rapidly in weakly basic conditions, but proceeded even at physiological pH, and could potentially result in the formation of adducts with cysteine-containing proteins or peptides even in the absence of enzymatic catalysis. None of the products from the reaction of DON with mercaptoethanol in which the thiol had been conjugated with the epoxy group showed any signs of toxicity in vitro.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b02864.

LC-HRMS chromatograms of all reactions described between DON, T-2 tetraol, de-epoxy-DON, and thiols; table including accurate mass data, calculated elemental compositions, and difference from the calculated exact mass; structures of **1a**, **1b**, **2a**, and **2b**, showing selected HMBC and NOE correlations; ^{1}H NMR spectra of two purified mixtures of **2a** and **2b**, showing their ratios; selected region of the ROESY NMR spectrum for **2a** and **2b** diagnostic for determining their stereochemistries; selected region of the HSQC NMR spectrum of **2a** and **2b** showing how coupling constants were measured for the signals overlapping in the ^{1}H spectrum; proposed fragmentation pathway of DON−mercaptoethanol Michael adducts; MS^n data for DON (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

COSY, correlation spectroscopy; DEPT, distortionless enhancement of polarization transfer; DON, deoxynivalenol; GSH, glutathione; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FBS, fetal bovine serum; HMBC, heteronuclear multiple-bond correlation; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; HSQC, heteronuclear single-quantum coherence:; IL-1β, interleukin-1 beta; JMOD, J-modulated spin−echo; LPS, lipopolysaccharide; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser spectroscopy; PMA, phorbol-12-myristate-13-acetate; ROESY, rotating frame Overhauser spectroscopy; SELTOCSY, selective total correlation spectroscopy; SPE, solid phase extraction; TOCSY, total correlation spectroscopy; TNF-α, tumor necrosis factor alpha

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