Characterization of the Amino Acid Adducts of the Enedial Derivative of Teucrin A

Alexandra Druckova and Lawrence J. Marnett*

Departments of Biochemistry, Chemistry, and Pharmacology, Vanderbilt Institute of Chemical Biology, Center in Molecular Toxicology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received June 24, 2006

The toxicity of germander, a herb used to treat obesity, is attributed to cytochrome P450 activation of the furan ring of its major diterpenoid component (teucrin A) into a reactive metabolite capable of adducting proteins. 1,4-Enedials have been proposed to be the reactive products of metabolism, possibly arising from a rearrangement of putative epoxide intermediates. We synthesized the enedial derivative of teucrin A as well as the enedial derived from a model furan, 3-(4-methoxy-benzyloxymethyl)-furan, by dimethyldioxirane oxidation and characterized the products of their reactions with amino acids and peptides. The reactions of the model enedial, 2-(4-methoxy-benzyloxymethyl)-but-2-enedial, with *N*-acetyl lysine (NAL) afforded regioisomeric *N*-alkyl-3-pyrrolin-2-ones, differing in the substitution on the double bond of the heterocyclic ring. Novel products formed in the reactions of the model enedial with *N*-acetyl cysteine (NAC) and both NAC/NAL uncovered the existence of tautomerization between the enedial and a hydroxyenal, which was manifest by the loss of 4-methoxybenzylalcohol and the incorporation of a second molecule of NAC. The reactions of teucrin A-enedial with NAC and NAL afforded analogues of the products observed with the model enedial, and the existence of the tautomeric equilibrium resulted in epimerization of the proton (H12) adjacent to the former furan ring. This work further illuminates the complex chemical behavior of unsaturated dialdehydes as an important class of toxic metabolites and lays the foundation for studies of the protein targets of teucrin A-enedial.

Introduction

Teucrin A is a major constituent of the *neo*clerodane diterpenoid fraction of the hydroalcoholic extract of germander (*Teucrium chamaedrys*) (Figure 1) (*1*, *2*). Germander has been used in folk medicine for its antiseptic and anticholeretic properties and in the liqueur industry as a bitter flavoring agent for wines and spirits. In the early 1990s, alcoholic extract preparations of germander and teas containing the dried plant were marketed in France as an adjuvant to weight control diets. Several reports of severe liver injury, including liver failure, resulted in the prohibition of germander-containing medicinal products in France in 1992, followed by Italy and Belgium, and prompted discussions of the safety of herbal medicinal preparations (*3*−*8*). Germander preparations are allowed for use only in alcoholic beverages by the FDA in the United States and similar regulations were suggested by the European Committee on Food (*9*, *10*).

The toxicity of teucrin A in mice has been attributed to metabolic activation of the 3-substituted furan ring to an electrophilic metabolite, as described for other furan-containing compounds, such as methylfuran, furosemide, 4-ipomeanol, and aflatoxin (*11*−*16*). Oral administration of teucrin A or germander extracts causes the depletion of intracellular glutathione and damage to cellular protein thiols, resulting in elevated plasma alanine amino transferase activity and significant centrilobular liver injury in mice (*17*, *18*). Isolated diterpenoid fractions from germander cause apoptosis in isolated rat hepatocytes (*19*).

1,4-Enedials have been proposed to be the reactive metabolic products of furans activated by cytochrome P450s, possibly arising from a rearrangement of putative epoxide intermediates (Figure 1) (*20*, *21*). 2-Methylbutene-1,4-dial and 4-oxo-2-pentenal have been identified as major metabolites of 3-methylfuran and 2-methylfuran, respectively, by trapping the reactive products *in situ* using a semicarbazide aldehyde trap (*20*). Amino acid, glutathione, and nucleoside conjugates of 1,4-butenedial have been characterized by chemical synthesis, and this presumed metabolite of furan has been reported to be mutagenic in *S. typhimurium* (*21*−*25*). Glutathione and methoxyamine conjugates of the furan-containing compound L-739,010 have been isolated from *in vitro* incubations with CYP 3A4 and characterized by LC/MS/MS and NMR (*26*). Similarly, an *N*-acetyl cysteine (NAC) and *N*-acetyl lysine (NAL) conjugate of a 4-ipomeanol metabolite has been identified from *in vitro* incubations, and its structure supports the existence of an 1,4-enedial metabolite of the furan ring (*15*). Protein adducts derived

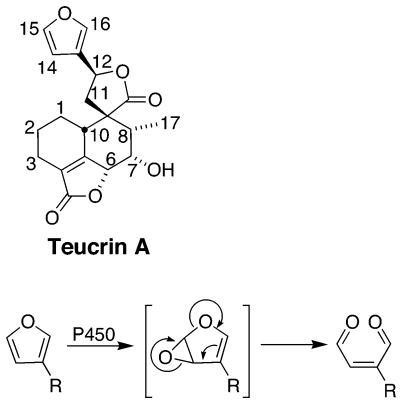


Figure 1. The structure of teucrin A and schematic representation of the proposed mechanism of furan activation by cytochrome P450.

* Corresponding author. Phone: (615) 343-7329. Fax: (615) 343-7534. E-mail: larry.marnett@vanderbilt.edu.

10.1021/tx060143k CCC: $33.50
Published on Web 09/14/2006

from L-739,010 have been detected following activation by P450 systems *in vitro* (*26*).

Our laboratory is interested in defining the chemistry of teucrin A metabolism and the biology that it triggers *in vivo*. As a first step in this program, we have synthesized the enedial product of teucrin A oxidation as well as the enedial derived from a model furan, **1**. We describe herein the products of the reactions of these enedials with amino acids and peptides.

Materials and Methods

Reagents and Solvents. HPLC grade solvents for column chromatography and HPLC were obtained from Fisher (Pittsburgh, PA) and used as received. Reagent grade chemicals were obtained from Aldrich (Milwaukee, WI). Anhydrous dimethylformamide and protected amino acid derivatives were purchased from Sigma-Aldrich (St. Louis, MO). The 5-mer peptides were purchased from Sigma-Genosys (The Woodlands, TX). Thin layer chromatography was performed on silica gel GF glass plates from Analtech (Newark, DE). The chromatograms were visualized under UV (254 nm), fluorescence, or by staining with sulfuric acid solution, followed by heating. Column chromatography was performed using silica gel 60−100 mesh from Fischer (Pittsburgh, PA). Teucrin A was a generous gift from Dr. Corrado Galli, University of Milan, Italy.

Instrumental Analysis. UV spectra were recorded using a Hewlett-Packard UV/VIS model 89500 spectrometer. Mass spectra were recorded on a Finnigan TSQ 7000 triple-quadrupole spectrometer under positive or negative ion mode. ^{1}H NMR spectra were recorded on Bruker 300 and 400 MHz NMR spectrometers using acetone-d_6, DMSO-d_6, and $CDCl_3$ as solvent and internal standard. HPLC analysis was performed on a Waters 1525 Binary HPLC Pump with Waters 2996 Photodiode Array Detector on the reversed phase Jupiter C18 5u 250 × 4.6 mm or 150 × 4.6 mm column at 1 mL/min (Phenomenex, CA). Semipreparative HPLC was performed using Jupiter C18 5u 250 × 10 mm column at 4 mL/min.

Dimethyldioxirane (DMDO). The title compound was prepared as previously reported (*27*). Briefly, peroxymonosulphate (Oxone, 50 g, 0.0813 mol) was added in one portion to a stirred mixture of water (20 mL), acetone (12 mL, 0.163 mol), and sodium bicarbonate (24 g) under a nitrogen atmosphere. The reaction was allowed to proceed for 15 min, and vacuum was applied to the reaction assembly. The yellow colored distillate was collected over 20 min in the receiving flask and cooled in a dry ice/acetone bath. The solution was stored over $MgSO_4$ at −20 °C for no more then 24 h prior to use. DMDO content was assayed using the iodometric titration, typically affording DMDO concentrations of 0.02−0.04 M. Acetone-d_6 was used to prepare deuterated DMDO.

3-(4-Methoxy-benzyloxymethyl)-furan (1). 3-Furanmethanol (300 mg, 3.06 mmol) was dissolved in 7 mL of anhydrous DMF in a flame-dried round-bottom flask equipped with a magnetic stirring bar under argon atmosphere, and the solution was cooled to 0 °C in an ice bath. Sodium hydride (95 mg, 3.98 mmol) was added in one portion and allowed to react for 30 min, followed by the addition of 4-methoxybenzylchloride (623 mg, 3.98 mmol) in 3 mL of DMF at 0 °C. The reaction mixture was allowed to warm up to room temperature and stirred overnight. The reaction was quenched with 12 mL of water and extracted with ethyl acetate (3 × 10 mL). Organic phases were combined and washed with 2 × 10 mL sat. $NaHCO_3$ and 1 × 10 mL water and dried over $MgSO_4$. The solvent was evaporated under reduced pressure, and the resulting yellow oil was subjected to column chromatography (50 g silica gel, hexane/ethyl acetate = 10:1. Desired product was isolated as colorless oil (550 mg, 82%). ^{1}H NMR (300 MHz, acetone-d_6) δ 7.52 (m, 2H, H5,H4), 7.26 (d, 2H, $J_{3',2'} = 8.67$ Hz, H3′), 6.89 (dd, 2H, $J_{2',7} = 2.07$ Hz, H2′), 6.45 (d, 1H, $J_{2,6} = 1.17$ Hz, H2), 4.43 (s, 2H, H6), 4.38 (s, 2H, H7), 3.78 (s, 3H, OCH_3). $C_{13}H_{14}O_3$ M 218.25.

2-(4-Methoxy-benzyloxymethyl)-but-2-enedial (2). Compound **1** (26.2 mg, 0.12 mmol) was dissolved in 1 mL of acetone-d_6 under argon atmosphere, and 4 mL of DMDO-d_6 was added at once. The reaction was allowed to proceed at room temperature for 30 min, and the ^{1}H NMR spectrum was obtained from 0.5 mL of the aliquot, showing the complete disappearance of starting material and the formation of *cis* and *trans* enedial products in a ratio of 6:1. The residual DMDO was bubbled off in the stream of nitrogen gas. The total volume was reduced to 1.5 mL (0.05 M), and the resulting pale yellow solution was stored at −20 °C. **2**: ^{1}H NMR (300 MHz, acetone-d_6) δ 10.65 (d, 1H, H4 *trans*), 10.62 (s, 1H, H1 *cis*), 10.59 (d, 1H, $J_{4,3} = 7.2$ Hz, H4 *cis*), 9.89 (s, 1H, H1 *trans*), 7.86 (d, 2H, $J_{3',2'} = 8.8$ Hz, H2′ *trans*), 7.31 (m, 2H, $J_{3',2'} = 8.7$ Hz, H2 *cis*), 7.10 (m, 2H, H3′ *trans*), 6.91 (m, 3H, H3′ *cis*/H3 *trans*), 6.75 (dt, 1H, $J_{3,4} = 7.2$ Hz, $J_{3,5} = 2.0$ Hz, H3 *cis*), 4.56 (s, 2H, H6), 4.33 (d, 2H, $J_{5,3} = 2.0$ Hz, H5), 3.91 (s, 3H, OCH_3 *trans*), 3.78 (s, 3H, OCH_3 *cis*). $C_{13}H_{14}O_4$ M 234.25.

Reaction of 2-(4-Methoxy-benzyloxymethyl)-but-2-enedial (2) with *N*-Acetyl Lysine (NAL). NAL (263.5 mg, 1.4 mmol) was dissolved in 3 mL of 0.2 M sodium phosphate buffer (pH 7.4), and 1 mL of a 2.1 M solution of **2** in acetone was added dropwise under stirring. The resulting solution was incubated at 37 °C overnight and extracted with dichloromethane, and the aqueous layer was analyzed by RP-HPLC. Two products, **3a** and **3b**, were isolated by HPLC using a gradient at 1 mL/min as follows: from 0 to 2 min, 75% A; from 2 to 12 min, linear gradient to 60% A; from 12 to 22 min, linear gradient to 30% A; and from 22 to 23 min, linear gradient to 75% A, where A = water + 0.1%acetic acid and B = acetonitrile + 0.1%acetic acid. The eluant was monitored at 254 nm. **3a**,**b**: ESI$^+$-MS (rel intensity) *m*/*z* 405.1 (M + H$^+$, 10), 427.2 (M + Na$^+$, 100); **3a**: ^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.31 (bs, 1H, NH), 7.27 (d, 2H, $J_{8(8'),9(9')} = 8.64$ Hz, H8(8′)), 7.00 (t, 1H, $J_{4,6} = 1.72$ Hz, H4), 6.88 (d, 2H, H9(9′)), 4.50 (s, 2H, H7), 4.27 (bs, 1H, Hα), 4.21 (d, 2H, $J_{6,4} = 1.88$ Hz, H6), 3.92 (m, 2H, H5), 3.79 (s, 3H, OCH_3), 3.65 (m, 1H, Hϵ), 3.32 (m, 1H, Hϵ'), 1.99 (s, 3H, COCH_3), 1.89 (m, 2H, Hβ), 1.64 (m, 1H, Hδ), 1.55 (m, 1H, Hδ'), 1.34 (m, 1H, Hγ); **3b**: ^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.33 (bs, 1H, NH), 7.25 (d, 2H, $J_{8(8'),9(9')} = 8.60$ Hz, H8(8′)), 6.88 (d, 2H, H9(9′)), 5.98 (t, 1H, $J_{3,6} = 1.72$ Hz, H3), 4.48 (s, 2H, H7), 4.31 (d, 2H, H6), 4.21 (m, 1H, Hα), 3.95 (m, 2H, H5), 3.79 (s, 3H, OCH_3), 3.60 (m, 1H, Hϵ), 3.26 (m, 1H, Hϵ'), 1.98 (s, 3H, COCH_3), 1.89 (m, 2H, Hβ), 1.62 (m, 1H, Hδ), 1.54 (m, 1H, H'), 1.32 (m, 1H, Hγ).

Reaction of 2-(4-Methoxy-benzyloxymethyl)-but-2-enedial (2) with *N*-Acetyl Cysteine (NAC). NAC (86 mg, 0.53 mmol) was dissolved in 3 mL of 0.2 M sodium phosphate buffer (pH 7.4), and 0.5 mL of a 0.5 M solution of **2** in acetone was added dropwise under stirring. The resulting solution was incubated at 37 °C overnight and extracted with dichloromethane, and the aqueous layer was analyzed by RP-HPLC. A single product **4** was isolated by HPLC using a gradient at 1 mL/min as follows: from 0 to 5 min, 100% A; from 5 to 15 min, linear gradient to 80% A; from 15 to 30 min, linear gradient to 40% A; and from 30 to 35 min, linear gradient to 100% A, where A = water + 0.1%acetic acid and B = acetonitrile + 0.1%acetic acid. The eluant was monitored at 254 nm. Elution time of product **4** was 19.5 min. **4**: ESI$^+$-MS (rel intensity) *m*/*z* 405.07 (M + H$^+$, 70), 427.17 (M + Na$^+$, 15); **4**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (d, 1H, $J_{NH,H\alpha} = 12.60$ Hz, $NH_{(1)}$), 8.19 (d, 1H, $J_{NH,H\alpha} = 12.88$ Hz, $NH_{(2)}$), 7.73 (d, 1H, $J_{5,4} = 1.96$ Hz, H5), 6.51 (d, 1H, H4), 4.39 (m, 1H, Hα_2), 4.21 (m, 1H, Hα_1), 3.57 (m, 2H, H6), 3.08 (m, 1H, Hβ_1), 2.92 (m, 1H, Hβ_1'), 2.75 (m, 1H, Hβ_2), 2.66 (m, 1H, Hβ_2'), 1.85 (s, 6H, COCH_3).

Reaction of 2-(4-Methoxy-benzyloxymethyl)-but-2-enedial (2) with NAC and NAL. NAC (86 mg, 0.53 mmol) and NAL (100 mg, 0.53 mmol) were dissolved in 3 mL of 0.2 M sodium phosphate buffer (pH 7.4), and 0.5 mL of a 0.5 M solution of **2** in acetone was added dropwise under stirring. The resulting solution was incubated at 37 °C overnight and extracted with dichloromethane, and the aqueous layer was analyzed by RP-HPLC. Products were separated using a gradient at 1 mL/min as follows: from 0 to 20 min, 95% A; from 20 to 24 min, linear gradient to 90% A; from 24 to 30 min, linear gradient to 40% A; and from 30 to 31 min, linear gradient to 95% A, where A = water + 0.1%acetic acid and

B = acetonitrile + 0.1%acetic acid. The eluant was monitored at 254 nm. Products eluted from the column as follows: **4** at 8.0 min, **5a** at 12.2 min, and **5b** at 13.5 min. **5a/b**: ESI$^+$-MS (relative intensity) *m/z* 475.26 (M + H$^+$, 100), 494.35 (M + NH$_4^+$, 20); **5a**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (d, 1H, $J_{NH,H\alpha}$ = 7.68 Hz, NH_{NAL}), 8.13 (d, 1H, NH_{NAC1}), 8.05 (d, 1H, $J_{NH,\ H\alpha}$ = 7.96 Hz, NH_{NAC2}), 6.93 (d, 1H, $J_{5,4}$ = 2.80 Hz, H5), 6.07 (d, 1H, H4), 4.38 (m, 1H, $H\alpha_{NAC1}$), 4.13−4.12 (m, 2H, $H\alpha_{NAL}$, $H\alpha_{NAC2}$), 3.93 (m, 2H, Hϵ), 3.65 (m, 2H, H6), 2.97 (m, 1H, $H\beta_{NAC2}$), 2.90−2.75 (m, 2H, $H\beta'_{NAC2}$, $H\beta_{NAC1}$), 2.66 (m, 1H, $H\beta'_{NAC1}$), 1.86 (s, 3H, COCH_3), 1.85 (s, 3H, COCH_3), 1.82 (s, 3H, COCH_3), 1.70−1.55 (m, 4H, $H\beta_{NAL}$, H), 1.25 (m, 2H, Hγ); **5b**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (d, 1H, $J_{NH,H\alpha}$ = 7.96 Hz, NH_{NAC1}), 8.14 (d, 1H, $J_{NH,H\alpha}$ = 7.96 Hz, NH_{NAC2}), 8.03 (d, 1H, $J_{NH,H\alpha}$ = 7.80 Hz, NH_{NAL}), 6.84 (d, 1H, $J_{5,3}$ = 2.20 Hz, H5), 6.73 (d, 1H, H3), 4.37 (m, 1H, $H\alpha_{NAC2}$), 4.19 (m, 1H, $H\alpha_{NAC1}$), 4.11 (m, 1H, $H\alpha_{NAL}$), 3.78 (m, 2H, Hϵ), 3.58 (s, 2H, H6), 2.93 (m, 1H, $H\beta_{NAC1}$), 2.79 (m, 1H, $H\beta_{NAC2}$), 2.70−2.64 (m, 2H, $H\beta'_{NAC1}$, $H\beta'_{NAC2}$), 1.85 (s, 6H, 2x COCH_3), 1.82 (s, 3H, COCH_3), 1.66−1.63 (m, 4H, $H\beta_{NAL}$, Hδ), 1.25 (m, 2H, Hγ).

Teucrin A Purification. Crude teucrin A was purified by column chromatography (50 g silica gel, hexane/ethyl acetate = 1:10). The desired compound was isolated as colorless crystals. ^{1}H NMR (400 MHz, acetone-d_6) δ 7.78 (dd, 1H, H16, $J_{16,15}$ = 1.52 Hz, $J_{16,14}$ = 0.76 Hz), 7.65 (t, 1H, H15, $J_{15,14}$ = 1.72 Hz), 6.59 (dd, 1H, H14, $J_{14,15}$ = 1.84 Hz), 5.84 (t, 1H, H12, $J_{12,11}$ = 8.88 Hz), 4.93 (m, 1H, H6), 4.60 (d, 1H, OH, $J_{OH,7}$ = 11.28 Hz), 4.10 (m, 1H, H7, $J_{7,8}$ = 2.56 Hz), 3.12 (m, 1H, H10), 2.80 (m, 2H, H11), 2.46−2.39 (m, 1H, H1), 2.33−2.28 (dq, 1H, H8, $J_{8,CH3}$ = 7.08 Hz), 2.24−2.15 (m, 1H, H3), 2.07 (m, 1H, H3′), 1.99 (m, 1H, H2), 1.65−1.55 (m, 1H, H2′), 1.50−1.40 (m, 1H, H1′), 1.24 (d, 3H, CH_3). $C_{19}H_{20}O_6$ M 344.36 *m/z* (relative intensity) 711.38 (100, [2M + Na]$^+$), 345.18 (20, [M + 1]$^+$).

Enedial Derivative of Teucrin A (6). Teucrin A (10 mg, 0.029 mmol) was dissolved in 0.5 mL of anhydrous acetone-d_6, and the solution was cooled in the acetone/dry ice bath. Cold DMDO-d_6 (1.2 mL, 0.034 mmol) was added under argon atmosphere. The reaction mixture was allowed to warm up to room temperature, and the progress of reaction was monitored by ^{1}H NMR. The reaction was complete in 2 h. Upon disappearance of the starting material, nitrogen was blown through the solution to eliminate unreacted DMDO-d_6, and the product was stored as acetone-d_6 solution at − 20 °C. **6**: ^{1}H NMR (400 MHz, acetone-d_6) δ 10.69 (s, 1H, H16), 10.65 (d, $J_{15,14}$ = 6.4 Hz, 1H, H15), 6.71 (dd, $J_{14,12}$ = 1.56 Hz, 1H, H14), 5.65 (td, $J_{12,11}$ = 8.88 Hz, 1H, H12), 4.92 (dd, $J_{6,7}$ = 4.62 Hz, $J_{6,8}$ = 1.38 Hz, 1H, H6), 4.30 (d, $J_{OH,7}$ = 11.50 Hz, 1H, OH), 4.07 (ddd, $J_{7,8}$ = 2.56 Hz, 1H, H7), 3.05 (m, 1H, H10), 2.90 (m, 2H, H11), 2.55 (m, 1H, H1), 2.41 (m, 1H, H8), 2.23 (m, 2H, H3, H2), 2.02 (m, 1H, H2′), 1.58 (m, 1H, H1′), 1.43 (m, 1H, H3′), 1.12 (d, $J_{CH3,H8}$ = 7.2 Hz, 3H, CH_3). $C_{19}H_{20}O_2$ M 360.12.

Reaction of 6 with NAL Methyl Ester. NAL methyl ester (34.6 mg, 0.15 mmol) was dissolved in 2 mL of 0.2 M sodium phosphate buffer at pH 7.4, and 1 mL of a 72.5 mM solution of **6** in acetone-d_6 was added dropwise. The reaction mixture was incubated at 37 °C overnight. Product formation was followed by UV/HPLC at 254 nm. Major products **7c** and **7d** were isolated using a gradient at 1 mL/min as follows: from 0 to 5 min, linear gradient from 85% A to 82% A; from 5 to 30 min, hold 82% A; from 30 to 40 min, linear gradient to 40% A; and from 40 to 41 min, linear gradient to 85% A; where A = water + 0.1%acetic acid; B = acetonitrile + 0.1%acetic acid. Products eluted from the column as follows: **7a** at 21.5 min, **7b** at 26.8 min, **7c** at 31.0 min, and **7d** at 31.8 min. **7a**−**d**: ESI$^+$-MS *m/z* (rel. intensity) 545.3 ([M + 1]$^+$, 100), 567.3 (([M + Na]$^+$, 10). **7c**,**d**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.21 (m, 1H, NH), 7.30 (s, 1H, H4), 5.50, 5.34 (t, 1H, $J_{12,11}$ = 8.4 Hz, $J_{12,11}$ = 8.55 Hz, H12), 4.98 (m, 1H, H6), 4.46 (d, 1H, $J_{OH,7}$ = 8.80 Hz, OH), 4.17 (m, 1H, Hα), 4.04 (m, 2H, H5), 3.98 (m, 1H, H7), 3.34 (bs, 2H, H, overlap with H_2O signal), 3.06 (m, 1H, H10), 2.8−2.5 (m, 2H, H11), 2.22 (m, 1H, H8), 2.2 − 2.05 (m, 2H, H1), 2.0 − 1.9 (m, 2H, H3), 1.83 (s, 3H, COCH_3), 1.7 − 1.5 (m, 2H, Hβ), 1.5 − 1.3 (m, 2H, Hδ), 1.3 − 1.2 (m, 2H, H2), 1.2 − 1.0 (m, 2H, Hγ), 1.19, 1.11 (d, 3H, $J_{CH3,8}$ = 6.9 Hz, CH_3).

Reaction of 6 with NAC. NAC (8.5 mg, 0.05 mmol) was dissolved in 2 mL of 0.2 M sodium phosphate buffer at pH 7.4, and 0.5 mL of a 35.0 mM solution of **6** in acetone-d_6 was added dropwise. The reaction mixture was incubated at 37 °C overnight. Product formation was followed by HPLC at 254 nm. Major product **8c** eluting at 21.0 min was isolated using a gradient at 1 mL/min as follows: from 0 to 4 min, linear gradient from 100% A to 90% A; from 4 to 18 min, linear gradient to 80% A; from 18 to 22 min, linear gradient to 30% A; and from 22 to 23 min, linear gradient to 100% A, where A = water + 0.1% acetic acid and B = acetonitrile + 0.1% acetic acid. **8c**: ESI$^-$-MS *m/z* (rel. intensity) 503.8 ([M − 1]$^-$, 100). **8c**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (m, 1H, NH), 7.91 (d, 1H, $J_{5,4}$ = 2.0 Hz, H5), 6.69 (d, 1H, H4), 5.61 (m, 1H, H12), 5.01 (m, 1H, H6), 4.52 (d, 1H, $J_{OH,7}$ = 10.88 Hz, OH), 4.01 − 3.99 (m, 2H, H7, Hα), 3.25 − 3.22 (m, 1H, Hβ), 3.10 (m, 1H, H10), 2.9 − 2.8 (m, 2H, Hβ', H11), 2.75 − 5.60 (m, 1H, H11′), 2.45 − 2.30 (m, 1H, H1), 2.25 − 2.23 (m, 1H, H8), 2.20 − 2.05 (m, 1H, H1′), 2.00 − 1.90 (m, 2H, H3), 1.84 (s, 3H, COCH_3), 1.55 − 1.40 (m, 1H, H2), 1.27 − 1.22 (m, 1H, H2′), 1.18 (d, 3H, $J_{CH3,8}$ = 7.04 Hz, CH_3).

Reaction of 6 with NAL Methyl Ester and NAC. NAL methyl ester (12.5 mg, 0.05 mmol) and NAC (8.5 mg, 0.05 mmol) were dissolved in 2 mL of 0.2 M sodium phosphate buffer at pH 7.4, and 0.5 mL of a 35.0 mM solution of **6** in acetone-d_6 was added dropwise. The reaction mixtures were incubated at 37 °C overnight. The formation of products was followed by HPLC/UV at 254 nm. Major product **9c** eluting at 25.3 min was isolated using a gradient at 1 mL/min as follows: from 0 to 4 min, linear gradient from 100% A to 90% A; from 4 to 14 min, hold 90% A; from 14 to 19 min, linear gradient to 80% A; and from 19 to 25 min, linear gradient to 40% A, where A = water + 0.1% acetic acid and B = acetonitrile + 0.1% acetic acid. **9c**: ESI$^-$-MS *m/z* (rel. intensity) 688.3 ([M − 1]$^-$, 100). **9c**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (m, 2H, NH), 7.16, 7.14 (d, 1H, $J_{3,5}$ = 2.18 Hz, H5), 7.01, 6.98 (d, 1H, H3), 5.67, 6.60 (t, 1H, $J_{12,11}$ = 9.12 Hz, $J_{12,11}$ = 8.72 Hz, H12), 5.04, 5.00 (m, 1H, H6), 4.66 (d, 1H, $J_{OH,7}$ = 10.80 Hz, OH), 4.23 − 4.14 (m, 2H, Hα), 3.98 (m, 1H, H7), 3.88 − 3.84 (m, 2H, Hϵ), 3.59 (s, 3H, COOCH_3), 3.10 (m, 0.5H, H10), 2.90 − 2.87 (m, 1.5H, H10, $H\beta_{NAC}$), 2.75 − 2.65 (m, 3H, $H\beta'_{NAC}$, H11), 2.3 − 2.4 (m, 0.5H, H1),, 2.4 − 2.2 (m, 1.5 H, H1, H8), 2.2 − 2.1 (m, 1H, H1′), 2.1 − 2.0 (m, 2H, H3), 1.82 (s, 6H, COCH_3), 1.7 − 1.6 (m, 3H, $H\beta_{NAL}$, Hδ), 1.55 − 1.3 (m, 1H, H2), 11.3 − 1.2 (m, 2H, Hδ', H2′), 1.24 (d, 3H, $J_{CH3,8}$ = 6.96 Hz, CH_3).

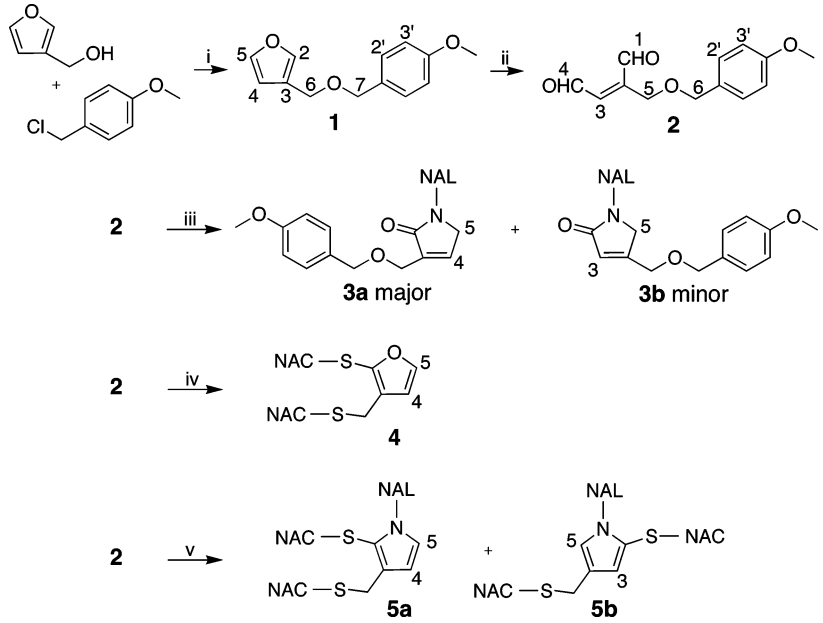
Reaction of 6 with RKDVY. Solution of 5-mer RKDVY in 100 mM HEPES buffer at pH 8.1 (100 μL, 1 mg/mL, 1.45 μmol) was diluted with 990 μL of 100 mM HEPES buffer at pH 8.1, and 67 μL of an equimolar solution of **6** in acetone was added. The reaction mixture was incubated at 37 °C overnight. The reaction was monitored by HPLC/UV looking at the disappearance of the starting peptides. Adducted peptides were analyzed by LC/MS/MS using a gradient at 1 mL/min as follows: from 0 to 20 min, linear gradient from 95% A to 20% A; and from 20 to 25 min, linear gradient from 20% A to 95% A, where A = water + 0.1% acetic acid and B = acetonitrile + 0.1% acetic acid.

Reaction of 6 with Btn-RKVDY. A solution of N-biotinylated 5-mer Btn-RKVDY in 100 mM HEPES buffer at pH 8.1 (100 μL, 1 mg/mL, 0.98 μmol) was diluted with 990 μL of 100 mM HEPES buffer at pH 8.1, and 40 μL of an equimolar solution of **6** in acetone was added. The reaction mixture was incubated at 37 °C overnight. The reaction was monitored by HPLC/UV, observing the disappearance of the starting peptides. Adducted peptides were analyzed by LC/MS/MS using a gradient at 1 mL/min as follows: from 0 to 1 min, hold 100% A; from 1 to 20 min, linear gradient to 50% A; and from 20 to 25 min, linear gradient to 100%A, where A = water + 0.1% acetic acid and B = acetonitrile + 0.1% acetic acid.

Results

Synthesis of a Model 1,4-Enedial. In order to optimize the conditions for oxidation of teucrin A by dimethyldioxirane

Scheme 1. Synthesis of Model α,β-Unsaturated Dialdehyde 2 and Its Conjugates with NAL and NAC[a]



[a] (i) NaH, DMP; (ii) Dimethyldioxirane, Acetone; (iii) NAL, 0.1 M Phosphate Buffer, pH 7.4; (iv) NAC, 0.1 M Phosphate Buffer, pH 7.4; (v) NAL, NAC, 0.1 M Phosphate Buffer, pH 7.4.

(DMDO), we prepared the model furan (**1**) by reaction of 3-furanmethanol with 4-methoxybenzylchloride in anhydrous DMF in the presence of sodium hydride (Scheme 1). Compound **1** was selectively oxidized to **2** by treatment with 1.5 equiv of DMDO in acetone at −20 °C. The stereochemistry of **2** was exclusively *cis*, although some conversion to the *trans* isomer occurred on warming to room temperature. An inspection of NMR spectra arising during the oxidation of **1** by DMDO revealed no signals attributable to an epoxide intermediate. The enedial (**2**) was unstable to manipulation; therefore, solutions were concentrated in a stream of nitrogen and used without further purification.

Reactions of 2 with Nucleophilic Amino Acids. Enedial **2** was reacted with 1.5 equiv of *N*-acetyllysine (NAL) in phosphate buffer at pH 7.4 at 37 °C overnight. The reaction afforded two products (**3a** and **3b**) with the same *m*/*z* of 405.6 Da, corresponding to the molecular ion $[M + 1]^+$ of a pyrroline-2-one adduct, arising from the conjugation of the lysine amino group with the enedial and the elimination of a water molecule. The products were readily separated by reverse-phase chromatography, and the resulting structures were assigned on the basis of ^{1}H NMR and COSY (Figure 2A). Table 1 summarizes the chemical shifts and coupling constants of the relevant proton signals in the major conjugates of **2** with amino acids. The ^{1}H NMR spectrum of the major isomer **3a** showed a new signal at 7.0 ppm and a new apparent quartet at 3.9 ppm, which exhibited a crosspeak in the COSY spectrum. The signal at 7 ppm was assigned to the H4 proton of the double bond of the 3-substituted pyrroline-2-one (**3a**). The signal at 3.9 ppm integrated to two protons and was attributed to the CH_2 group of the ring, adjacent to proton 4, as revealed by their interaction in the COSY spectrum. The ^{1}H NMR spectrum of the minor isomer **3b** revealed the presence of a new signal at 6.0 ppm and an apparent quartet at 3.9 ppm as observed in the spectrum of **3a**. However, the COSY spectrum did not show any interaction between the two signals. Thus, the minor adduct was assigned as a regioisomer of **3a**, the 4-substituted pyrroline-2-one **3b**.

The reaction of **2** with 1.5 equiv of *N*-acetylcysteine (NAC) in phosphate buffer at pH 7.4 at 37 °C overnight afforded a single product **4** with an *m*/*z* of 405.07 Da. This ion did not correspond to the formation of a 1:1 conjugate of **2** with NAC. The product was purified by HPLC, and ^{1}H NMR analysis revealed the presence of two cysteines in the molecule but the absence of the aromatic ring of **2**. The new doublets in the downfield region of the ^{1}H NMR spectrum at 6.5 and 7.8 ppm were assigned to the furan ring protons H4 and H5, respectively, and exhibited a cross peak in the COSY spectrum. The product

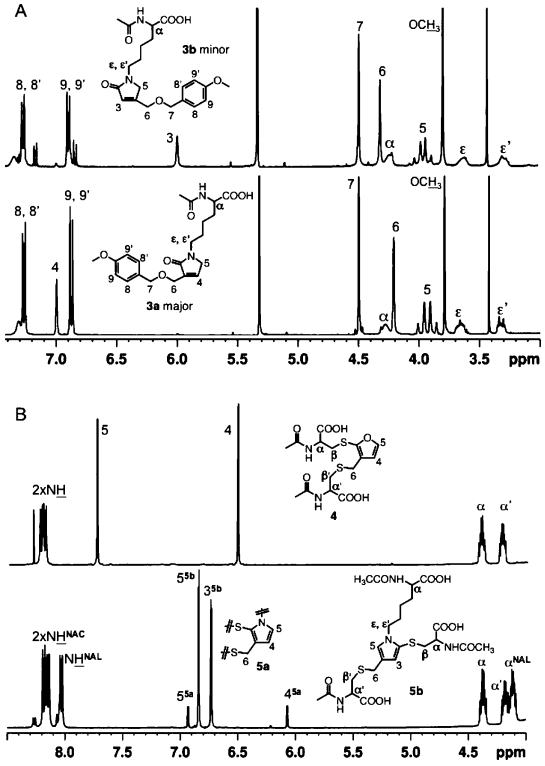
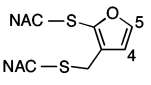
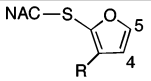
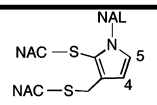
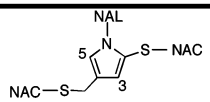
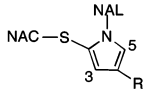


Figure 2. ^{1}H NMR spectra of the conjugates of the model enedial **2** with NAC and NAL containing the labeled structures showing the position of each proton. (A) Comparison of **3a** and **3b** conjugates of **2** with NAL at 400 MHz in CD_2Cl_2. (B) Comparison of the NAC conjugate of **2** (**4**) and NAC/NAL conjugates of **2** (**5a** and **5b**) at 400 MHz in DMSO-d_6.

Table 1. Chemical Shifts and Coupling Constants of Relevant Protons in the Major Conjugates of α,β-Unsaturated Enedial Derivatives 2 and 6 with NAL and NAC

Ring structure	Compound	Proton position	Number of protons	Splitting pattern	Chemical shift (ppm)	Coupling Constants (Hz)
	**3a**	4	CH	s	7.0	-
		5	CH2	aq	3.9	-
	7c,d	4	CH	s	7.3	-
		5	CH2	m	4.0	-
	**3b**	3	CH	s	6.0	-
		5	CH2	aq	3.9	-
	7a,b	3	CH	s	6.1	-
		5	CH2	m	4.0	-
	**4**	4	CH	d	6.5	1.96
		5	CH	d	7.8	
	**8c**	4	CH	d	6.7	1.99
		5	CH	d	7.9	
	**5a**	4	CH	d	6.0	2.80
		5	CH	d	6.9	
	**5b**	3	CH	d	6.7	2.20
		5	CH	d	6.8	
	**9c**	3	CH	d	7.0	2.18
		5	CH	d	7.1	

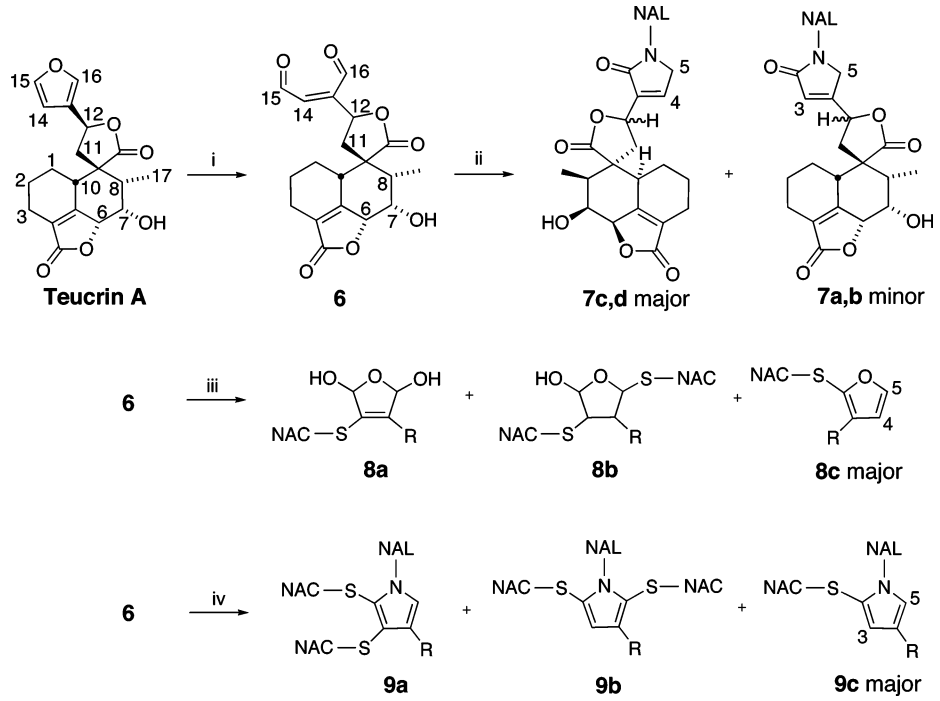
of this reaction was assigned as the 2,3-substituted furan, **4**. An explanation for the formation of this product involves an equilibrium between the enedial and a γ-hydroxy-1,3-dien-3-al, with the latter undergoing Michael addition with the sulfhydryl group of NAC, followed by the elimination of the 4-methoxybenzylalcohol. One of the aldehyde functionalities reacts with the second equivalent of NAC via 1,2-addition, affording the final product upon cyclization and elimination of water (Scheme 3).

Enedial **2** was incubated with both NAC and NAL under the conditions described above. Four products were detected and isolated by HPLC and subjected to mass spectral analysis. The first eluting peak corresponded to the cysteine adduct (**4**) described above. The following three peaks eluted close to each other and displayed the same *m*/*z* of 575.26 Da, consistent with the incorporation of 2 molecules of cysteine, 1 molecule of lysine, and the loss of 4-methoxybenzylalcohol. The first two of these products were separated by HPLC and analyzed by ^{1}H NMR and COSY. The ^{1}H NMR spectrum of the conjugate **5a** contained two new doublets in the downfield region at 6.0 and 6.9 ppm and exhibited a cross peak in the COSY spectrum. The product **5a** was assigned as a 1,2,3-substituted pyrrole derivative. The spectrum of the most abundant product **5b** showed two doublets at 6.7 and 6.8 ppm that did not produce a cross peak in the COSY spectrum. The product **5b** was, thus, assigned to be a 1,2,4- substituted pyrrole. The third product was produced in low yields; therefore, we were unable to characterize it by NMR.

Synthesis of the Enedial Derivative of Teucrin A. The enedial derivative of teucrin A (**6**) was prepared by treatment of teucrin A with 2 equiv of DMDO in acetone at −20 °C overnight (Scheme 2). The reaction quantitatively produced the *cis* isomer (**6**), without evidence for *cis/trans* isomerization. The ^{1}H NMR spectrum showed two resonances at low field: a singlet at 10.69 ppm and a doublet at 10.65 ppm, corresponding to the *cis*-enedial protons H16 and H15, respectively (Figure 3B). A signal at 6.71 ppm had a coupling constant of 6.4 Hz, identical to the coupling constant of the doublet at 10.65 ppm, and was assigned to the H14 proton of the enedial double bond. NMR analysis of the reaction mixture did not reveal any indication of an epoxide. The enedial **6** was unstable to manipulation; therefore, solutions were concentrated in a stream of nitrogen and used without further purification.

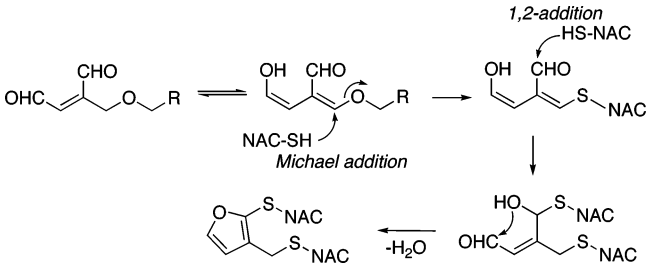
Reactions of 6 with Nucleophilic Amino Acids. Enedial **6** was reacted with 1.5 equiv of NAL methyl ester in phosphate buffer at pH 7.4 at 37 °C overnight. The reaction afforded four products (**7a**−**d**) with the same *m*/*z* of 545.3 Da, corresponding to the molecular ion $[M + 1]^+$ of the pyrroline-2-one adduct (Figure 4A). The products were separated by HPLC, and the structures were assigned on the basis of ^{1}H NMR, COSY, and TOCSY analyses. The ^{1}H NMR spectra of the two major isomers **7c** and **7d** were virtually identical, with minor changes in the chemical shifts of few resonances. Both compounds revealed singlet resonances at 7.3 ppm and multiplet signals at 4.0 ppm, which exhibited a cross peak in the COSY spectrum. This suggested that **7c** and **7d** are diastereomers of 3-substituted pyrroline-2-one (Table 1). The formation of the diastereomers likely results from racemization of the H12 proton of **6** as a result of the equilibrium between the enedial and β-hydroxy-1,3-dien-3-al. Indeed, the H12 signals integrated to a 1:1 ratio. Products **7a** and **7b** were generated in low yields, which precluded rigorous NMR analysis. The spectra showed a singlet

Scheme 2. Synthesis of α,β-Unsaturated Enedial Derivative of Teucrin a 6 and Its Conjugates with NAL and NAC[a]



[a] (i) Dimethyldioxirane, Acetone; (ii) NAL, 0.1M Phosphate Buffer, pH 7.4; (iii) NAC, 0.1M Phosphate Buffer, pH 7.4; (iv) NAL, NAC, 0.1M Phosphate Buffer, pH 7.4.

Scheme 3. Proposed Mechanism for the Formation of NAC Conjugates with the Enedial Derivatives



resonance at 6.1 ppm and a multiplet resonance at 3.9 ppm, which were attributed to the H3 and H5, respectively, of the 4-substituted pyrroline-2-one ring. The COSY spectrum lacked a cross peak between the two signals. Thus, **7a** and **7b** were assigned as diastereomers resulting from the racemization of the H12 proton. NAL methyl ester was used in the reaction with **6** instead of NAL because of the difficulties with product purification. The conjugates of **6** with NAL were highly polar, and we were unable to separate the diastereomers formed.

The reaction of **6** with 1.5 equiv of NAC in phosphate buffer at pH 7.4 at 37 °C overnight resulted in a complex mixture of products as illustrated by HPLC (Figure 4B). The major peaks were collected and analyzed by mass spectrometry in the negative ion mode. The first broad peak eluting between 10 and 11 min showed an *m*/*z* of 538.16 Da, corresponding to the $[M - 1]^-$ ion of **8a**, which could form by Michael addition of NAC to **6**, followed by hydration of the aldehyde and hemiacetal formation. The peak eluting at 11.5 min had an *m*/*z* of 685.15 Da, corresponding to the $[M - 1]^-$ ion **8b**, potentially arising from the stepwise addition of two molecules of NAC. Both **8a** and **8b** were unstable to concentration and solvent removal; therefore, we were unable to further purify them for NMR analysis. The product with the elution time of 21.0 min had an *m*/*z* of 504.16 Da, corresponding to the $[M - 1]^-$ ion of the dehydrated conjugate of one molecule of NAC and **6** (**8c**). The

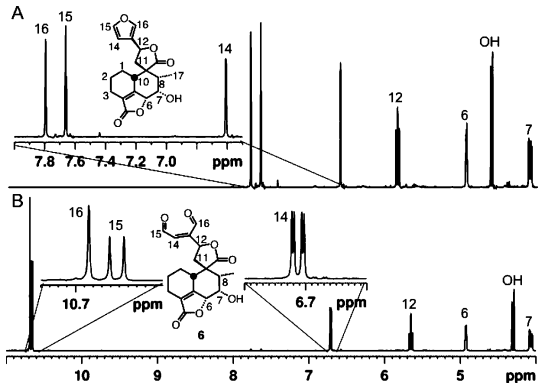


Figure 3. Comparison of the relevant ^{1}H NMR signals in the spectra of teucrin A and its *cis* enedial derivative **6** at 400 MHz in acetone-d_6 containing the labeled structures showing the position of each proton.

^{1}H NMR spectrum revealed two sets of doublets, one at 7.90 and 6.69 ppm, the second at 7.87 and 6.65 ppm, with a coupling constant of 1.99 Hz (Figure 5B). A COSY experiment showed a cross peak between each of the two resonances; therefore, **8c** was assigned as the diastereomeric 2,3-substituted furan resulting from the epimerization of H12 proton. Integration of the signals provided a ratio of the diastereomers of 2:1.

The reaction of **6** with 1.5 equiv of both NAL methyl ester and NAC was performed under the same conditions as those described above and afforded a mixture of five products that were separated by HPLC (Figure 4C). Collected fractions were analyzed by mass spectrometry using the negative ion mode. The first eluting peak at 9.5 min corresponded to the NAC conjugate **8c** with an *m*/*z* of 504.16 Da. The following peaks at 13.0 and 14.5 min (**9a**, **9b**) had an $[M - 1]^-$ ion with the same *m*/*z* of 851.34 Da. This mass resulted from the reaction of two molecules of NAC and one molecule of NAL methyl ester with **6** and was attributed to the regioisomers of the tetrasubstituted pyrrole (Scheme 2). The product eluting at 22 min corresponded to the $[M - 1]^-$ ion of

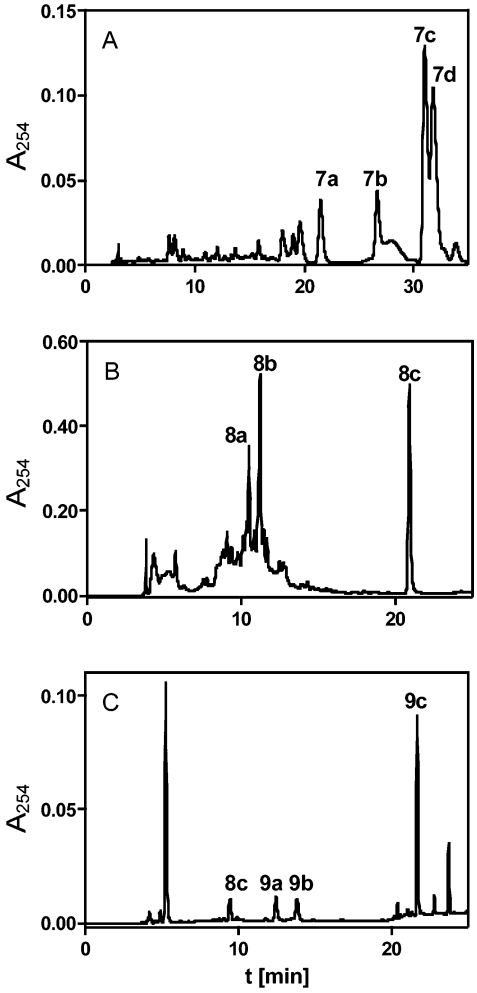


Figure 4. Representative HPLC chromatograms with UV monitoring of the reaction mixtures of **6** with NAL, NAC, and NAC/NAL containing labeled major products corresponding to the structures in Scheme 2. (A) HPLC trace of the reaction mixture of **6** with NAL. (B) HPLC trace of the reaction mixture of **6** with NAC. (C) HPLC trace of the reaction mixture of **6** with NAC/NAL.

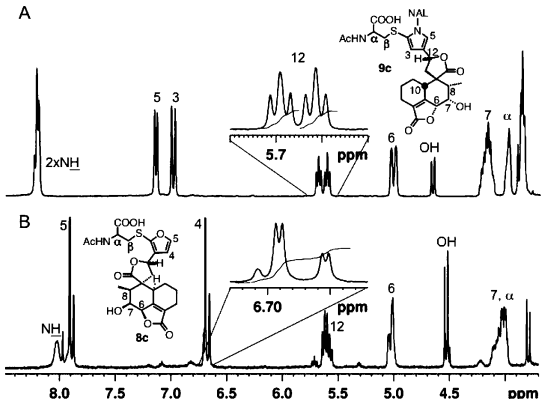


Figure 5. Comparison of the ^{1}H NMR spectra of the conjugates of **6** with NAC and NAC/NAL (**9c** and **8c**) at 400 MHz in DMSO-d_6 containing the labeled structures showing the position of each proton. (A) Evidence for the formation of diastereomeric products in the reaction of **6** with NAC and NAL observed as split signals corresponding to protons H5, H3, H12 (close-up), and H6 in the spectrum of **9c**. (B) Evidence for the formation of diastereomeric products in the reaction of **6** with NAC observed as split signals of protons H5 and H4 in the spectrum of **8c**.

the trisubstituted pyrrole (**9c**) with an *m*/*z* of 688.25 Da. Product **9c** was purified by HPLC, and the structure was assigned on the basis of NMR experiments. ^{1}H NMR analysis revealed the presence of two sets of signals attributable to two diastereomers resulting from the racemization of proton H12 as described above (Figure 5A). Integration of the signals for each isomer afforded a ratio of 1:1. Two new resonances in the aromatic region with chemical shifts of 7.16 and 7.14 ppm were assigned to the H5 proton of a 1,2,4-trisubstituted pyrrole. Proton H3 had a resonance at 7.01 and 6.98. Protons H5 and H3 showed a weak cross peak in the COSY spectrum, and their coupling constant was determined to be 2.18 Hz, supporting the assigned structure of **9c**.

Reactions of 6 with Peptides. Enedial **6** was reacted with one equivalent of the lysine-containing peptide RKDVY with a free amino terminus in HEPES buffer at pH 8.1 at 37 °C overnight. The reaction afforded three products that were purified by HPLC and sequenced by tandem mass spectral analysis (Figure 6A). The peak eluting at 12.6 min showed a molecular ion $[M + 1]^+$ with an *m*/*z* of 1022.59 Da, corresponding to the pyrroline-2-one adduct. The site of modification was determined from the MS/MS fragmentation spectrum of the base peak $[M + 2]^{2+}$ with an *m*/*z* of 511 (Figure 6B). MS/MS sequencing showed all y ions corresponding to the parent peptide (y_1 through y_4), confirming the position of the modification on the *N*-terminus. Additionally, all of the b* ions were found in the fragmentation spectrum with masses corresponding to the addition of the teucrin A core (+344 Da).

The second peak eluting at 13.3 min showed the same molecular ion $[M + 1]^+$ at an *m*/*z* of 1022.59 Da. The fragmentation pattern in the MS/MS spectrum was very different (Figure 6C). An unmodified y_4 ion was no longer observed, whereas y_3-y_1 ions were present. Ions b_2-b_4 were found in the spectrum to be modified by the addition of 344.5 Da. The presence of the unmodified c_1 ion with an *m*/*z* of 172 confirmed the second product as pyrroline-2-one with the adducted ϵ-NH_2 group of the lysine residue. The minor peak eluting at 14.9 min had an *m*/*z* of 1068.66 Da, which corresponded to $[1022.7 + 2Na]^{+1}$, but because of the lack of the material, we were not able to obtain a fragmentation spectrum of good quality.

The reaction of **6** with the *N*-terminal biotinylated peptide Btn-RKVDY under the same conditions as those described above afforded a single product that was isolated by HPLC and characterized by MS/MS analysis (Figure 7A). The molecular ion $[M + 1]^+$ of the peak eluting at 20 min had an *m*/*z* of 1361.7 Da, corresponding to the formation of the pyrroline-2-one with the ϵ-NH_2 group of lysine. The fragmentation pattern of this ion in the MS/MS spectrum revealed the presence of all b ions and the y_4* ion modified by the addition of 344 Da, confirming the modification of the lysine residue (Figure 7B). The formation of additional products in this case was not observed, ruling out the possibility of the reaction of **6** with arginine. The peak eluting at 18 min was the unreacted biotinylated peptide.

Discussion

Furan and its derivatives are present in the environment as pollutants from industrial processes, as additives in foods and beverages, and in many natural products (*28*). It has been recognized for several decades that the reactive metabolites generated by cytochrome P450s are responsible in part for the observed organ-selective toxicity of furans (*11*, *20*, *29*−*33*). Furan itself is metabolized by CYP 2E1 into *cis*-2-butene-1,4-enedial, a reactive metabolite shown to bind covalently to liver proteins and to be mutagenic in *S. typhimurium* (*21*, *25*, *32*). The reactivity and products of the reactions of *cis*-2-butene-1,4-enedial with model nucleophiles, such as NAC, NAL, and GSH, have been reported as well as the structure and synthesis

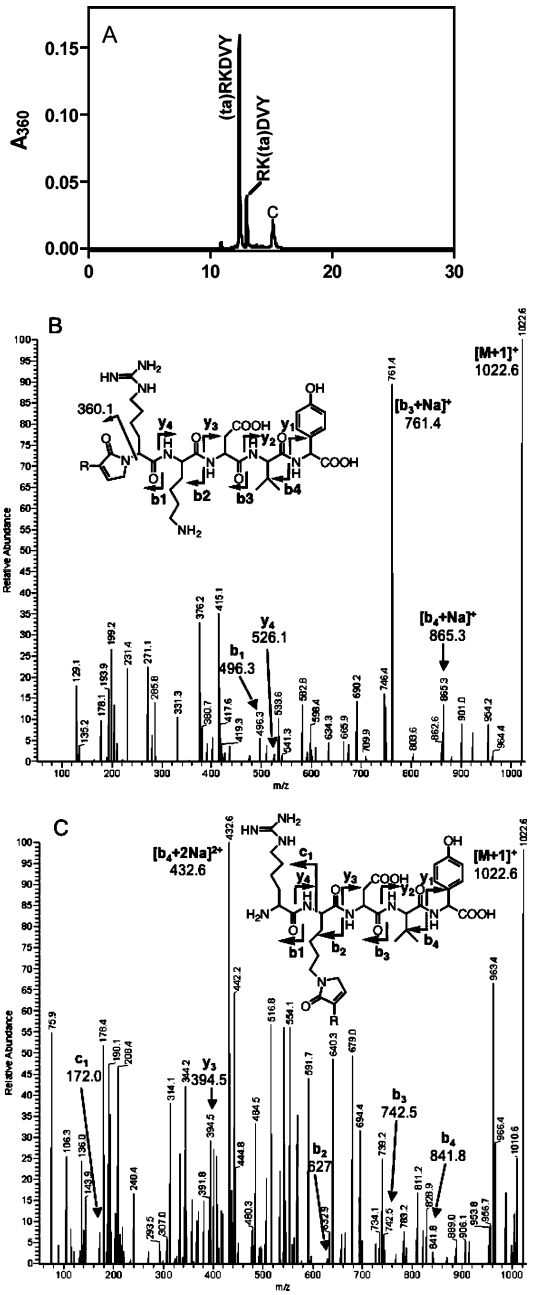


Figure 6. Representative HPLC chromatogram with UV monitoring of the reaction mixture of **6** with peptide RKDVY (A), a representative MS/MS spectrum showing the daughter ion with an *m*/*z* of 1022.59, corresponding to the major *N*-terminal conjugate of **6** (B), and a representative MS/MS spectrum showing the daughter ion with an *m*/*z* of 1022.59, corresponding to the minor conjugate of the lysine side chain amine with **6** (C).

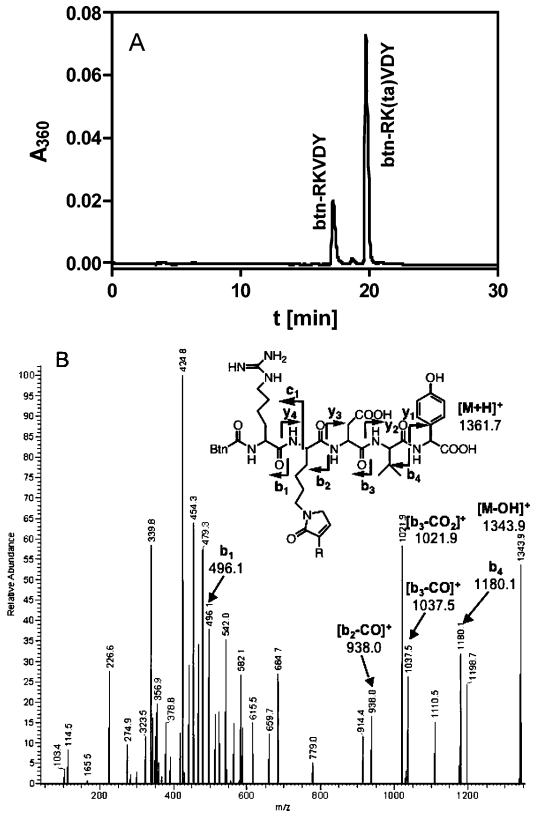


Figure 7. Representative HPLC chromatogram with UV monitoring of the reaction mixture of **6** with *N*-terminal biotinylated peptide RKVDY (A) and a representative MS/MS spectrum showing the daughter ion with an *m*/*z* of 1361.7, corresponding to the conjugate of the lysine side chain amine with **6** (B).

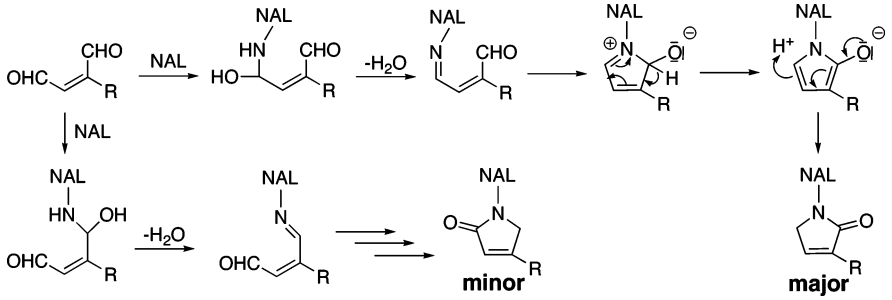
of DNA adducts it forms with dC, dA, and dG (*22*−*24*, *34*). However, the characterization of conjugates of more complex furans with model nucleophiles is incomplete, with only sparse reports appearing in the literature (*15*, *26*). The bioactivation of heterocyclic compounds including furans has been reviewed recently (*16*). Our present results are consistent with the previously reported chemistry of 1,4-enedials and add a comprehensive analysis of conjugates formed by enedials **2** and **6** derived from 3-substituted furans.

Enedials **2** and **6** were prepared by DMDO oxidation of the model furan **1** and teucrin A, respectively (Scheme 1 and 2). In both cases, *cis* isomers were formed exclusively, with slow isomerization to the more stable *trans* isomer observed in the case of **2**. The enedial products were unstable to isolation and sensitive to hydration. There was no evidence for the formation of an epoxide in the course of the DMDO oxidation in the NMR spectra of the reaction mixtures *in situ*. However, these observations should not be taken as evidence against epoxide intermediacy in these reactions because such an intermediate would likely be extremely unstable. The *exo*-epoxide of aflatoxin B_1 has been prepared by DMDO oxidation, and its $t_{1/2}$ in aqueous buffer is 1.0 s (*13*, *35*). In our hands, DMDO oxidation of aflatoxin B_1 generated the *exo*-epoxide in approximately 10% yield on the basis of ^{1}H NMR (Druckova, A., unpublished results). Thus, if epoxides were formed from either **1** or teucrin A, they would be extremely unstable and may rearrange to the enedials, thereby, eluding detection.

We conducted a series of reactions with a model enedial, **2**, and amino acid nucleophiles and characterized the products of these reactions. The results of these experiments were essential to understanding the chemistry of the more complex enedial derivative **6**, the presumed hepatotoxic metabolite of teucrin A. Indeed, a novel product observed in the reaction of the model enedial, **2**, with NAC, **4**, uncovered the potential for the existence of an equilibrium between the enedial and hydroxyenal tautomers (Scheme 3). Product **4** most likely arises after 1,2-addition of NAC to the C1 carbonyl of **2** following Michael addition of the first molecule of NAC to the double bond of the hydroxyenal tautomer, resulting in the displacement of the 4-methoxybenzylalcohol leaving group (Scheme 3). Subsequent ring closure and dehydration leads to the formation of the single product **4**.

The realization of the equilibration potential prompted the careful examination of the possibility of diastereomers arising from the reactions of **6** with NAC or NAL. Indeed, the ^{1}H NMR spectra of all isolated products showed evidence for the

Scheme 4. Proposed Mechanism for the Formation of NAL Conjugates with the Enedial Derivatives



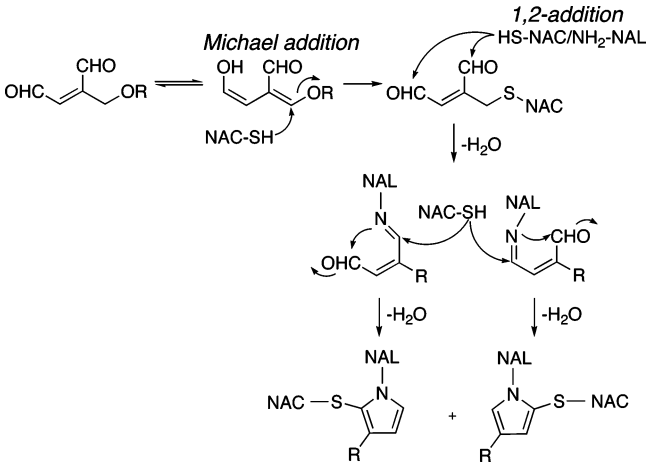
epimerization of the H12 proton and the formation of diastereomers. The reaction of **6** with NAC in aqueous buffer afforded a mixture of unstable products with the exception of **8c**, which was successfully purified and analyzed by NMR. The formation of unstable products in the reaction of *cis*-2-butene-1,4-dial with NAC has been previously attributed to the reversibility of cysteine 1,2-addition to the aldehyde group or 1,4-addition to the double bond in the aqueous environment (*24*). However, the reaction afforded *N*-acetyl-*S*-2-furanyl-L-cysteine with acetone as solvent. The analysis of the NMR spectrum of **8c** revealed the presence of two diastereomers in a 2:1 ratio. The formation of the 2-substituted furan in this reaction can occur through the mechanism depicted in the Scheme 3. The epimerization of the H12 proton of **8c** to afford diastereomeric products in a 2:1 ratio suggests a kinetically favored reaction in which the C1 carbonyl of the enol tautomer reacts with the thiol group, and tautomerization back to carbonyl on C4 is preferentially driven in favor of one isomer by steric influences.

The products of the reaction of model enedial **2** with NAL were identified as a pair of regioisomers of the *N*-alkyl-3-pyrrolin-2-one ring, **3a** and **3b**. The formation of the more sterically hindered **3a** as the major product can be rationalized by the mechanism depicted in Scheme 4. The initial nucleophilic attack by the amine of NAL occurs via 1,2-addition to the less sterically hindered carbonyl carbon C4. Subsequent dehydration to form the Schiff base and cyclization affords the major pyrrolin-2-one isomer **3a**. The displacement of the 4-methoxybenzylalcohol by the second molecule of NAL did not occur, likely because the amino group of lysine is not a sufficiently strong Michael donor for this reaction to occur, and efficient cyclization precludes Michael addition. Structurally identical derivatives of **3a** and **3b** have been shown to be the thermodynamically favored products of the reaction of *cis*-2-butene-1,4-dial with NAL, where an equilibrium between *N*-alkyl-4-pyrrolin-2-one and *N*-alkyl-3-pyrrolin-2-one was observed (*24*).

This mechanism is applicable to the reaction of **6** with NAL, which almost exclusively afforded the major regioisomer (**7c**,**d**, Scheme 2). The enedial derivative of teucrin A (**6**) presents substantially larger steric bulk to the incoming nucleophile than **2**, directing the attack preferentially to the C4 carbonyl. Despite the fact that the Michael addition did not occur in the reaction of **2** with NAL, the proposed equilibrium between the enedial and hydroxyenal species was established, resulting in the racemization of the H12 proton as confirmed by the ^{1}H NMR of the products of **6** with NAL. The diastereomers were formed in a 1:1 ratio.

The reaction of **2** in the presence of both NAL and NAC afforded a mixture of the regioisomeric pyrroles **5a** and **5b** as well as a minor amount of the adduct **4**. The products of the reaction with NAL alone (**3a**,**b**) were not observed. This is consistent with the proposal that a rapid addition of cysteine occurs prior to a reaction of the amine group with the aldehyde

Scheme 5. Proposed Mechanism for the Formation of NAC/NAL Conjugates with the Enedial Derivatives



(*24*). Furthermore, the benzylalcohol moiety of **2** was displaced in both pyrrole products (**5a**,**b**) by the second molecule of NAC, confirming that the thiol group was a superior Michael donor in the reactions with unsaturated aldehydes in an aqueous buffer of pH 7.4. Subsequent reaction of NAL with one of the free carbonyls followed by 1,2-addition of the thiol group of NAC would explain the formation of both regioisomeric pyrroles **5a** and **5b** (Scheme 5).

The reaction of **6** in the presence of both NAL and NAC afforded a mixture of pyrroles (**9a**−**c**) and the cysteine adduct **8c**. The products **9a** and **9b** were produced in low yields and characterized only by mass spectral analysis. The data suggested the formation of tetrasubstituted pyrroles produced by the reaction of NAL and two molecules of NAC with **6**. The major product, **9c**, was isolated and characterized by NMR. NMR analysis revealed the presence of two diastereomers in a 1:1 ratio as in the case of **7c** and **7d**. The formation of the 1,2,4-substituted pyrrole ring of **9c** is outlined by the mechanism depicted in Scheme 5, in which the 1,2-addition of the thiol group to the imine carbon follows the addition of NAL to the more accessible carbonyl C4. The reaction is completed by cyclization and dehydration to form **9c**. In the presence of the amine, the slow steps of the Schiff base formation allow for complete epimerization of H12. The 1,2,4-substituted pyrrole was isolated as the major NAC/NAL adduct formed in microsomal incubations of the 3-substituted furan 4-ipomeanol, providing evidence for the enedial metabolite generated by cytochrome P450 *in vitro* (*15*).

The formation of the stable lysine adducts of teucrin A enedial derivative **6** led us to investigate the reactions of **6** with lysine-containing peptides. The formation of the pyrroline-2-one adducts was observed; as expected, the *N*-terminal amine was more reactive than the ϵ-amine. The *N*-terminal protected peptide afforded a single pyrrolinone product formed by the reaction

of the amino group of the lysine side chain. The pyrrolinone adduct of **6** with an *N*-terminal biotinylated peptide was purified and characterized by mass spectral analyses. The ability to efficiently synthesize stable structurally characterized peptide adducts of reactive metabolites provides a tool for the development of selective antibodies against these epitopes (*36*). Efforts in our laboratory are currently under way to identify the protein targets of teucrin A metabolites *in vivo* using this approach. Autoantibodies against microsomal epoxide hydrolase are present in the sera from patients who drank germander tea for extended periods. This observation supports the idea of covalently modified proteins present *in vivo* upon exposure to germander, and we are in the process of identifying the targets and confirming the presence of teucrin A modifications in the hepatic proteins isolated from rats treated with teucrin A. The sensitivity of epoxide hydrolase to autoantibody generation may indicate the involvement of an epoxide intermediate as the modifying agent, although there may be other reasons for the generation of autoantibodies unrelated to modification by a germander derivative.

In summary, we describe a detailed characterization of the products of the reaction of amino acids and peptides with 2-substituted 1,4-enedials. Identification of the products uncovered the potential for an equilibrium between the enedial and hydroxyenal tautomers in aqueous solutions, providing insight into the mechanism of their formation. This work further illuminates the complex chemical behavior of unsaturated dialdehydes as an important class of toxic metabolites and provides important background information that will be useful in defining the chemistry and biology of teucrin A toxicity.

Acknowledgment. We are grateful to T. Adams of the Flavor and Extract Manufacturers Association for helpful discussions during preparation of this article. This work was supported by research grants from the National Cancer Institute (CA87819) and the National Foundation for Cancer Research.

Supporting Information Available: ^{1}H NMR and COSY spectra of compounds **3a**, **3b**, **5a**, **5b**, **7c**,**d**, **8c**, and **9c**. This material is avaliable free of charge via the Internet at http://pubs.acs.org.

References

(1) De Vincenzi, M., Maialetti, F., and Silano, M. (2003) Constituents of aromatic plants: teucrin A. *Fitoterapia 74*, 746−749.

(2) Bedir, E., Manyam, R., and Khan, I. A. (2003) Neo-clerodane diterpenoids and phenylethanoid glycosides from Teucrium chamaedrys L. *Phytochemistry 63*, 977−983.

(3) Castot, A., and Larrey, D. (1992) Hepatitis observed during a treatment with a drug or tea containing Wild Germander. Evaluation of 26 cases reported to the Regional Centers of Pharmacovigilance. *Gastroenterol. Clin. Biol. 16*, 916−922.

(4) Diaz, D., Ferroudji, S., Heran, B., Barneon, G., Larrey, D., and Michel, H. (1992) Fulminant hepatitis caused by wild germander. *Gastroenterol. Clin. Biol. 16*, 1006−1007.

(5) Legoux, J. L., Maitre, F., Labarriere, D., Gargot, D., Festin, D., and Causse, X. (1992) Cytolytic hepatitis and wild Germander: a new case with reintroduction. *Gastroenterol. Clin. Biol. 16*, 813−815.

(6) Mattei, A., Bizollon, T., Charles, J. D., Debat, P., Fontanges, T., Chevallier, M., and Trepo, C. (1992) Liver damage induced by the ingestion of a product of phytotherapy containing wild germander. Four cases. *Gastroenterol. Clin. Biol. 16*, 798−800.

(7) Pauwels, A., Thierman-Duffaud, D., Azanowsky, J. M., Loiseau, D., Biour, M., and Levy, V. G. (1992) Acute hepatitis caused by wild germander. Hepatotoxicity of herbal remedies. Two cases. *Gastroenterol. Clin. Biol. 16*, 92−95.

(8) Ben Yahia, M., Mavier, P., Metreau, J. M., Zafrani, E. S., Fabre, M., Gatineau-Saillant, G., Dhumeaux, D., and Mallat, A. (1993) Chronic active hepatitis and cirrhosis induced by wild germander. 3 cases. *Gastroenterol. Clin. Biol. 17*, 959−962.

(9) *Opinion of the Scientific Committee on Food on Teucrin A* (2003) Scientific Committee on Food, European Commission, Health & Consumer Directorate-General.

(10) Racha, J. K., Rettie, A. E., and Kunze, K. L. (1998) Mechanism-based inactivation of human cytochrome P450 1A2 by furafylline: detection of a 1:1 adduct to protein and evidence for the formation of a novel imidazomethide intermediate. *Biochemistry 37*, 7407−7419.

(11) Boyd, M. R., Grygiel, J. J., and Minchin, R. F. (1983) Metabolic activation as a basis for organ-selective toxicity. *Clin. Exp. Pharmacol. Physiol. 10*, 87−99.

(12) Falzon, M., McMahon, J. B., Schuller, H. M., and Boyd, M. R. (1986) Metabolic activation and cytotoxicity of 4-ipomeanol in human non-small cell lung cancer lines. *Cancer Res. 46*, 3484−3489.

(13) Baertschi, S. W., Raney, K. D., Stone, M. P., and Harris, T. M. (1988) Preparation of the 8, 9-epoxide of the mycotoxin aflatoxin B_1: the ultimate carcinogenic species. *J. Am. Chem. Soc. 110*, 7929−7931.

(14) Smela, M. E., Currier, S. S., Bailey, E. A., and Essigmann, J. M. (2001) The chemistry and biology of aflatoxin B(1): from mutational spectrometry to carcinogenesis. *Carcinogenesis 22*, 535−545.

(15) Baer, B. R., Rettie, A. E., and Henne, K. R. (2005) Bioactivation of 4-ipomeanol by CYP4B1: adduct characterization and evidence for an enedial intermediate. *Chem. Res. Toxicol. 18*, 855−864.

(16) Dalvie, D. K., Kalgutkar, A. S., Khojasteh-Bakht, S. C., Obach, R. S., and O'Donnell, J. P. (2002) Biotransformation reactions of five-membered aromatic heterocyclic rings. *Chem. Res. Toxicol. 15*, 269−299.

(17) Kouzi, S. A., McMurtry, R. J., and Nelson, S. D. (1994) Hepatotoxicity of germander (Teucrium chamaedrys L.) and one of its constituent neoclerodane diterpenes teucrin A in the mouse. *Chem. Res. Toxicol. 7*, 850−856.

(18) Lekehal, M., Pessayre, D., Lereau, J. M., Moulis, C., Fouraste, I., and Fau, D. (1996) Hepatotoxicity of the herbal medicine germander: metabolic activation of its furano diterpenoids by cytochrome P450 3A depletes cytoskeleton-associated protein thiols and forms plasma membrane blebs in rat hepatocytes. *Hepatology 24*, 212−218.

(19) Fau, D., Lekehal, M., Farrell, G., Moreau, A., Moulis, C., Feldmann, G., Haouzi, D., and Pessayre, D. (1997) Diterpenoids from germander, an herbal medicine, induce apoptosis in isolated rat hepatocytes. *Gastroenterology 113*, 1334−1346.

(20) Ravindranath, V., Burka, L. T., and Boyd, M. R. (1984) Reactive metabolites from the bioactivation of toxic methylfurans. *Science 224*, 884−886.

(21) Chen, L. J., Hecht, S. S., and Peterson, L. A. (1995) Identification of cis-2-butene-1, 4-dial as a microsomal metabolite of furan. *Chem. Res. Toxicol. 8*, 903−906.

(22) Byrns, M. C., Predecki, D. P., and Peterson, L. A. (2002) Characterization of nucleoside adducts of cis-2-butene-1, 4-dial, a reactive metabolite of furan. *Chem. Res. Toxicol. 15*, 373−379.

(23) Byrns, M. C., Vu, C. C., and Peterson, L. A. (2004) The formation of substituted 1,N6-etheno-2′-deoxyadenosine and 1,N2-etheno-2′-deoxyguanosine adducts by cis-2-butene-1,4-dial, a reactive metabolite of furan. *Chem. Res. Toxicol. 17*, 1607−1613.

(24) Chen, L. J., Hecht, S. S., and Peterson, L. A. (1997) Characterization of amino acid and glutathione adducts of cis-2-butene-1,4-dial, a reactive metabolite of furan. *Chem. Res. Toxicol. 10*, 866−874.

(25) Peterson, L. A., Naruko, K. C., and Predecki, D. P. (2000) A reactive metabolite of furan, cis-2-butene-1, 4-dial, is mutagenic in the Ames assay. *Chem. Res. Toxicol. 13*, 531−534.

(26) Zhang, K. E., Naue, J. A., Arison, B., and Vyas, K. P. (1996) Microsomal metabolism of the 5-lipoxygenase inhibitor L-739, 010: evidence for furan bioactivation. *Chem. Res. Toxicol. 9*, 547−554.

(27) Murray, R. W., and Jeyaraman, R. (1985) Dioxiranes: Synthesis and reactions of methyldioxiranes. *J. Org. Chem. 50*, 2847−2853.

(28) Maga, J. A. (1979) Furans in foods. *Crit. Rev. Food Sci. Nutr. 11*, 355−366.

(29) Boyd, M. R., and Burka, L. T. (1978) In vivo studies on the relationship between target organ alkylation and the pulmonary toxicity of a chemically reactive metabolite of 4-ipomeanol. *J. Pharmacol. Exp. Ther. 207*, 687−697.

(30) Boyd, M. R., and Dutcher, J. S. (1981) Renal toxicity due to reactive metabolites formed in situ in the kidney: investigations with 4-ipomeanol in the mouse. *J. Pharmacol. Exp. Ther. 216*, 640−646.

(31) Wolf, C. R., Statham, C. N., McMenamin, M. G., Bend, J. R., Boyd, M. R., and Philpot, R. M. (1982) The relationship between the catalytic activities of rabbit pulmonary cytochrome P-450 isozymes and the lung-specific toxicity of the furan derivative, 4-ipomeanol. *Mol. Pharmacol. 22*, 738−744.

(32) Burka, L. T., Washburn, K. D., and Irwin, R. D. (1991) Disposition of [14C]furan in the male F344 rat. *J. Toxicol. Environ. Health 34*, 245−257.

(33) Nelson, S. D., McClanahan, R. H., Thomassen, D., Gordon, W. P., and Knebel, N. (1992) Investigations of mechanisms of reactive metabolite formation from (R)-(+)-pulegone. *Xenobiotica 22*, 1157−1164.

(34) Vu, C. C., and Peterson, L. A. (2005) Synthesis of a 2′-deoxyguanosine adduct of cis-2-butene-1,4-dial, a reactive metabolite of furan. *Chem. Res. Toxicol. 18*, 1012−1017.
(35) Johnson, W. W., Ueng, Y. F., Harris, T. M., and Guengerich, F. P. (1996) Reactions of aflatoxin B-1 exo-8,9-epoxide with water and biological nucleophiles. *FASEB J. 10*, 609.
(36) Davies, S. S., Talati, M., Wang, X., Mernaugh, R. L., Amarnath, V., Fessel, J., Meyrick, B. O., Sheller, J., and Roberts, L. J., II (2004) Localization of isoketal adducts in vivo using a single-chain antibody. *Free Radical Biol. Med. 36*, 1163−1174.

TX060143K