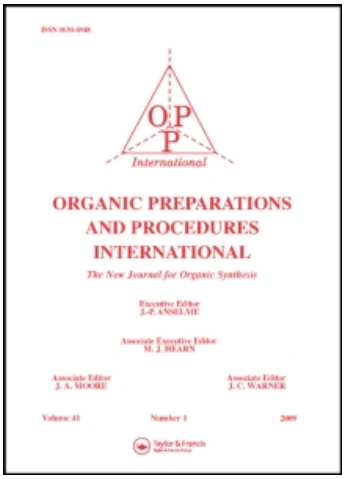

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SYNTHESIS OF *S*-ARYL-D,L-CYSTEINES AND INCORPORATION INTO KERATIN SEQUENCES

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SYNTHESIS OF *S*-ARYL-D,L-CYSTEINES AND INCORPORATION INTO KERATIN SEQUENCES

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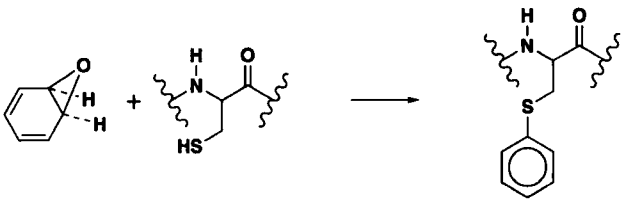
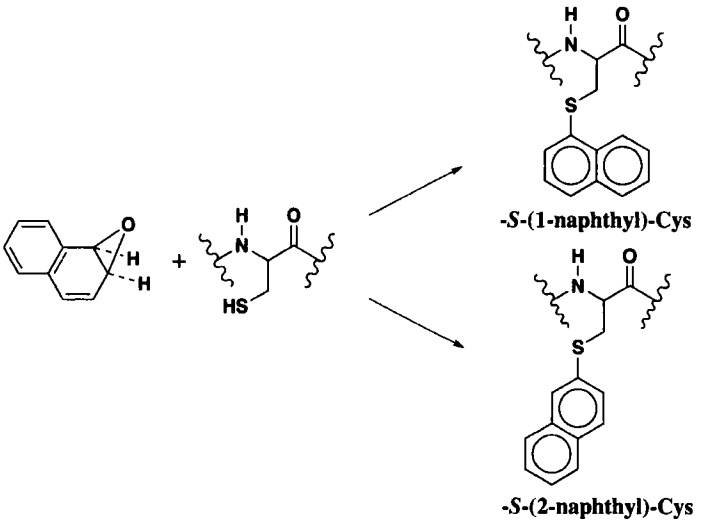
Although there is a significant potential for benzene and naphthalene toxicity through dermal exposure in occupational settings, no satisfactory method for quantitation of dermal dose has been developed nor have possible mechanisms of action been extensively investigated. The epoxide metabolites of both aromatics form protein adducts, targeting the nucleophilic sulfhydryl group of cysteine residues. In dermal exposures, the most accessible cysteines will be residues in the head region of the keratin proteins K1 and K10, which are associated in intermediate filaments[1] in *stratum corneum*. Keratin adducts may serve as biomarkers and may also mediate dermal toxicity. Benzene oxide is symmetrical and only a single product, *S*-phenylcysteine, is generated by nucleophilic addition to the cysteine sulfhydryl group. The C1-C2 bond of naphthalene is epoxidized and the 1,2-oxide may undergo nucleophilic attack at either position 1 or 2 to yield *S*-(1-naphthyl)cysteine and *S*-(2-naphthyl)cysteine, respectively.

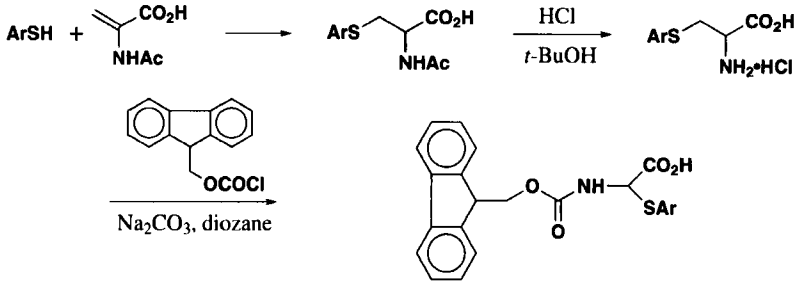


-*S*-phenyl-Cys-

Both K1 and K10 contain cysteine residues that are close to the protein *N*-terminus[2] which should be readily accessible for adduct formation. We selected the10-mer sequences GGGRFSS(*S*-aryl-C)GG of K1 and GGG(*S*-aryl-C)GGGGG of K10 for use as haptens in raising antibodies to keratin adducts. An analogous strategy has been successfully applied in developing immunochemical analyses for keratin adducts of sulfur mustards.[3] The approach adopted for obtaining haptens was the synthesis of head sequences by 9-fluorenylmethylcarbonyl (FMOC) chemistry incorporating the N^{α}-FMOC-protected *S*-aryl cysteines.



This strategy enables us to prepare the pure, rigorously characterized haptens in quantity. While the synthetic route to the N^{α}-FMOC-protected cysteines was based on published procedures, the low solubility of the intermediates required modification of the reaction conditions. *S*-aryl-**D,L**-mercapturic acids were obtained by a high-yield Michael addition of the arylthiols to α-acetamidoacrylic acid,[4] but could not be purified by crystallization or chromatography. It was possible to obtain very pure products by washing 5% bicarbonate solutions of the reaction mixtures with ether, and then precipitating the mercapturic acid by carefully lowering the pH to 3 with concentrated hydrochloric acid.



An interesting feature of the ^{1}H NMR spectra of the mercapturic acids is the clear resolution of the diastereotopic methylene protons of cysteine enantiomers. Deacetylation of the

mercapturic acids proved to be challenging, because the *S*-arylcysteine products, like the *S*-arylmercapturic acids, could not be recrystallized or conveniently purified in quantity by chromatography. Methanolic ammonia was not effective and the use of refluxing with methanolic or ethanolic HCl was precluded by the likelihood of esterification of the carboxylic acid function. However, this problem was circumvented by extended refluxing in 1:1 *t*-butanol/concentrated HCl under an inert atmosphere, with progress monitored by ^{1}H NMR spectrometry. From this hydrolysis medium, the *S*-arylcysteines could be isolated directly in ≥ 90% purity (by ^{1}H NMR) by evaporating the reaction solution.

The published procedure[5] for preparation of the FMOC derivatives required modification because of the low solubility of the *S*-aryl cysteines. The FMOC derivatization was done on the cysteine adducts as a slurry in 10% sodium carbonate/dioxane 3:2 and the reaction time was doubled to 32 hours. By-products arising from side-reactions of the 9-fluorenylchloroformate reagent were removed from the aqueous reaction mixture by ether extraction. The pure FMOC derivatives could then be isolated as crystalline solids by careful adjustment of the pH to 3 with hydrochloric acid.

Acceptable mass measurements were obtained for all previously unreported aryl cysteines and derivatives, and are given in the Experimental Section. The FMOC derivatives were used for incorporation into the K1 and K10 sequences by automated solid-phase peptide synthesis.[6] The synthesis of the oligopeptides was accomplished with no modification of the standard procedure.[7] Since the modified cysteines were racemic mixtures, each oligopeptide synthesis yielded pairs of diastereomeric products. Under the chromatographic conditions applied to purify the oligopeptides, the diastereomer pairs were not separated, but appeared as a single Gaussian-shaped peak in the HPLC Chromatogram, exemplified by *Fig. 1*. The combined yield of the diastereomeric modified oligopeptide pairs was comparable to that obtained for the unmodified oligopeptide synthesized in each case for reference. Thus the *S*-arylcysteines were incorporated with efficiencies similar to those of the natural amino acids. The purity of all modified peptides (determined as unresolved diastereomeric mixtures) was determined to be > 98% by HPLC analysis. Parallel series of oligomers with lysine substituted for *N*-terminal glycine have also been synthesized for conjugation of the naphthalene-modified peptides to keyhole limpet hempcyanin (KLH) by the glutaraldehyde procedure.

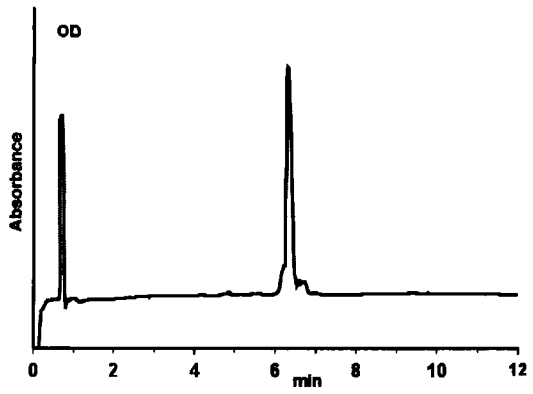


Fig.1. HPLC chromatogram of diastereomeric mixture of K1 oligopeptides containing S-phenylcysteine (λ = 229 nm).

As all of the compounds in this study are obtained as microcrystalline solids containing variable amounts of water of crystallization, elemental composition was verified by exact mass measurements of the protonated molecular ions using a fast atom bombardment source. The synthetic approach described here lends itself readily to incorporation of modified amino acids into selected target sequences, and can readily be adapted for preparation of isotopically labeled standards.

EXPERIMENTAL SECTION

2-Acetamidoacrylic acid, thiophenol, 2-naphthalenethiol and 9-fluorenylmethyl chloroformate were purchased from Sigma-Aldrich and used as received. 1-Naphthalenethiol was purchased from Fischer Scientific and used as received. Dioxane was distilled from CaH_2; all other solvents were reagent grade and used as received. ^{1}H NMR spectra were acquired on a Varian Inova NMR spectrometer at 500 MHz in Me_2SO-d_6. Low resolution mass spectra were obtained either on a VG70-250 SEQ hybrid mass spectrometer equipped with a FAB ionization source operating in a positive ion mode or a Finnigan TSQ 7000 equipped with an ESI source operating in a positive ion mode. High-resolution mass spectra were determined on a VG70-250 SEQ hybrid or JEOL HX110HF mass spectrometer using a FAB ionization source and operating in a positive ion mode.

S-Phenylmercapturic Acid.- To a mixture of 2-acetamidoacrylic acid (3.0 g, 23.3 mmol) and thiophenol (2.79 g, 25.3 mmol) was added dioxane (20 mL) followed by twenty drops of piperidine. The reaction was refluxed for 2 h, cooled to room temperature and 5% $NaHCO_3$ (50 mL) added. The aqueous solution was washed with ether (40 mL) and the organic extract washed with 5% $NaHCO_3$ (20 mL). The combined aqueous layers were washed an additional two times with ether and then carefully acidified to pH 3 with conc. HCl. After cooling in an ice bath for 30 min, the resulting precipitate was collected, washed with water and dried under vacuum to give 1.08 g (39%) of a pale yellow microcrystalline solid. mp 152-153°C, *lit.*[4] 151°C. ESI LRMS: m/z 198 [MH^+], 181 [M-OH].

^{1}H NMR: δ 12.98 (bs, 1H, COO*H*); 8.31 (d, 1H, N*H*, $J_{NH\text{-}C}\alpha_H$= 7.7 Hz); 7.35-7.30 (m, 4H, phenyl *o*-H, *m*-H); 7.20 (t, 1H, phenyl *p*-H, $J_{m\text{-}pH}$=7.4 Hz); 4.33 (ddd, 1H, C$^{\alpha}$*H*, $J_{C}\alpha_{H\,-\,C}\beta_{H2}$=8.7, $J_{C}\alpha_{H\,\text{-}NH}$=7.7, $J_{C}\alpha_{H\,-\,C}\beta_{H2}$=4.9 Hz); 3.33 (dd, 1H, C*H*$^{\beta}_2$, $J_{C}\beta_{H2\text{-}\,C}\beta_{H2}$=13.6, $J_{C}\alpha_{H\,-\,C}\beta_{H2}$=4.9 Hz); 3.12 (dd, 1H, C*H*$^{\beta}_2$, $J_{C}\beta_{H2\text{-}\,C}\beta_{H2}$=13.6, $J_{C}\alpha_{H\,-\,C}\beta_{H2}$=8.7 Hz); 1.81 (s, 3H, COC*H*$_3$).

S-(1-Naphthyl)mercapturic Acid.- 2-Acetamidoacrylic acid (1.68 g, 13.0 mmol) and 1-naphthalenethiol (2.19 g, 13.7 mmol) were treated as in the above procedure to yield 1.78 g (47%) of a white microcrystalline solid, mp 127-129°C. Exact mass, FABMS as MH^+: expected for $C_{15}H_{16}NO_3S$, 290.085090; found, 290.080269.

^{1}H NMR: δ 12.90 (bs, 1H, COO*H*); 8.37 (d, 1H, N*H*, $J_{NH\text{-}\,C}\alpha_H$ = 7.8 Hz); 8.24 (d, 1H, Ar-H, J=9.2 Hz); 7.95 (d, 1H, Ar-H, J=7.8 Hz); 7.84 (d, 1H, Ar-H, J=9.1 Hz); 7.64 (d, 1H, Ar-H, J=6.7 Hz); 7.61-7.55 (m, 2H, Ar-H); 7.48 (ψt, 1H, Ar-H, J_{av}=7.7 Hz); 4.35 (ddd, 1H, C$^{\alpha}$*H*, $J_{C}\alpha_{H\,-}$

$_C\beta_{H2}$=8.8, $J_{C}\alpha_{H\text{-}NH}$=7.8, $J_{C}\alpha_{H-C}\beta_{H2}$=5.0 Hz); 3.38 (dd, 1H, C$^{\beta}H_2$, $J_{C}\beta_{H2\text{-}C}\beta_{H2}$=13.7, $J_{C}\alpha_{H-C}\beta_{H2}$=5.0 Hz); 3.21 (dd, 1H, C$^{\beta}H_2$, $J_{C}\beta_{H2\text{-}C}\beta_{H2}$=13.7, $J_{C}\alpha_{H-C}\beta_{H2}$=8.8 Hz); 1.82 (s, 3H, COCH_3).

***S*-(2-Naphthyl)mercapturic Acid**.- 2-Acetamidoacrylic acid (2.5 g, 19.4mmol) and 2-naphthalenethiol (3.26 g, 20.4 mmol) were treated as in the above procedure to yield 3.44 g (61%) of a pale yellow microcrystalline solid. mp 103-105°C. Exact mass, FAB MS as MH$^+$: expected for $C_{15}H_{16}NO_3S$, 290.085090; found, 290.087790.

^{1}H NMR: δ 12.92 (bs, 1H, COO*H*); 8.36 (d, 1H, N*H*, $J_{NH\text{-}C}\alpha_H$ = 8.9 Hz); 7.87-7.81 (m, 4H, Ar-H); 7.52-744 (m, 3H, Ar-H); 4.41 (ddd, 1H, C$^{\alpha}H$, $J_{C}\alpha_{H\text{-}NH}$=8.9, $J_{C}\alpha_{H-C}\beta_{H2}$=7.6, $J_{C}\alpha_{H-C}\beta_{H2}$=5.0 Hz); 3.45 (dd, 1H, C$^{\beta}H_2$, $J_{C\,C}\beta_{H2\text{-}C}\beta_{H2}$=13.6, $J_{C}\alpha_{H\text{-}C}\beta_{H2}$=5.0 Hz); 3.25 (dd, 1H, C$^{\beta}H_2$, $J_{C}\beta_{H2\text{-}C}\beta_{H2}$=13.6, $J_{C}\alpha_{H-C}\beta_{H2}$=7.6 Hz); 1.81 (s, 3H, COCH_3).

***S*-Phenylcysteine•HCl**.- *S*-Phenylmercapturic acid (1.08 g, 4.51 mmol) was stirred for 4 days at 70°C with 15 mL conc. HCl and 20 mL *t*-BuOH. At this time, ^{1}H NMR showed complete hydrolysis. The solution was evaporated under a stream of N_2 and the residual solid dried *in vacuuo* to give 1.0 g (95%) of a microcrystalline yellow solid, mp 188-190°C dec. (*lit.*[8] 190°C dec.) ESI LRMS, m/z: 198 [MH$^+$], 181 [M-OH].

^{1}H NMR: δ 8.61 (b, 2H, NH_2); 7.44 (d, 2H, phenyl *o*-H, $J_{o\text{-}m}$=6.8 Hz); 7.36 (ψt, 2H, phenyl *m*-H, J_{av}=7.7 Hz); 7.27 (t, 1H, phenyl *p*-H, $J_{p\text{-}m}$=7.3 Hz); 4.03 (ψt, 1H, C$^{\alpha}H$, J_{av}=5.4 Hz); 3.44 (m [ABX], 2H, C$^{\beta}H_2$).

***S*-(1-Naphthyl)cysteine•HCl**.- *S*-(1-Naphthyl)mercapturic acid (980 mg, 3.39 mmol) was stirred for 70 h with 17 mL conc. HCl and 40 mL *t*-BuOH at 70°C. Treatment as above yielded 899 mg (93%) of a microcrystalline yellow solid. Exact mass, FAB MS as MH$^+$: expected for $C_{13}H_{14}NO_2S$, 248.074526; found, 248.076320. mp 170-172°C.

^{1}H NMR: δ 8.73 (b, 2H, NH_2); 8.30 (d, 1H, Ar-H, J=7.6 Hz); 7.98 (d, 1H, Ar-H, J=8.0 Hz); 7.90 (d, 1H, Ar-H, J=7.5 Hz); 7.78 (d, 1H, Ar-H, J=7.9 Hz); 7.63 (ψt, 1H, Ar-H, J_{av}=7.2 Hz); 7.59 (ψt, 1H, Ar-H, J_{av}=7.4 Hz); 4.03 (ψt, 1H, C$^{\alpha}H$, J_{av}=5.9 Hz); 3.51 (m [ABX], 2H, C$^{\beta}H_2$).

***S*-(2-Naphthyl)cysteine•HCl**.- *S*-(2-Naphthyl)mercapturic acid (980 mg, 3.39 mmol) was stirred for 102 h with 17 mL conc. HCl and 40 mL *t*-BuOH at 70°C. Treatment as above yielded 813 mg (96%) of a microcrystalline yellow solid. Exact mass, FAB MS as MH$^+$: expected for $C_{13}H_{14}NO_2S$, 248.074526; found, 248.073447. mp 180-182°C.

^{1}H NMR: δ 8.60 (b, 2H, NH_2); 7.99 (s, 1H, naphthyl-H-1); 7.91-7.85 (m, 3H, Ar-H); 7.54-7.46 (m, 3H, Ar-H); 4.13 (ψt, 1H, C$^{\alpha}H$, J_{av}=6.1 Hz); 3.56 (m [ABX], 2H, C$^{\beta}H_2$).

N^{α}-FMOC-*S*-Phenyl-D,L-Cysteine.- *S*-Phenylcysteine•HCl (706 mg, 3.58 mmol) and 9-fluorenylmethyl chloroformate (945 mg, 3.65 mmol) were added to a mixture of 10% Na_2CO_3 (15 mL) and freshly distilled dioxane (10 mL), and the reaction stirred for 32 h. Following the addition of water (80 mL), the reaction mixture was washed three times with ether. The aqueous layer was carefully acidified with HCl to pH 3, and the resulting precipitate was cooled at 4°C overnight. The solid was stirred for 30 min at 4°C, collected and washed with cold water. Drying

in vacuo yielded 1.04 g (69.2%) of tan solid. mp 157-159°C. Exact mass, FAB MS as MH^+: expected for $C_{24}H_{22}ON_4S$, 420.126955; found, 420.127652.

^1H NMR: δ 7.88 (d, 1H, fluorene H-1(8), $J_{1,2}$=7.5 Hz); 7.70 (d, 1H, fluorene H-4(5), $J_{4,3}$=6.8 Hz); 7.41 (ψt, 1H, phenyl *m*-H, J_{av}=7.3 Hz); 7.36-7.30 (m, 7H, fluorenyl H-2(7),H3(6), phenyl *o*-H, N*H*); 7.20 (t, 1H, phenyl *p*-H, $J_{p\text{-}m}$=6.8 Hz); 4.27-4.07 (m, 2H, fluorenyl CH_2); 4.21 (ψq, 1H, fluorenyl H-9, J_{av}=6.9 Hz); 4.05 (m, 1H, C$^{\alpha}H$); 3.39 (dd, 1H, C$^{\beta}H_2$, $J_{C}\beta_{H2\text{-}C}\beta_{H2}$=13.6, $J_{C}\alpha_{H\text{-}C}\beta_{H2}$=4.6 Hz); 3.25 (dd, 1H, C$^{\beta}H_2$, $J_{C\,C\,C}\beta_{H2\text{-}C}\beta_{H2}$=13.6, $J_{C}\alpha_{H\text{-}C}\beta_{H2}$=9.7 Hz).

N^{α}-FMOC-*S*-(1-Naphthyl)-D,L-Cysteine.- Treatment of *S*-(1-Naphthyl)cysteine•HCl (885 mg, 3.12 mmol) and 9-fluorenylmethyl chloroformate (823 mg, 3.18 mmol) by the above procedure yielded 1.0 g (68%) of tan microcrystalline solid. mp 93-95°C. Exact mass FAB MS as M^+: expected for $C_{28}H_{23}NO_4S$, 469.134780; found, 469.135523.

^1H NMR: δ 8.26 (dd, 1H, naphthyl H-6 or H-7, J=7.1, J=2.9 Hz); 7.95 (dd, 1H, naphthyl H-7 or H6, J=6.1, J=3.3 Hz); 7.88 (d, 2H, fluorenyl H-4(5), $J_{3,4}$=8.0 Hz); 7.87 (b, 1H, N*H*); 7.84 (d, 1H, naphthyl H, J=7.5 Hz); 7.72 (d, 2H, fluorenyl H-1(8), $J_{1,2}$= 7.0 Hz); 7.64 (d, 1H, naphthyl H, J=7.9 Hz); 7.65 (d, 1H, naphthyl H, J=5.5 Hz); 7.55 (d, 1H, naphthyl H, J=7.59 Hz), 7.48 (ψt, 1H, naphthyl H-3, J_{av}=7.8 Hz); 7.40 (ψt, 2H, fluorenyl H-3(6), J_{av}=7.3 Hz); 7.31 (dd, 2H, fluorenyl H-2(7), $J_{2,1}$=7.0, $J_{2,3}$=6.8 Hz); 4.30-4.20 (m, 3H, fluorenyl H-9, CH_2); 4.12-4.07 (m, 1H, C$^{\alpha}H$); 3.43 (dd, 1H, C$^{\beta}H_2$, $J_{C}\beta_{H2\text{-}C}\beta_{H2}$=13.7, $J_{C}\alpha_{H\text{-}C}\beta_{H2}$=4.6 Hz); 3.24 (dd, 1H, C$^{\beta}H_2$, $J_{C}\beta_{H2\text{-}C}\beta_{H2}$=13.7, $J_{C}\alpha_{H\text{-}C}\beta_{H2}$=10.5 Hz).

N^{α}-FMOC-*S*-(2-Naphthyl)-D,L-Cysteine.- Treatment of *S*-(2-Naphthyl)cysteine•HCl (813 mg, 2.86 mmol) and 9-fluorenylmethyl chloroformate (755 mg, 2.92 mol) by the above procedure yielded 742 mg (55%) of tan microcrystalline solid, mp. 154-156°C. Exact mass, FAB as MNa^+: expected for $C_{28}H_{23}NO_4SNa$, 492.124550; found, 492.117451.

^1H NMR: δ 7.88-7.80 (m, 7H, 4 naphthyl-H, 2 fluorenyl-H, N*H*); 7.68 (d, 2H, fluorenyl H-1(8), J1,2= 6.5 Hz); 7.50-7.43 (m, 3H, naphthyl H); 7.40 (ψt, 2H, fluorenyl H-3(6), J_{av}=7.7 Hz); 7.30 (ψt, 2H, fluorenyl H-2(7), $J_{2,1}$=6.5, $J_{2,3}$=8.2 Hz); 4.24-4.16 (m, 3H, fluorenyl H-9, CH_2); 4.12-4.07 (m, 1H, C$^{\alpha}H$); 3.53 (dd, 1H, C$^{\beta}H_2$, $J_{C}\beta_{H2\text{-}C}\beta_{H2}$=13.8, $J_{C}\alpha_{H\,\text{-}\,C\,C}\beta_{H2}$=4.0 Hz); 3.15 (dd, 1H, C$^{\beta}H_2$, $J_{C}\beta_{H2\text{-}C}\beta_{H2}$=13.8, $J_{C}\alpha_{H\text{-}C}\beta_{H2}$=8.8 Hz).

Modified Peptide Sequences.- The modified K1 and K10 sequences were synthesized by the UNC Microprotein Core Facility using a Protein Technologies/Rainin Instrument multiple peptide synthesizer (Woburn, MA). FMOC chemistry was performed using O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) activation and assembly was on PEG-polystyrene resin. Cleavage and deprotection was accomplished over two hours with 95% TFA, 2.5% H_2O, and 2.5% triisopropylsilane as a scavenger. After cold ether precipitation and lyophilization, the peptides were purified by reverse phase HPLC utilizing a gradient from 100% water (0.1% TFA) to 50% acetonitrile:50% water (0.1% TFA) over 30 min, monitoring the effluent at 229 nm. Purified peptide fractions were pooled, lyophilized, and subjected to both

analytical HPLC evaluation as well as time-of-flight mass spectrometry (Kratos MALDI III, Kratos Analytical, Chestnut Ridge, NY). HPLC analysis confirmed purity in excess of 98% in all cases. Both because the peptides were obtained as microcrystalline solids with variable amounts of water of crystallization and were highly valuable materials, elemental compositions were confirmed by exact mass measurement.

GGGRFSS(*S*-phenyl-C)GG (*S*-phenylcysteine-K1): Exact mass calc for $C_{40}H_{58}N_{13}O_{13}S$ $(MH)^+$, 960.3998; found, 960.404.

GGGRFSS(*S*-(1-naphthyl)-C)GG (S-(1-naphthylcysteine)-K1): Exact mass calc for $C_{44}H_{60}N_{13}O_{13}S$ $(MH)^+$, 1010.4154; found, 1010.408.

KGGRFSS(*S*-(1-naphthyl)-C)GG (lysyl-S-(1-naphthylcysteine)-K1): Exact mass calc for $C_{48}H_{69}N_{14}O_{13}S$ $(MH)^+$, 1081.4889; found, 1081.491.

GGGRFSS(*S*-(2-naphthyl)-C)GG (S-(2-naphthylcysteine)-K1): Exact mass calc for $C_{44}H_{60}N_{13}O_{13}S$ $(MH)^+$, 1010.4154; found, 1010.415.

KGGRFSS(*S*-(2-naphthyl)-C)GG (lysyl-S-(2-naphthylcysteine)-K1): Exact mass calc for $C_{48}H_{69}N_{14}O_{13}S$ $(MH)^+$, 1081.4889; found, 1081.489.

GGGG(*S*-phenyl-C)GGGGG (*S*-phenylcysteine-K10): Exact mass calc for $C_{27}H_{39}N_{10}O_{11}S$ $(MH)^+$, 711.2520; found, 711.254.

KGGG(*S*-(1-naphthyl)-C)GGGGG (lysyl-*S*-(1-naphthyl)-cysteine-K10): Exact mass calc for $C_{35}H_{50}N_{11}O_{11}S$ $(MH)^+$, 832.3412; found, 832.347.

GGGG(*S*-(2-naphthyl-C)GGGGG (*S*-(2-naphthylcysteine)-K10): Exact mass calc for $C_{31}H_{41}N_{10}O_{11}S$ $(MH)^+$, 761.2677; found, 761.276.

KGGG(*S*-2-naphthyl-C)GGGGG (lysyl-(*S*-2-naphthylcysteine)-K10): Exact mass calc for $C_{35}H_{50}N_{11}O_{11}S$ $(MH)^+$, 832.3412; found, 832.321.

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