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Amino acid adducts of PAH *o*-quinones: model studies with naphthalene-1,2-dione

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Abstract—Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants which may cause cancer and require metabolic activation to exert their carcinogenic effects. As a step toward identifying the spectrum of PAH *o*-quinone-amino acid adducts that may form in biological systems, several naphthalene-1,2-dione-amino acid adducts were synthesized. Each adduct was formed as a 1,4-Michael addition product and spectral data corroborate that these adducts were either *o*-quinones or catechols. PAH *o*-quinone adducts can be used as standards to identify their presence in vitro and in vivo. © 2000 Elsevier Science Ltd. All rights reserved.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants and may be causative agents in human lung cancer. They are considered pro-carcinogens because they require metabolic activation to electrophiles to exert their deleterious effects.[1,2] The most extensive studies have been performed on the metabolic activation of benzo[*a*]pyrene (BP). Major metabolites of BP arise due to the action of monoxygenases (CYP450) and include phenols and diones, BP-1,6-dione, BP-3,6-dione and BP-6,12-dione.[1] Additionally arene oxides such as BP-4,5-oxide and BP-7,8-oxide also form. However, none of these metabolites are recognized as the electrophiles that modify DNA to cause mutation. Instead three alternative routes of metabolic activation have been proposed for PAH.

The first route involves the formation of *anti*- and *syn*-diol epoxides (via the combined action of CYP1A1 and epoxide hydrolase).[1,2] CYP1A1 catalyzes the initial step by forming BP-7,8-oxide, enzymatic addition of water catalyzed by epoxide hydrase forms the non-K-region *trans*-dihydrodiol, (−)7*R*,8*R*-dihydroxy-7,8-dihydro-BP (a proximate carcinogen), which then undergoes a second monoxygenation event to form (+)-*anti*-diol-epoxide, (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-BP (an ultimate carcinogen). The diol-epoxides of BP and other PAHs readily form stable N^2-deoxyguanosine adducts in DNA which are mutagenic.[3,4]

The second route of activation is the formation of radical cations formed in the peroxidase cycle of CYP450.[5] Cleavage of a peroxidase results in the formation of intermediate I and intermediate II, these higher oxidation states of heme-iron are reduced by the removal of an electron from the most electrophilic carbon of the PAH. For BP, this results in the formation of a radical cation at C6 which can form depurinating adducts with DNA.

The third route of activation involves the $NADP^+$-dependent oxidation of the non-K-region *trans*-dihydrodiols to reactive and redox-active *o*-quinones catalyzed by dihydrodiol dehydrogenase members of the aldo-keto reductase (AKR) superfamily (Fig. 1).[6–8]

Homogeneous rat liver dihydrodiol dehydrogenase AKR1C9 efficiently oxidizes racemic *trans*-dihydrodiols of benzene, naphthalene, phenanthrene, chrysene and benz[*a*]pyrene to their corresponding *o*-quinones. The identification of the *o*-quinone products suggested that enzymatic oxidation of the *trans*-dihydrodiol initially produced a ketol which tautomerizes to the catechol and that subsequent air oxidation generated the fully oxidized *o*-quinone (Fig. 2).[8] Initial attempts to isolate *o*-quinones as the products of these reactions failed due to their high reactivity, but they were subsequently isolated as thioether conjugates of mercaptoethanol which were characterized by EIMS and ^1H NMR.[8]

Because the resultant non-K region PAH *o*-quinones are reactive Michael acceptors, they have the potential to alkylate cellular nucleophiles, including DNA, RNA, protein, and GSH. We have previously characterized the formation of PAH *o*-quinone stable (N^2-deoxyguanosine) and depurinating (N7-guanine) DNA adducts, and the formation of *S*-glutathionyl-PAH *o*-quinone conjugates.[9–11] The formation of these DNA adducts in vivo may ultimately provide a dosimeter of PAH exposure.

Keywords: naphthalene-1,2-dione; polycyclic aromatic hydrocarbons, amino acids; aldo-keto reductase.

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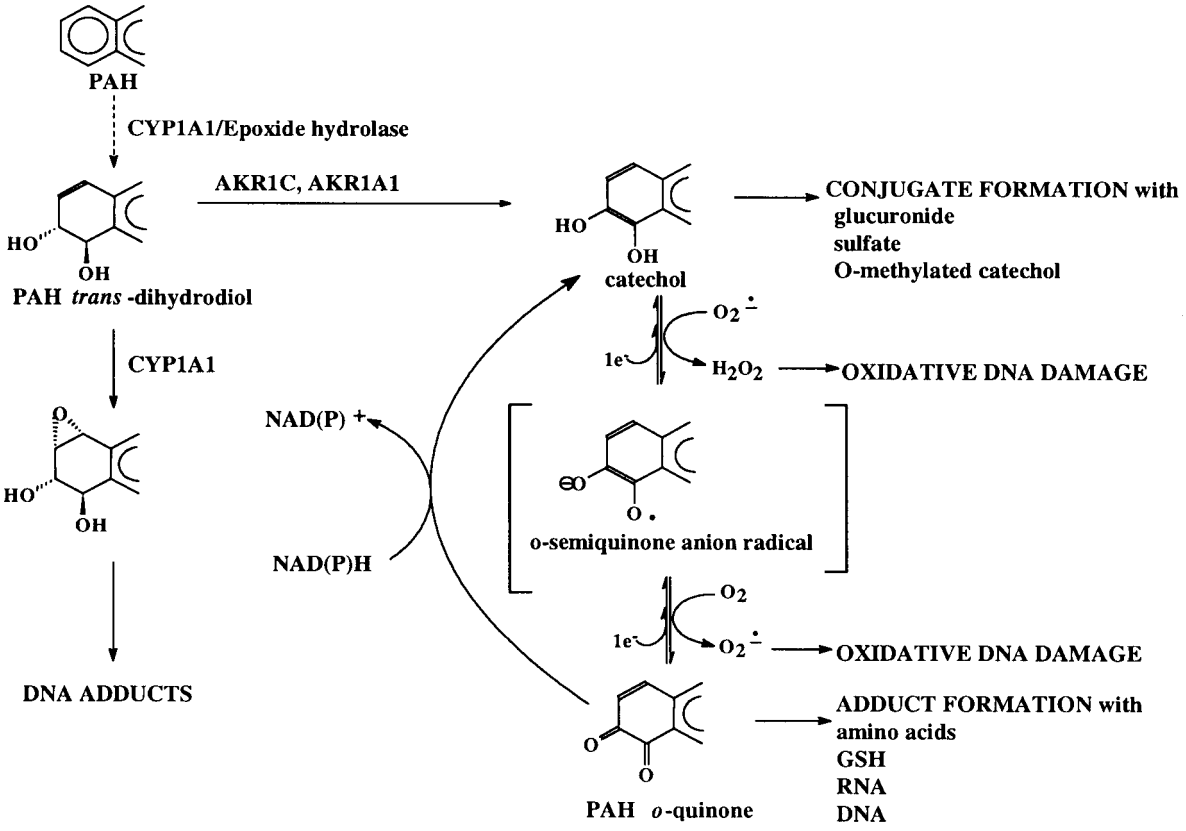

Figure 1. Cytochrome P450 (CYP) and aldo-keto reductase (AKR) isoforms compete for non-K region PAH *trans*-dihydrodiols.

The resultant non-K-region *o*-quinones are also redox active and in the presence of cellular reducing equivalents enter into futile-redox cycles in which the production of reactive oxygen species (ROS) is amplified multiple times.[12,13] The ability of these *o*-quinones to redox-cycle can be measured spectrally in the presence of NADPH with the concomitant production of ROS. The pro-oxidant state produced could lead to oxidatively damaged bases in DNA (tumor initiation) and the activation of protein kinase C (tumor promotion), providing an explanation by which PAH could act as complete carcinogens.

Catechol estrogens also give rise to structurally related *o*-quinones which have been shown to undergo Michael-addition reactions with thiols, primary amines and nucleophilic amino acids.[14–16] However, little is known about the ability of PAH *o*-quinones to alkylate proteins and form adducts with the side-chains of nucleophilic amino acids.

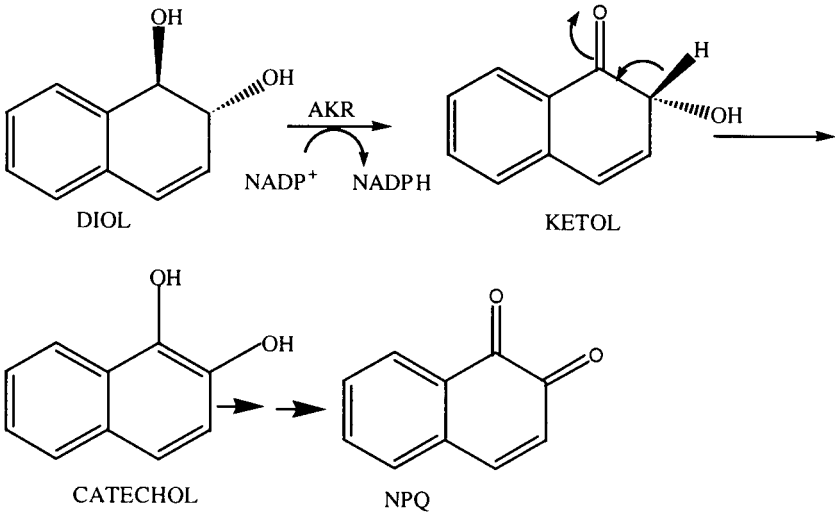

Figure 2. Mechanism of diol transformation into *o*-quinones.

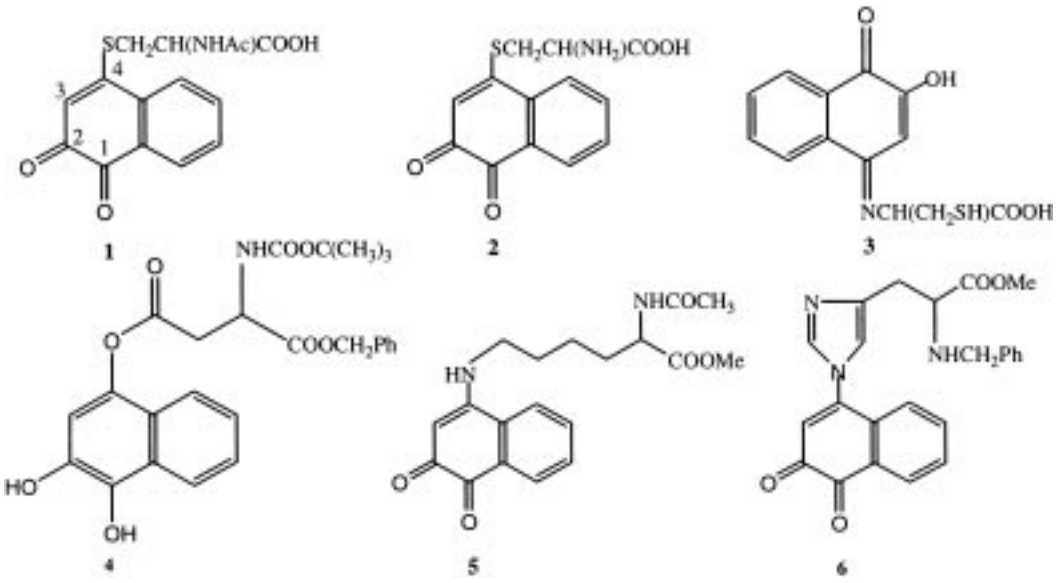

Figure 3. Amino-acid adducts of naphthalene-1,2-dione.

To develop standards for measuring the covalent modification of proteins by PAH *o*-quinones, we now report the synthesis and characterization of amino acid adducts observed with naphthalene-1,2-dione (NPQ).

1. Results and discussion

NPQ was used as a model PAH *o*-quinone in these studies primarily due to its ease of synthesis and relatively high solubility in aqueous systems. NPQ was synthesized according to the method of Fieser.[17] Reactions of NPQ with amino acids were restricted to those with reactive side-chains, e.g., L-cysteine, *N*-acetyl-L-cysteine and *N*-protected-L-histidine, L-aspartate and L-lysine methyl or benzyl esters. These reactions often gave a complex spectrum of products, but emphasis was placed on isolating the major adduct by chromatographic methods, e.g. TLC, RP-HPLC and gel exclusion chromatography. Products were characterized by NMR, LC/MS and HRMS.

The reaction of NPQ with *N*-acetyl-L-cysteine was performed in 50 mM potassium phosphate buffer (pH 7.0) with a 5-fold molar excess of *N*-acetyl-L-cysteine and products were isolated. Within a few minutes the reaction was complete and the mixture was lyophilized, redissolved in water and subjected to Sephadex G10 column chromatography. Although the reaction mixture contained one major orange adduct (63% yield) which eluted first from the column, an additional 7–8 products also eluted from the column and the mixture was not further characterized. Significant features in ^{13}C NMR spectrum of the major adduct include a large downfield shift of the C1 (161.3 ppm) vs. the C2 (120.37 ppm) carbon resonance indicating that the position of substitution is at C1. Substitution at C1 is supported by the ^{1}H NMR spectrum of the adduct at 253 K, which showed the presence of an unexpected isolated proton at δ 6.69. This aromatic proton is assigned to 2-C*H* and should be contrasted with the expected value for an isolated aromatic proton if substitution were at C2, 1-C*H* (δ=7.65). This isolated aromatic proton is consistent with a hydroquinone adduct but it can only be seen at low temperatures and indicates that the formation of one stable conformer of the hydroquinone only occurs under these conditions. Since the naphthalene-1,2-dione adduct was substituted at C1 and not C2, the adduct must correspond to a 1,4- rather than a 1,6-Michael addition product (Fig. 3).

The ^{13}C NMR spectrum also shows the presence of four carbon resonances that correspond to two sets of carbonyl carbons and are diagnostic of the structure. One set of resonances correspond to the carbonyl carbons of *N*-acetyl-L-cysteine and the other set of resonances correspond to the carbonyl carbons of NPQ. Thus, the adduct is a 1,4-Michael addition product obtained at the level of *o*-quinone oxidation and it is not a hydroquinone. ^{1}H and ^{13}C NMR spectra therefore corroborate the structure of the stable adduct as *N*-acetyl-*S*-(3,4-dihydro-3,4-dioxo-1-naphthyl)-L-cysteine (**1**).

Please note that in this discussion IUPAC nomenclature requires the naphthalene ring system to be re-numbered from the site of substitution so that C1 of the adduct corresponds to C4 of NPQ.

Reaction of L-cysteine and NPQ was conducted in a manner identical to that described for *N*-acetyl-L-cysteine. As before a number of reaction products could be isolated by Sephadex G10 chromatography, the major orange product (**2**) was obtained in 63 % yield which permitted its further spectral characterization. Like adduct (**1**), this adduct gave a significant downfield shift in the carbon resonance observed at C1 indicating substitution at this position. ^{1}H NMR (298 K) showed the presence of both an isolated vinyl proton (5.73 ppm) and an isolated aromatic proton (6.64 ppm) in the ratio of 9:1. The ^{13}C NMR spectrum also showed three carbon resonances at 175.6, 176.2 and 181.0, corresponding to the one carbonyl carbon of L-cysteine and the two carbonyl carbons of NPQ, respectively. Spectral data obtained

for this adduct are thus consistent with a 1,4-Michael addition of the thiol nucleophile. The ^{1}H and ^{13}C NMR spectra corroborate the structure of the final adduct as *S*-(3,4-dihydro-3,4-dioxo-1-naphthyl)-L-cysteine (**2**). Additionally sufficient quantities of a purple adduct were obtained which permitted its characterization by LRMS and NH_4^+CI, MS. The MH^+ 278, and fragmentation pattern of the *p*-iminoquinone ring which results from a *retro*-Diels–Alder (*m*/*z*=159, 160) corroborates the structure as a *N*-(3-hydroxy-4-oxo-1-naphthyl)imino-L-cysteine (**3**).[18]

Encouraged by these findings, we conducted reactions of *N*-protected esters of L-aspartic acid, L-histidine and L-lysine with NPQ. Reaction of *N*-*t*-Boc-L-aspartic acid-α-benzyl ester with NPQ in CH_2Cl_2 gave one major adduct along with several minor products. The major adduct was purified by preparative TLC, which was isolated as a dark-red compound (**4**). This adduct was the least stable of those synthesized. It was characterized by ^{1}H NMR and mass spectrometry. This adduct gave an isolated aromatic proton, 2-C*H* (δ=6.62 ppm) indicative of a C1 substituted hydroquinone adduct. HRMS, corroborated the hydroquinone structure since the calculated mass was 2 amu higher than that expected for the quinone adduct. Based on the ^{1}H NMR and HRMS we assign the structure of the adduct as *O*-(3,4-dihydroxy-1-naphthyl)-*N*-*t*-Boc-L-aspartyl benzyl ester (**4**).

Reactions of *N*-α-acetyl-L-lysine methyl ester and *N*-α-benzyl-L-histidine methyl ester with NPQ did not go to completion. Approximately 20–25% of the NPQ was consumed under optimal conditions. In the case of the L-lysine derivative the final stable adduct was characterized by LC/MS and HRMS. LC/MS gave the correct MH^+=359, and HR-EIMS indicated that the elemental composition of the adduct (358.357) corresponded to that for ϵ*N*-(3,4-dihydro-3,4-dioxo-1-naphthyl)-*N*-α-acetyl-L-lysyl-methyl ester (**5**), i.e., there was no evidence for M^++2H or the catechol adduct. In the case of the L-histidine adduct, LC/MS gave the desired MH^+=416 and HR-EIMS gave 414 ($M^{+\cdot}$). Based on the structures of the other adducts it is likely that the histidine adduct corresponds to 1-*N*-(3,4-dihydro-3,4-dioxo-1-naphthyl)-*N*α-benzyl-L-histidyl methyl ester (**6**).

Formation of PAH *o*-quinones from *trans*-dihydrodiols (Fig. 1) is now considered a major potential pathway of PAH activation.[6,19] PAH *o*-quinones can alkylate macromolecules e.g., DNA and proteins by Michael addition. This study shows that the amino acid side-chains of L-cysteine, L-aspartate, L-lysine, and L-histidine react with NPQ to form the stable 1,4-Michael addition products. Evidence is provided that the *N*-acetyl-L-cysteine, L-cysteine, and L-lysine adducts are obtained at the level of the fully oxidized *o*-quinone. By contrast, the L-aspartyl-adduct was obtained as a hydroquinone. Similar adducts are expected with other PAH *o*-quinones formed by AKRs.

The synthesis of the *N*-acetyl-L-cysteine adduct of NPQ has been described previously.[20] The structure of the adduct was assigned to a catechol or hydroquinone, based on the presence of singlet at 6.62 ppm in the ^{1}H NMR, which is consistent with our low temperature NMR study. However, ^{13}C NMR was not performed to determine the number of carbonyl carbons in this adduct. Reactions of 2-substituted 1,4-naphthoquinones with glutathione were studied over 25 years ago, and evidence was obtained that the oxidized thio-substituted quinone is formed without prior accumulation of the thio-substituted hydroquinone under conditions of aeration.[21]

Amino acid adduction of *o*-quinones involves 1,4-Michael addition which proceeds via the formation of a ketol which rearranges to the catechol adduct. In these instances the catechol prefers to undergo air-oxidation to give the autoxidized quinone adduct. This autoxidation will lead to the consumption of molecular oxygen and the production of ROS e.g. H_2O_2, $HO^{\cdot}$, and superoxide anion. Local production of ROS in a protein environment is likely to cause further oxidative damage of the protein, for example the formation of cysteic acid and methionine sulfoxide may occur.[22] Thus, the formation of quinone-amino acid adducts may lead to secondary modification of the polypeptide chain. Because of this propensity to redox-cycle NPQ-protein adducts can be trapped by alkaline permethylation following treatment of Clara cells with naphthalene.[23]

The *N*- and *C*- protected amino acid adducts described in this study will only be useful as standards to monitor covalent modification of proteins by PAH *o*-quinones if they are deprotected. The cytotoxicity of PAH could depend to a large degree on their metabolic conversion to non-K region PAH *o*-quinone/semiquinone intermediates and their subsequent interaction with amino acid side chains in proteins. This may occur with NPQ which has been implicated as a causative agent in the development of naphthalene-induced cataract formation by its interactions with lens proteins.[24,25]

2. Experimental

2.1. General

Amino acids and their derivatives were purchased from Sigma Chemical Co. All reagents and solvents were commercially available unless otherwise indicated. NPQ was synthesized according to published procedures.[13] Reversed-phase HPLC was performed using a Beckman 125 pump and Beckman 166 variable-wavelength UV/vis detector set to 254 nm; gradient elution was achieved using a water: acetonitrile mixture. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-500 or Bruker AM-250 spectrometer. Samples were dissolved in CD_3OD, D_2O $CDCl_3$ or DMSO-d_6. ^{1}H NMR chemical shifts are expressed relative to TMS and dioxane was employed as an external standard for ^{13}C experiments. ESMS data were acquired on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with a Finnigan electrospray source. The mass spectrometer was operated in the positive ion mode with a potential of 4.25 kV applied to the electrospray needle. Nitrogen was used as the sheath (60 units) and auxiliary (5 units) gas to assist with nebulization. The capillary temperature was held at 200°C. Full scanning analyses were performed in the range of *m*/*z* 100 to *m*/*z* 800. Collision induced dissociation experiments coupled with multiple

tandem mass spectrometry employed helium as the collision gas. The relative collision was set at 20% of maximum (1 V). NH_4^+ CI MS were performed on a VG-2AB-E high resolution mass spectrometer. HRMS were performed in the Department of Chemistry, University of Pennsylvania.

Caution: The work described involves the synthesis and handling of hazardous agents and was therefore conducted in accordance with the NIH Guidelines for the Laboratory Use of Chemical Carcinogens.

2.1.1. Reaction of *N*-acetyl-L-cysteine with NPQ. *N*-acetyl-L-cysteine (0.75 mmol) was added to a bright yellow solution of NPQ (0.15 mmol) in 150 ml potassium phosphate buffer (50 mM, pH 7.0) containing 1% acetonitrile. The reaction turned orange immediately and was stirred for 1 h at 25°C in air. The progress of the reaction was monitored by RP-HPLC using a linear gradient of 5–95% acetonitrile containing 0.1% trifluoroacetic acid. This analysis indicated the presence of one major product and several minor products. The reaction mixture was lyophilized and subjected to Sephadex G10 column chromatography in water, which led to the isolation of one major product. The major product was orange in color and was obtained in 63% (30 mg, **1**). ^{1}H NMR (500 MHz, CD_3OD at 253°K): δ 2.0 (s, 3H, $-CH_3$), 3.40 (dd, 1H, Cys β), 3.70 (dd, 1H, Cys β), 4.67 (t, 1H, Cys α), 6.64 (s, 1H, 2C*H*), 7.65 (t, 1H, 6C*H*), 7.75 (t, 1H, 7C*H*), 7.94–8.12 (d, 2H, 8C*H* & 5C*H*). ^{13}C NMR (125 MHz, CD_3OD): δ 22.69 ($-CH_3$), 35.49 ($-CH_2$), 53.95 (–CH), 120.37 (–C2), 161.38 (–C1), 126.42, 129.75, 131.56, 132.35, 134.83, 136.31 (6C, 5-10), 173.30, 175.90 (2C, carbonyls of *N*-acetyl-L-cysteine) 177.55, 180.64 (2C, carbonyls of NPQ). Spectral data were consistent with *N*-acetyl-*S*-(3,4-dihydro-3,4-dioxo-1-naphthyl)-L-cysteine (**1**).

2.1.2. Reaction of L-cysteine with NPQ. L-cysteine (0.34 mmol) was added to a solution containing NPQ (0.065 mmol) in 70 ml potassium phosphate buffer (50 mM, pH 7.0) containing 1% acetonitrile. The reaction turned orange immediately and was stirred for 1 h at 25°C in air. The progress of the reaction was monitored by RP-HPLC using a linear gradient of 5–95% acetonitrile containing 0.1% trifluoroacetic acid. This analysis indicated the presence of one major adduct and several minor products. The reaction mixture was lyophilized and subjected to Sephadex G10 column chromatography in water and led to the isolation of an orange material (11.4 mg, 62.5%, **2**). ^{1}H NMR (500 MHz, DMSO-d_6 at 298 K): δ 3.40 (dd, 1H, Cys β), 3.70 (dd, 1H, Cys β), 4.67 (t, 1H, Cys α), 5.9 (b, 2H, $-NH_2$, D_2O exchangeable), 6.64 (s, 1H, 2C*H*), 7.2–8.7(4H, aromatic). 10.2 (s, 1H, –COO*H*, D_2O exchangeable). ^{13}C NMR (125 MHz, D_2O): δ 41.12 ($-CH_2$), 59.47 (–CH), 99.34 (–C2), 183.0 (–C1), 124.21, 129.28, 129,28, 130.65, 133.35, 136.1, (6C, C5-10) 175.6, 176.2, 181.0, (3 carbonyls). Spectral data were consistent with *S*-(3,4-dihydro-3,4-dioxo-1-naphthyl)-L-cysteine (**2**).

A purple product was also purified from the Sephadex G10 chromatography (1.6 mg, 8.8%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.59 (t, 1H, H-6), 7.77 (t, 1H, H-7), 8.02 (d, 1H, H-8), 8.13 (d, 1H H-5). There was no amino proton at δ 5.9. The diagnostic aliphatic protons were obscured by the DMSO and water peaks, and unfortunately the purple product was only soluble in DMSO. NH_4^+CI MS: (*m*/*z*) 278 (MH^+), 277 ($M^{+\cdot}$), 246, 245, (MH^+ & $M^{+\cdot}$ minus –SH), 232, 231, (MH^+ and $M^{+\cdot}$ minus $-CH_2SH$,) 202, 201, (MH^+ & $M^{+\cdot}$ minus –SH and $-CO_2$), 160, 159 (*m*/*z*=minus 202, 210 minus HC≡C–OH by *retro* Diels–Alder) and 134,133 (*m*/*z*=160, 159 minus HC≡CH). The ring fragmentation in NH_4^+CI MS is explained by *N*-attack to give a *p*-iminoquinone [*N*-(3-hydroxy-4-oxo-1-naphthyl)-imino-L-cysteine] (**3**).[18]

Reactions of the protected amino acids with NPQ were conducted in either dichloromethane or a water and THF mixture. This change in reaction conditions was used because reactions conducted in phosphate buffer gave complex mixtures and recently reactions of estrogen *o*-quinones with amino acid mimetics were successfully performed in water organic solvent mixtures.[26]

2.1.3. Reaction of *N*-*t*-boc-L-aspartic acid-α-benzyl ester with NPQ. The L-aspartic acid derivative (0.1 mmol) was added to a solution containing NPQ (0.1 mmol) in 15 ml of CH_2Cl_2 and the mixture was stirred at room temperature for 18 h. The methylene chloride solution was dried in vacuo, and showed the presence of one major product and several minor products on TLC as well as RP-HPLC. The major product was purified by preparative TLC (solvent system: ethyl-acetate), which yielded a dark-red compound (**4**), which was very unstable. ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.4 (s, 9H), 3.5–3.74 (m, 3H), 5.31 (s, 2H), 6.62 (s, 1H), 7.35–8.12 (m, aromatic protons). ESMS (*m*/*z*): 480.9 (M+1), 363, 292, 231, 230. HR-EIMS: calcd for $C_{26}H_{25}NO_8$: 479.1597, found: 481.2088.

2.1.4. Reaction of *N*-α-acetyl-L-lysine methyl ester with NPQ. The L-lysine derivative (0.1 mmol) was added to a solution containing NPQ (0.1 mmol) in 10 ml of THF/Water (1:1, v/v) and the mixture was stirred at room temperature for 18 h to yield (**5**). ESMS (*m*/*z*): 359 (M+1), 341, 331, 327. HR-EIMS: calcd for $C_{19}H_{22}N_2O_5$:358.1528, found: 358.3570.

2.1.5. Reaction of *N*-α-benzyl-L-histidine methyl ester with NPQ. The L-histidine derivative (0.1 mmol) was added to a solution containing NPQ (0.1 mmol) in 10 ml of THF/water (1:1, v/v) and the mixture was stirred at room temperature for 24 h to yield (**6**). ESMS (*m*/*z*): 416.3 (M+1), 381, 332. 272, 246. HR-EIMS: calcd for $C_{24}H_{21}N_3O_4$: 415.1532, found: 414.3809 ($M^{+\cdot}$).

Registry nos. *N*-acetyl-*S*-(3,4-dihydro-3,4-dioxo-1-naphthyl)-L-cysteine 132493-15-3; *S*-(3,4-dihydro-3,4-dioxo-1-naphthyl)-L-cysteine 132493-13-1.

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