Tripeptide Probes for Tripeptidyl Protease I Production via Gene Transfer

MeeKyoung Kim,[†] Qinwen Mao,[‡] Beverly L. Davidson,[‡,§,||] and David F. Wiemer*[,†]

Department of Chemistry, University of Iowa, Iowa City, Iowa 52242-1294 and Program in Gene Therapy, Departments of Internal Medicine, Neurology, Physiology & Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242-1294

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Tripeptides derived from 5-chloroanthraquinone hydrazide and anthraquinone hydrazide have been prepared as potential reagents to probe cellular expression of tripeptidyl protease I (TPP-I). Attempted chemical synthesis of Gly-L-Pro-L-Ala-chloroanthraquinone hydrazide, a compound that had been reported to serve as a substrate for this enzyme, was complicated by formation of a pyrazoloquinone. In contrast, formation of pyrazoloquinones was not observed during coupling reactions with anthraquinone hydrazide, and several tripeptide derivatives of this compound were prepared. The most attractive probe for TPP-I activity in tests with mouse kidney tissue sections proved to be Gly-L-Pro-L-Ser anthraquinone hydrazide.

Introduction

Lesions in the *CNL2* gene result in neuronal ceroid lipofuscinoses (NCL's), a group of rare but devastating neurodegenerative diseases.[1] One type of NCL, known variously as classical late-infantile neuronal ceroid lipofuscinosis (LINCL), Jansky−Bielschowsky disease, CLN2 deficiency, or pepinase deficiency, presents as an acute seizure disorder at 4−6 years of age and includes rapid loss of visual, motor, and cognitive functions.[2] Recent studies have shown that this disease is characterized by a deficiency in tripeptidyl protease I (TPP-I), a soluble lysosomal protease,[3] and that inactivating mutations result in accumulation of storage material in the lysosome.[4] Despite the increased understanding of the molecular basis of this disease, current therapy for LINCL can only relieve the seizure disorder and cannot address the underlying cause or the progression of the disease.[1,3] TPP-I is a nonmembrane bound lysosomal enzyme that can be secreted from cells overexpressing the enzyme and taken up by deficient cells.[5] This characteristic, together with the fact that only partial enzyme replacement will likely ameliorate the storage deficit, makes TPP-I an attractive candidate for the development of gene-based therapies.

A major limitation in the development of gene-based therapies for neurodegenerative diseases, and more specifically TPP-I deficiency, is an assay to assess the distribution of the secreted enzyme product. At present, CLN2 activity in the CNS is monitored by a fluorometric assay using Ala-Ala-Phe 7-amido-4-methlycoumarin in tissue lysates.[6] This assay allows evaluation of overall levels of enzyme in dissected regions of the brain but is somewhat insensitive in that it does not yield specific information on enzyme distribution within sections of brain.

One strategy for histochemical analysis of TPP-I activity in tissue sections was recently reported by Dikov et al.[7] These investigators reported preparation of tripeptides linked to various aromatic moieties through hydrazide linkages. For example, 5-chloroanthraquinone hydrazide (**1**) reportedly was converted to the alanine derivative **2** through DCC-mediated coupling with tBOC-L-alanine, and the product was converted to the tripeptide derivative **3** through a sequence of coupling and deprotection steps (Scheme 1). This tripeptide also was reported to serve as a TPP-I substrate, presumably yielding an intermediate hydrazine that was converted to a solid, colored hydrazone in tissue sections through reaction with an aromatic aldehyde. To assess the utility of new CLN2 gene transfer vectors, we investigated both similar and novel strategies for detection of TPP-I activity. In this paper, we report the preparation and characterization of several tripeptidyl anthraquinone derivatives and histochemical analyses of these compounds in mouse tissue sections known to harbor high levels of endogenous enzyme activity.

Chemical Synthesis. 5-Chloroanthraquinone hydrazide (**1**) was readily prepared from commercial 1,5-dichloroanthraquinone (**4**) through reaction with hydrazine as previously described (Scheme 2).[8] Coupling of this hydrazine with tBOC-L-Ala was first conducted through reaction with DCC, apparently as described by Dikov et al.,[7] but in our hands this procedure gave a coupled product only in low yield. The same material was obtained in much better yield through a coupling mediated with the DCC substitute 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl).[9] The product initially was assumed to be the amide **2** as previously described.[7] However, careful analysis of the NMR data for this series of compounds did not support assignment of the structures previously proposed for the coupled products. Of special significance is the ^{13}C NMR data. The symmetrical anthraquinone **4** shows a single resonance at 180.9 ppm that can be assigned to the quinone carbonyl carbons. In the hydrazine derivative **1** this symmetry is lost, and two quinone resonances are observed at 183.8 and 182.5

* To whom correspondence should be addressed. E-mail: davidwiemer@uiwoa.edu.
[†] Department of Chemistry, University of Iowa.
[‡] Department of Internal Medicine, University of Iowa College of Medicine.
[§] Department of Neurology, University of Iowa College of Medicine.
[||] Department of Physiology & Biophysics, University of Iowa College of Medicine.

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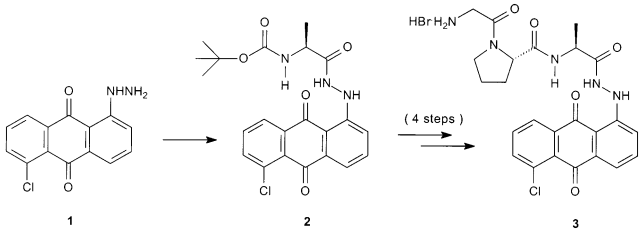
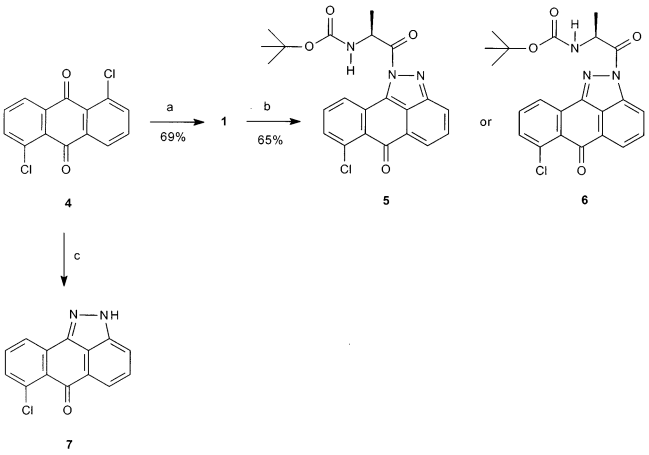
Scheme 1



Scheme 2[a]



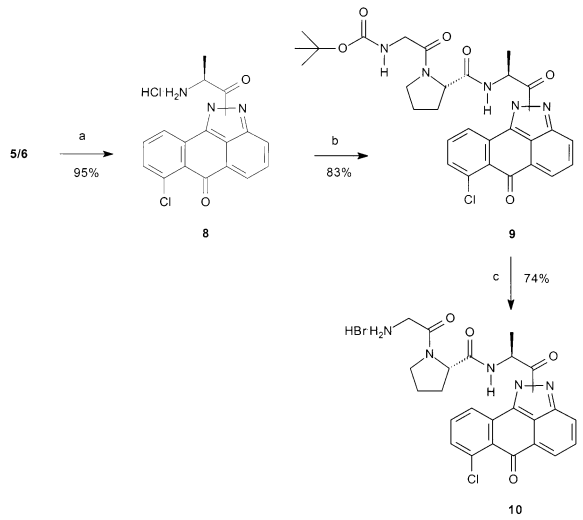
[a] Reagents and conditions: (a) $NH_2NH_2 \cdot H_2O$, Pyr, reflux; (b) N-tBOC-L-Ala, EDC·HCl, HOBt, NMM, THF; (c) $NH_2NH_2 \cdot H_2O$, iPr$_2$NEt, THF, reflux.

ppm. However, in the product of the DCC and EDC·HCl coupling reactions with tBOC-L-Ala, only one carbonyl resonance is apparent (181.1 ppm) and, because the expected total of 16 aromatic or carbonyl resonances is observed, it is not possible that an accidental overlap of two resonances has occurred. Instead, it appears probable that cyclization to a pyrazole derivative occurred under these conditions, as previously observed with alkyl-substituted hydrazines derived from related anthraquinones.[10] Further coupling with tBOC-L-Ala might afford an N-acylated derivative such as compound **5** or its regioisomeric N-acylated product **6**. To explore the possibility of pyrazole formation, quinone **4** was treated with hydrazine for an extended period, and the pyrazole **7**[10] was obtained. As might be expected,[11] only a single carbonyl resonance was observed in the ^{13}C spectrum of this product.

Because the previous report[7] contained no spectral data and only a very brief summary of the reactions employed, it is not possible to establish whether the reported biological evaluations were based on a tripeptide derived from a quinone or a pyrazole. In this study, the pyrazole derivative **5** (or **6**) was carried on to the corresponding tripeptide (Scheme 3). After hydrolysis of the tBOC group gave the free amine **8**, an EDC·HCl-mediated coupling with tBOC-Gly-L-Pro afforded the protected tripeptide **9** and final cleavage of the tBOC group gave the tripeptide **10**.

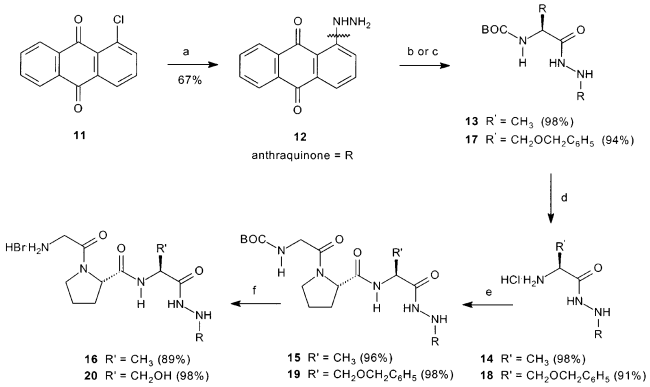
Additional tripeptides for these studies were derived from commercial 1-chloroanthraquinone **11** (Scheme 4). Treatment of compound **11** with hydrazine gave the expected hydrazine derivative **12**.[8] Treatment of compound **12** with EDC·HCl and tBOC-L-Ala clearly gave the protected peptide **13** rather than a pyrazole type

Scheme 3[a]



[a] Reagents and conditions: (a) 6 N HCl/dioxane; (b) N-tBOC-Gly-L-Pro, EDC·HCl, HOBt, NMM, THF; (c) 30% HBr/AcOH.

Scheme 4[a]

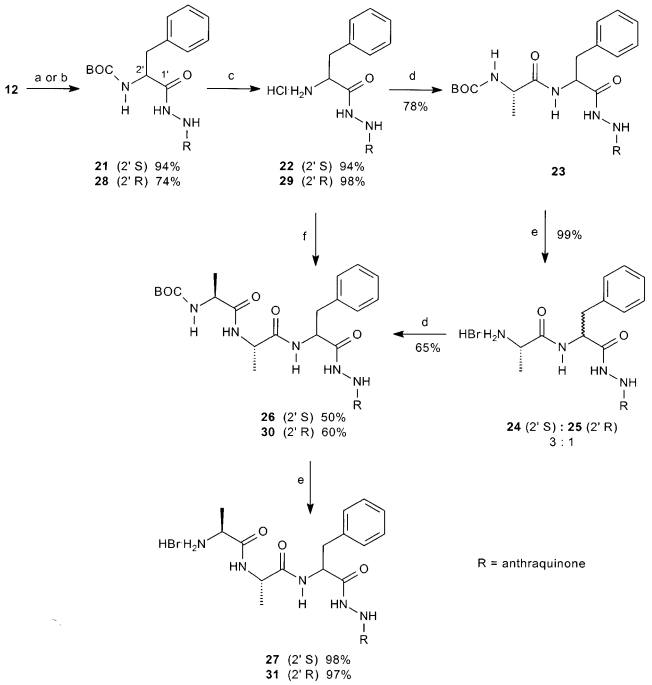


[a] Reagents and conditions: (a) $NH_2NH_2 \cdot H_2O$, pyr, reflux; (b) N-tBOC-L-Ala, EDC·HCl, HOBt, NMM, THF; (c) N-tBOC-O-benzyl-L-Ser, EDC·HCl, HOBt, NMM, THF; (d) 6 N HCl/dioxane; (e) N-tBOC-Gly-L-Pro, EDC·HCl, HOBt, NMM, THF; (f) 30% HBr/AcOH.

product, as evidenced by observation of two quinone carbons at ~180 ppm. Hydrolysis of the tBOC group gave the desired amine **14** in good yield. Standard EDC·HCl coupling with tBOC-Gly-L-Pro and subsequent reaction with HBr to cleave the tBOC group gave the desired tripeptide **16**. A third tripeptide was prepared through use of tBOC-*O*-Bz-L-Ser in the first coupling reaction to obtain the protected amino acid hydrazide **17**. A parallel reaction sequence involving hydrolysis of the tBOC group to afford the amino acid **18**, coupling with tBOC-Gly-L-Pro to obtain the protected tripeptide **19**, and final deprotection gave the tripeptide **20**.

A recent report indicated that L-Ala-L-Ala-L-Phe derivatives were particularly good substrates for TPP-I.[12] Therefore the hydrazine **12** was treated with tBOC-L-Phe to obtain the coupled product **21** (Scheme 5). After removal of the tBOC group gave the free amine **22**, coupling with tBOC-L-Ala gave the protected dipeptide **23**. In this case, deprotection was accomplished in good yield by reaction with HBr, but partial racemization occurred to give a 3:1 mixture of two diastereomers. The major diastereomer (**24**) was allowed to react with tBOC-L-Ala to obtain the protected tripeptide **26**, and final deprotection gave the desired target compound **27**.

Scheme 5[a]



[a] Reagents and conditions: (a) N-tBOC-L-Phe, EDC·HCl, HOBt, NMM, THF; (b) N-tBOC-D-Phe, EDC·HCl, HOBt, NMM, THF; (c) 6 N HCl/dioxane; (d) N-tBOC-L-Ala, EDC·HCl, HOBt, NMM, THF; (e) 30% HBr/AcOH (f) N-tBOC-L-Ala-L-Ala, EDC·HCl, HOBt, NMM, THF.

Table 1. Intensity and Color of the Final Reaction Product of TPP-I Histochemical Staining with Tripeptides

tripeptide	*p*-anisaldehyde	*p*-nitrobenzaldehyde	benzaldehyde
10	+++ yellow-green autofluorescent	+++ yellow-green autofluorescent	++ yellow-green autofluorescent
16	–	–	–
20	+++ dark purple	+++ brown	+ brown
27	+++ yellow-green	+++ yellow-green	++ yellow-green
31	–	–	–

Instead of conducting a parallel sequence with the minor diastereomer **25**, tBOC-D-Phe was coupled with hydrazine **12** to obtain the *R* isomer **28**. After deprotection to the free amine **29**, EDC·HCl-mediated coupling with tBOC-L-Ala-L-Ala gave the protected tripeptide **30**, and final cleavage of the tBOC group gave the tripeptide derivative **31**.

Biological Evaluation. Five different substrates, compounds **10**, **16**, **20**, **27**, and **31** were tested histochemically on frozen sections of murine tissues (see the Experimental Section for the preparation method). The sections were incubated in substrate solutions containing 1 mM of the substrate and 1 mg/mL of benzaldehyde (BA), *p*-anisaldehyde (*p*-AA), or *p*-nitrobenzaldehyde (*p*-NBA) in 0.1 M acetate buffer (pH 4.5) at 37 °C for 4 h. The substrates were systematically compared for sensitivity to TPP-I activity using conditions to control the rate of crystalline formation and diffusion of product, and the results are summarized in Table 1. Among the five substrates, compounds **10**, **20**, and **27** were readily hydrolyzed by endogenous TPP-I in murine tissues, revealing lysosomal accumulation of precipitates. There was no evidence of hydrolysis of tripeptides **16** and **31** by TPP-I. For compounds **10**, **20**, and **27**, the aldehydes *p*-NBA and *p*-AA were more effective than benzaldehyde itself as regards precipitate density and color. The combination of tripeptide **20** and *p*-AA was most sensitive, resulting in purple precipitates in areas of high endogenous enzyme activity (e.g., kidney; Figure 1). Together the data support that compounds **10**, **20**, and **27** were specific for TPP-I, with low to no background (sections incubated with tripeptides 16 and 31). This combination of reagents was used to assess CLN2 expression in murine brain following viral-mediated gene transfer. Recombinant protein was readily detectable in areas of low endogenous activity, demonstrating the utility of histochemical evaluation of CNS therapies for TPP-I deficiency (data not shown).

Conclusions

Five tripeptide derivatives of anthraquinone have been prepared and tested as histochemical reagents for detection of TPP-I activity. One, derived from 5-chloroanthraquinone hydrazide, was identified as a tetracyclic pyrazoanthrane based on analysis of ^{13}C NMR data, while the other four were tripeptide derivatives of the tricyclic anthraquinone hydrazide. Of these compounds, the tripeptide Gly-L-Pro-L-Ser anthraquinone hydrazide was the most effective histochemical reagent, particularly when combined with *p*-anisaldehyde.

Experimental Section

THF and diethyl ether were freshly distilled from sodium/benzophenone, while pyridine was freshly distilled from calcium hydride. All reactions in nonaqueous solvents were conducted in oven-dried or flame-dried glassware, under a positive pressure of argon, with magnetic stirring. Flash column chromatography was carried out on ICN silica gel with 40 μm average particle diameter. NMR spectra were recorded on either a Brucker AC-300 or a DPX-300 instrument (^1H at 300 MHz and ^{13}C at 75 MHz) or on a Brucker DPX-400 MHz instrument (^1H at 400 MHz and ^{13}C at 100 MHz). Spectra were obtained in $CDCl_3$ solution unless otherwise noted, with $(CH_3)_4$-Si (^1H, 0.0 ppm) or $CDCl_3$ (^{13}C, 77.0 ppm) as internal standards. Low resolution electron impact (EI) mass spectra were recorded with a Hewlett-Packard 6890 instrument operating at 70 eV; only selected ions are reported here. High-resolution mass spectra were obtained at the University of Iowa Mass Spectrometry Facility. Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). All reagents that are not otherwise described were obtained from commercial sources and used without further purification.

General Procedure for Preparation of Hydrazinoanthraquinones 1 and 12. To a stirred solution of the anthraquinone (**4** or **11**, 19.8 mmol) in pyridine (90 mL) was added hydrazine monohydrate (59.9 mmol) dropwise, and the reaction mixture was heated at reflux for 1−3 h. After the reaction mixture had cooled to room temperature, it was concentrated in vacuo. To obtain compound **12**, this residue was purified by column chromatography ($CHCl_3$). Compound **1** was purified by crystallization from xylene followed by column chromatography ($CHCl_3$).

General Procedure for Peptide Bond Formation with EDC·HCl. A solution of 1-hydroxybenzotriazole (365 mg, 2.7 mmol), an N-tBOC-protected amino acid (2.7 mmol), and EDC·HCl (537 mg, 2.8 mmol) in THF (11 mL) was cooled in an ice bath and stirred for 10 min. To this reaction mixture, hydrazinoanthraquinone (643 mg, 2.7 mmol) and *N*-methylmorpholine (NMM, 273 mg, 2.7 mmol) were added, and the resulting mixture was stirred for 1 h at 0 °C. After removal of the ice bath, the reaction mixture was allowed to stand at room temperature for 14−19 h, then washed with water and extracted with chloroform. After the combined organic layers were dried ($MgSO_4$ or Na_2SO_4), concentration in vacuo and

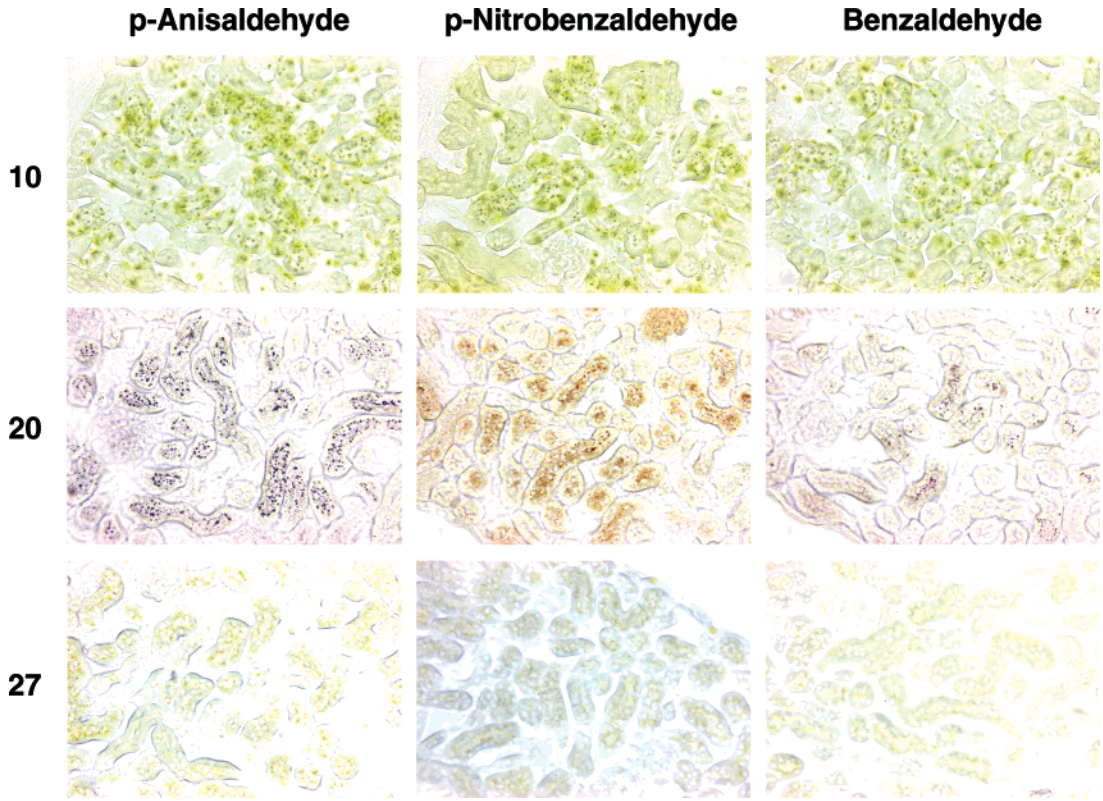


Figure 1. Murine kidney sections treated with a tripeptide conjugate and an aldehyde.

purification of the residue by flash column chromatography ($CHCl_3$ or $CHCl_3$:EtOAc:MeOH 47:50:3) gave the desired product.

General Procedure for Removal of N-tBOC Groups.[13] **a. Hydrochloric Acid in Dioxane.** The N-tBOC-aminoanthraquinonylhydrazide (1.2 mmol) was placed into a round-bottom flask and 6 N HCl in dioxane (5 mL) was added. The suspension was stirred for 1 h at room temperature. After removal of solvent by evaporation, the residue was crystallized from ethanol/ether. **b. Hydrobromic Acid in Acetic Acid.** The N-tBOC-tripeptidyl-anthraquinonylhydrazide (1.18 mmol) was dissolved in a 30% solution of HBr in acetic acid (3 mL) and stirred for 1 h at room temperature. After the reaction mixture was diluted with ether, the precipitate was collected by filtration, washed with ether, and finally purified by column chromatography ($CHCl_3$:EtOAc:MeOH, 45:50:5) or crystallization from ethanol/ether.

5-Chloro-1-anthraquinonylhydrazine (1). Purple solid; ^{1}H NMR δ 8.25 (dd, J = 7.7, 1.3 Hz, 1H), 7.99 (dd, J = 8.5, 1.3 Hz, 1H), 7.70−7.55 (m, 4H); ^{13}C NMR δ 183.8, 182.5, 150.1, 148.4, 137.4, 136.9, 135.3, 135.1, 134.9, 133.5, 129.5, 126.5, 119.5, 118.3; EIMS, *m*/*z* (relative intensity) 274 ($[M + 2]^+$, 3), 272 (M^+, 8), 257 (10), 242 (8), 229 (20), 187 (4), 173 (8), 159 (17), 145 (11), 132 (100), 119 (41), 105 (24), 95 (41), 81 (17), 69 (16), 55 (17).

N-tBOC-Ala-5-chloro-1-anthraquinonylhydrazide (2). HOBt (28 mg, 0.21 mmol), EDC·HCl (42 mg, 0.22 mmol), and N-tBOC-L-Ala (40 mg, 0.21 mmol) were suspended in THF (0.9 mL) and stirred for 15 min at 0 °C. 7-Chloropyrazoloanthrone (**7**, 54 mg, 0.2 mmol) and *N*-methylmorpholine (23 μL) were added, and the reaction mixture was stirred for 2 h at 0 °C. After removal of the ice bath, the reaction mixture was allowed to warm to room temperature and stirred for 14 h. The reaction mixture was diluted with water and extracted with chloroform. The combined organic layers were washed with brine and dried over $MgSO_4$. Final purification by column chromatography (silica gel, 9:1, chloroform/EtOAc) gave compound **2** (48 mg, 0.11 mmol, 51%) as a dark red solid and compound **5**/**6** (2.2 mg, 5.2 μmol, 2%) as a dark red solid: ^{1}H (DMSO) δ 10.35 (s, 1H, exchanges with D_2O), 10.28 (s, 1H, exchanges with D_2O), 8.25 (dd, J = 7.59, 1.69 Hz, 1H), 7.92 (dd, J = 8.1, 1.49 Hz, 1H), 7.87 (dd, J = 7 Hz, 86, 7.75, 1H), 7.67 (t, J = 8.11 Hz, 1H), 7.53 (d, J = 6.44 Hz, 1H), 7.36 (d, J = 8.53 Hz, 1H), 7.20 (d, J = 6.61 Hz, 1H, exchanges with D_2O), 4.12−4.02 (m, 1H), 1.42 (s, 9H), 1.29 (d, J = 7.23 Hz, 3H); ^{13}C NMR (DMSO) δ 183.2, 181.4, 172.6, 155.4, 151.4, 136.9, 136.9, 136.7, 135.7, 135.0, 134.5, 133.1, 128.7, 126.4, 118.1, 117.4, 112.5, 79.2, 48.8, 28.2 (3C), 17.4; HRMS (FAB) calcd for $C_{22}H_{23}N_3O_5Cl$ $(M + H)^+$ 444.1326, found 444.1315.

N-tBOC-Ala-5-chloro-1-anthra[1,9-*cd*]pyrozol-6(2*H*)-one (5 or isomer 6). A solution of 1-hydroxybenzotriazole (50 mg, 0.37 mmol), N-tBOC-L-Ala (69 mg, 0.37 mmol), and EDC·HCl (74 mg, 0.37 mmol) in THF (1.7 mL) was cooled in an ice bath and stirred for 10 min. To this reaction mixture were added compound **1** (100 mg, 0.37 mmol) and *N*-methylmorpholine (0.04 mL, 0.37 mmol), and the resulting mixture was stirred for 1 h at 0 °C. After removal of the ice bath, the reaction mixture was allowed to stand at room temperature for 14 h, then washed with water and extracted with chloroform. After the combined organic layers were dried ($MgSO_4$ or Na_2SO_4), concentration in vacuo and purification of the residue by flash column chromatography ($CHCl_3$) gave compound **5**/**6** as a dark red solid (101 mg, 65%): ^{1}H δ 8.39 (d, J = 8.1 Hz, 1H), 8.24 (dd, J = 6.6, 2.2 Hz, 1H), 8.04 (d, J = 7.3 Hz, 1H), 7.77 (t, J = 7.8 Hz, 1H), 7.64−7.57 (m, 2H), 5.76−5.66 (m, 1H), 5.44 (d, J = 8.1 Hz, 1H), 1.65 (d, J = 6.9 Hz, 3H), 1.49 (s, 9H); ^{13}C NMR δ 181.1, 173.6, 155.1, 144.1, 137.9, 137.3, 135.0, 133.2, 132.2, 131.6, 129.6, 126.4, 124.6, 123.6, 123.1, 120.7, 80.1, 49.2, 28.2 (3C), 19.2; HRMS (FAB) calcd for $C_{22}H_{21}N_3O_4Cl$ $(M + H)^+$ 426.1221, found 426.1226.

7-Chloropyrazoloanthrone (7). To a stirred suspension of 1,5-dichloroanthraquinone (343 mg, 1.2 mmol) and iPr$_2$NEt (240 mg, 1.9 mmol) in THF (5.5 mL) was added hydrazine monohydrate (186 mg, 3.7 mmol) dropwise, and the reaction mixture was heated at reflux for 2 h. After cooling, the mixture was washed with water and extracted with chloroform. The combined organic layers were washed with brine and dried over $MgSO_4$. Then the residue was separated by column chromatography (silica gel) with chloroform/EtOAc (1/1) as an eluent to give 7-chloropyrazoloanthrone (**7**) as a brown solid (57 mg, 18%); ^{1}H NMR (DMSO) δ 8.23 (dd, J = 7.11, 1.86 Hz, 1H), 7.88−7.82 (m, 2H), 7.78−7.62 (m, 2H), 7.34 (dd, J = 7.25,

0.69 Hz, 1H); ^{13}C NMR (DMSO) δ 181.7, 153.7, 137.1, 136.3, 135.5, 134.7, 134.3, 133.0 (2C), 128.6, 126.2, 118.2, 115.2, 110.0.

Ala-5-chloro-1-anthra[1,9-*cd*]pyrozol-6(2*H*)-one (8). Red solid; ^{1}H NMR (CD_3OD) δ 8.24 (d, J = 8.1 Hz, 1H), 8.04 (dd, J = 3.2, 6.0 Hz, 1H), 7.82 (d, J = 7.4 Hz, 1H), 7.70 (t, J = 8.0 Hz, 1H), 7.55−7.49 (m, 2H) 5.21 (q, J = 7.1 Hz, 1H), 1.80 (d, J = 7.1 Hz, 3H); ^{13}C NMR (CD_3OD) δ 181.9, 170.9, 146.3, 139.1, 138.2, 136.7, 134.9, 133.5, 132.9, 130.7, 127.7, 125.8, 124.8, 124.4, 121.4, 50.7, 17.3; HRMS (FAB) calcd for $C_{17}H_{13}N_3O_2Cl$ $(M - Cl)^+$ 326.0696, found 326.0721.

N-tBOC-Gly-L-Pro-L-Ala-5-chloro-1-anthra[1,9-*cd*]pyrozol-6(2*H*)-one (9). Yellow solid; ^{1}H NMR δ 8.42 (d, J = 8.1 Hz, 1H), 8.28 (dd, J = 7.1, 1.8 Hz, 1H), 8.08 (d, J = 7.3 Hz, 1H), 7.80 (t, J = 7.8 Hz, 1H), 7.67−7.59 (m, 2H), 5.87−5.82 (m, 1H), 5.52 (s, 1H), 4.70 (dd, J = 6.1, 1.6 Hz, 1H), 4.01 (dd, J = 5.0, 5.0 Hz, 2H), 3.64−3.57 (m, 1H), 3.50−3.41 (m, 1H), 2.42−1.91 (m, 4H), 1.67 (d, J = 7.0 Hz, 3H), 1.47 (s, 9H); ^{13}C NMR δ 181.3, 172.7, 170.6, 168.7, 155.8, 144.3, 138.0, 137.4, 135.1, 133.2, 132.3, 131.7, 129.8, 126.5, 124.7, 123.8, 123.2, 120.8, 79.8, 60.6, 48.5, 46.4, 43.1, 28.3 (3C), 27.6, 24.9, 18.5; HRMS (FAB) calcd for $C_{29}H_{31}N_5O_6Cl$ $(M + H)^+$ 580.1963, found 580.1949.

Gly-L-Pro-L-Ala-5-chloro-1-anthra[1,9-*cd*]pyrozol-6(2*H*)-one (10). Yellow solid; ^{1}H NMR (CD_3OD) δ 8.28 (d, J = 7.9 Hz, 1H), 8.19 (dd, J = 5.4, 1.6 Hz, 1H), 7.92 (d, J = 7.4 Hz, 1H), 7.74 (t, J = 7.6 Hz, 1H), 7.63−7.59 (m, 2H) 5.63 (q, J = 6.9 Hz, 1H), 4.51 (dd, J = 8.2, 3.7 Hz, 1H), 3.78 (s, 2H), 3.51−3.45 (m, 2H), 2.26−1.88 (m, 4H), 1.59 (d, J = 7.3 Hz, 3H); ^{13}C NMR ($(CD_3)_2SO$) δ 180.7, 172.7, 171.4, 164.3, 143.3, 137.6, 136.0, 135.1, 134.3, 132.3, 132.0, 129.3, 126.2, 124.1, 123.4, 123.2, 120.5, 59.0, 47.8, 47.7, 45.9, 29.4, 24.1, 16.8; HRMS (FAB) calcd for $C_{24}H_{23}N_5O_4Cl$ $(M - Cl)^+$ 480.1439, found 480.14.

1-Hydrazinoanthraquinone (12).[9] Dark brown solid; ^{1}H NMR δ 10.41 (s, 1H, exchanges with D_2O), 8.26−8.22 (m, 2H), 7.82−7.67 (m, 3H), 7.63−7.54 (m, 2H); ^{13}C NMR δ 184.9, 183.7, 154.3, 135.2, 134.7, 134.2, 133.9, 133.0, 133.0, 126.8 (2), 118.3 (2), 116.5.

N-tBOC-Ala-1-anthraquinonylhydrazide (13). Dark red solid; ^{1}H NMR δ 10.48 (s, 1H, exchanges with D_2O), 8.77 (s, 1H, exchanges with D_2O), 8.12−8.08 (m, 2H), 7.66−7.58 (m, 3H), 7.43 (t, J = 8.2 Hz, 1H), 7.17 (d, J = 8.4 Hz, 1H), 5.37 (d, J = 7.6 Hz, 1H), 4.22−4.38 (m, 1H), 1.49 (s, 9H), 1.46 (d, J = 7.2 Hz, 3H); ^{13}C NMR δ 185.1, 182.7, 172.7, 155.9, 150.9, 135.0, 134.1, 133.9, 133.7, 133.2, 132.5, 126.7, 126.5, 118.3, 117.6, 114.2, 80.5, 48.6, 28.3 (3C), 17.7; HRMS (FAB) calcd for $C_{22}H_{24}N_3O_5$ $(M + H)^+$ 410.1716, found 410.1731. Anal. Calcd for $C_{22}H_{23}O_5N_3$: C, 64.52; H, 5.67; N, 10.27. Found: C, 64.45; H, 5.71; N, 10.21.

Ala-1-anthraquinonylhydrazide (14). Red solid; ^{1}H NMR (CD_3OD) δ 8.25 (dd, J = 6.8, 2.0 Hz, 1H), 8.17 (dd, J = 7.4, 1.7 Hz, 1H), 7.87−7.65 (m, 4H), 7.36 (dd, J = 7.4, 1.2 Hz, 1H), 4.24−4.17 (m, 1H), 1.69 (d, J = 6.9 Hz, 3H); ^{13}C NMR (CD_3OD) δ 186.7, 184.4, 171.2, 152.6, 136.7, 135.8, 135.7, 135.5, 135.0, 134.2, 128.1, 127.8, 119.5, 119.3, 115.9, 64.4, 17.6; HRMS (FAB) calcd for $C_{17}H_{16}N_3O_3$ $(M - Cl)^+$ 310.1192, found 310.1206.

N-tBOC-Gly-L-Pro-L-Ala-1-anthraquinonylhydrazide (15). Dark red solid; ^{1}H NMR δ 10.51 (s, 1H, exchanges with D_2O), 9.06 (s, 1H, exchanges with D_2O), 8.23−8.17 (m, 2H), 7.75−7.66 (m, 3H), 7.55 (t, J = 8.0 Hz, 1H), 7.26 (d, J = 8.7 Hz, 1H), 7.20 (d, J = 7.3 Hz, 1H, exchanges with D_2O), 5.45 (s, 1H), 4.65−4.52 (m, 2H), 4.02 (dd, J = 16.6, 4.7 Hz, 1H), 3.78−3.71 (m, 2H), 3.55−3.47 (m, 1H), 2.22−1.98 (m, 4H), 1.50 (d, J = 7.3 Hz, 3H), 1.46 (s, 9H); ^{13}C NMR δ 185.3, 183.3, 171.7, 171.6, 169.8, 156.4, 151.6, 135.4, 134.4, 134.2, 133.9 (2C), 133.3, 132.8, 127.0, 126.7, 118.5, 118.4, 80.5, 61.2, 48.5, 47.1, 43.4, 28.8, 28.3 (3C), 24.9, 16.9; HRMS (FAB) calcd for $C_{29}H_{33}N_5O_7Na$ $(M + Na)^+$ 586.2278, found 586.2277.

Gly-L-Pro-L-Ala-1-anthraquinonylhydrazide (16). Dark red solid; ^{1}H NMR (CD_3OD) δ 8.31 (dd, J = 7.5, 1.5 Hz, 1H), 8.34 (dd, J = 7.0, 1.8 Hz, 1H), 7.90−7.80 (m, 2H), 7.75−7.64 (m, 2H), 7.43 (d, J = 8.1 Hz, 1H), 4.53−4.44 (m, 2H), 3.78−3.53 (m, 4H), 2.39−1.95 (m, 4H), 1.54 (d, J = 7.1 Hz, 3H); ^{13}C NMR (CD_3OD) δ 186.7, 184.6, 175.1, 175.1, 174.8, 153.1, 136.6, 135.8, 135.7, 135.6, 135.0, 134.3, 128.1, 127.8, 119.9, 119.3, 115.7, 67.1, 61.9, 60.0, 47.9, 30.9, 25.8, 17.6; HRMS (FAB) calcd for $C_{24}H_{26}N_5O_5$ $(M - Br)^+$ 464.1934, found 464.1950.

N-tBOC-*O*-Benzyl-L-Ser-1-anthraquinonylhydrazide (17). Dark red solid; ^{1}H NMR δ 10.54 (s, 1H, exchanges with D_2O), 8.31−8.21 (m, 3H), 7.78−7.71 (m, 3H), 7.42−7.30 (m, 5H), 7.23 (d, J = 8.5 Hz, 1H), 5.46 (d, J = 6.7 Hz, 1H), 4.64 (d, J = 11.7 Hz, 1H), 4.59 (d, J = 11.7 Hz, 1H), 4.51−4.48 (m, 1H), 4.00 (dd, J = 8.8, 3.6 Hz, 1H), 3.70 (dd, J = 9.0, 6.4 Hz, 1H), 1.49 (s, 9H); ^{13}C NMR δ 185.3, 182.9, 170.4, 155.5, 151.0, 137.1, 135.1, 134.1, 134.0, 133.8, 133.3, 132.6, 128.5 (2C), 128.0, 127.9 (2C), 126.8, 126.6, 118.4, 118.0, 114.4, 80.7, 73.7, 69.7, 53.4, 28.3 (3C); HRMS (FAB) calcd for $C_{29}H_{30}N_3O_6$ $(M + H)^+$ 516.2135, found 516.2129.

***O*-Benzyl-L-Ser-1-anthraquinonylhydrazide (18).** Red solid; ^{1}H NMR (CD_3OD) δ 8.18 (dd, J = 8.9, 3.3 Hz, 1H), 8.10 (dd, J = 7.3, 1.8 Hz, 1H), 7.88−7.67 (m, 3H), 7.60 (dd, J = 7.5, 1.1 Hz, 1H), 7.42−7.29 (m, 5H), 7.21 (dd, J = 8.5, 1.3 Hz, 1H), 4.67 (d, J = 11.9 Hz, 1H), 4.61 (d, J = 11.9 Hz, 1H), 4.30 (t, J = 4.4 Hz, 1 H), 4.01−3.93 (m, 2H); ^{13}C NMR (CD_3OD) δ 186.8, 184.4, 168.4, 152.5, 138.5, 136.6, 135.8, 135.7, 135.5, 135.0, 134.2, 129.8 (2C), 129.4 (3C), 128.1, 127.8, 119.5, 119.5, 115.9, 74.9, 69.1, 53.8; HRMS (FAB) calcd for $C_{24}H_{22}N_3O_4$ $(M - Cl)^+$ 416.1610, found 416.1607.

N-tBOC-Gly-L-Pro-*O*-benzyl-L-Ser-1-anthraquinonylhydrazide (19). Dark red solid; ^{1}H NMR δ 10.56 (s, exchanges with D_2O), 8.84 (s, exchanges with D_2O), 8.29−8.16 (m, 2H), 7.79−7.70 (m, 4H), 7.47−7.22 (m, 6H), 4.76−4.52 (m, 3H), 4.09 (dd, J = 9.4, 3.6 Hz, 1H), 4.00−3.90 (m, 2H), 3.72 (dd, J = 10.0, 4.9 Hz, 1H), 3.69−3.61 (m, 1H), 3.53−3.48 (m, 2H), 2.25−2.01 (m, 4H), 1.43 (s, 9H); ^{13}C NMR δ 186.8, 183.3, 171.1, 169.6, 169.2, 156.8, 151.4, 137.3, 135.4, 134.4, 134.3, 133.9, 133.4, 132.9, 128.6 (2C), 128.1, 127.7 (2C), 127.0, 126.8, 118.5, 118.3, 114.6, 80.1, 73.5, 68.9, 61.2, 52.8, 46.8, 43.2, 28.5, 28.2 (3C), 24.9; HRMS (FAB) calcd for $C_{36}H_{39}N_5O_8Na$ $(M + Na)^+$ 692.2696, found 692.2693.

Gly-L-Pro-L-Ser-1-anthraquinonylhydrazide (20). Dark red solid; ^{1}H NMR (CD_3OD) δ 8.32 (dd, J = 8.9, 3.3 Hz, 1H), 8.24 (dd, J = 7.3, 1.8 Hz, 1H), 7.88−7.81 (m, 2H), 7.76−768 (m, 2H), 7.53 (dd, J = 7.5, 1.1 Hz, 1H), 4.61−4.53 (m, 2H), 3.69−3.54 (m, 4H), 2.37−2.32 (m, 2H, 2.14−2.06 (m, 2H); ^{13}C NMR (CD_3OD) δ 186.8, 184.6, 167.7, 166.8, 166.4, 153.2, 136.6, 135.8, 135.8, 135.6, 135.0, 134.3, 128.1, 127.8, 120.1, 119.3, 113.4, 62.1, 60.7, 47.5, 41.6, 30.3, 26.1, 20.9; HRMS (FAB) calcd for $C_{24}H_{27}N_5O_6$ $(M - Br + H)^+$ 481.1961, found 481.1961.

N-tBOC-L-Phe-1-anthraquinonylhydrazide (21) and N-tBOC-D-Phe-1-anthraquinonylhydrazide (28). Golden orange solid; ^{1}H NMR δ 10.47 (s, 1H), 8.27−8.21 (m, 2H), 7.76−7.70 (m, 3H), 7.45−7.21 (m, 6H), 6.78 (d, J = 8.25 Hz, 1H), 5.16 (br, 1H), 4.53−4.44 (m, 1H), 3.14 (d, J = 7.31 Hz, 2H), 1.48 (s, 9H); ^{13}C NMR δ 185.6, 183.2, 171.0, 151.1, 136.2, 135.3, 134.4, 134.3, 134.0, 133.5, 132.9, 129.4 (3C), 129.0 (2C), 127.2, 127.0, 126.9, 118.7, 118.0, 114.6 81.9, 54.6, 37.8, 28.3 (3C); **21**, HRMS (FAB) calcd for $C_{28}H_{28}N_3O_5$ $(M + H)^+$ 486.2029, found 486.2036; **28**, HRMS (FAB) calcd for $C_{28}H_{28}N_3O_5$ $(M + H)^+$ 486.2029, found 486.2032.

L-Phe-1-anthraquinonylhydrazide (22) and *D*-Phe-1-anthraquinonylhydrazide (29). Dark orange solid; ^{1}H NMR (CD_3OD) δ 8.23 (dd, J = 7.2, 1.3 Hz, 1H), 8.15 (dd, J = 7.2, 1.9 Hz, 1H), 7.82−7.74 (m, 2H), 7.63 (dd, J = 7.4, 1.0 Hz, 1H), 7.45−7.31 (m, 6H), 6.53 (dd, J = 8.5, 1.0 Hz, 1H), ?3.98 (t, J = 7.2 Hz, 1H), 3.13 (d, J = 1.5 Hz, 1H), 3.11 (d, J = 2.9 Hz, 1H); ^{13}C NMR δ 186.7, 184.5, 173.7, 152.4, 137.6, 136.6, 135.8, 135.6, 135.5, 134.9, 134.3, 130.8 (2C), 130.2 (2C), 128.7, 128.1, 127.7, 119.6, 119.2, 115.6, 56.0, 41.1; **22**, HRMS (FAB) calcd for $C_{23}H_{20}N_3O_3$ $(M + H)^+$ 386.1505, found 386.1509; **29** HRMS (FAB) calcd for $C_{23}H_{20}N_3O_3$ $(M + H)^+$ 386.1505, found 386.1504.

N-tBOC-L-Ala-L-Phe-1-anthraquinonylhydrazide (23). Dark orange solid; ^{1}H NMR δ 10.40 (s, 1H), 8.13 (dd, J = 5.3, 5.3 Hz, 2H), 7.76−7.57 (m, 4H), 7.32−7.29 (m, 6H), 6.49 (d, J = 6.6 Hz, 1H), 5.71 (d, J = 7.6 Hz, 1H), 4.89−4.87 (m, 1H), 4.19−4.14 (m, 1H), 3.18 (br, 2H), 1.42 (s, 9H), 1.24 (d, J =

16.4 Hz, 3H); ^{13}C NMR δ 185.1, 183.1, 173.5, 170.8, 155.7, 150.8, 150.6, 136.0, 135.0, 134.0, 133.8, 133.2, 132.5, 129.3 (2C), 128.3 (2C), 127.0, 126.7, 126.6, 118.2, 117.9, 113.9, 80.3, 52.7, 50.1, 37.7, 28.1 (3C), 17.7; HRMS calcd for $C_{31}H_{33}N_4O_6$ $(M + H)^+$ 557.2400, found 557.2404.

L-Ala-L-Phe-1-anthraquinonylhydrazide (24). Dark orange solid; ^{1}H NMR (CD_3OD) δ 8.26−8.15 (m, 2H), 7.86−7.62 (m, 3H), 7.52−7.36 (m, 6H), 6.59 (d, J = 8.5 Hz, 1H), 4.39−4.33 (m, 1H), 4.24−4.21 (m, 1H), 3.29 (d, J = 8.0 Hz, 2H), 1.56 (dd, J = 10.0, 7.3 Hz, 3H); ^{13}C NMR (CD_3OD) δ 186.7, 184.4, 173.1, 169.8, 152.6, 152.6, 137.8, 136.7, 135.6, 135.5, 134.9, 134.2, 130.9 (2C), 130.4 (2C), 128.4, 128.1, 127.8, 119.5, 119.3, 115.7, 55.4, 54.7, 38.6, 17.8; HRMS (FAB) calcd for $C_{26}H_{25}N_4O_4$ (M^+) 457.1876, found 457.1887.

N-tBOC-L-Ala-L-Ala-L-Phe-1-anthraquinonylhydrazide (26). Orange solid; ^{1}H NMR δ 8.47 (d, J = 7.4 Hz, 1H), 8.45 (d, J = 8.0 Hz, 1H), 8.33 (d, J = 7.8 Hz, 1H), 8.17 (d, J = 7.4 Hz, 1H), 7.85−7.79 (m, 2H), 7.75−7.67 (m, 2H), 7.29−7.10 (m, 5H), 6.72−6.94 (m, 1H), 6.24−6.18 (m, 1H), 4.97−4.94 (m, 1H), 4.55−4.50 (m, 1H), 4.14−4.04 (m, 1H), 3.49 (dd, J = 14.2, 5.5 Hz, 1H), 3.29 (dd, J = 13.6, 7.2 Hz, 1H), 1.46 (s, 9H), 1.38 (d, J = 6.9 Hz, 3H), 1.34 (d, J = 7.2 Hz, 3H); ^{13}C NMR δ 184.4, 182.6, 172.5, 171.6, 171.2, 155.0, 144.6, 138.2, 135.6, 134.2, 133.6, 131.5, 131.0, 129.4, 129.3, 129.2 (2C), 128.5 (2C), 127.0, 124.2, 123.8, 121.0, 115.7, 80.5, 53.4, 48.9, 48.9, 38.7, 28.3 (3C), 18.0, 17.9; HRMS calcd for $C_{34}H_{38}N_5O_7$ $(M + H)^+$ 628.2771, found 628.2774.

L-Ala-L-Ala-L-Phe-1-anthraquinonylhydrazide (27). Dark orange solid; ^{1}H NMR (CD_3OD) δ 8.31 (dd, J = 6.7, 1.7 Hz, 1H), 8.23 (dd, J = 7.0, 1.3 Hz, 1H), 7.88 (dt, J = 7.4, 1.7 Hz, 2H), 7.70 (dd, J = 7.5, 0.9 Hz, 1H), 7.52 (dd, J = 8.4, 7.7 Hz, 1H), 7.40−7.22 (m, 5H), 6.78 (dd, J = 8.6, 0.8 Hz, 1H), 4.72 (t, J = 8.0 Hz, 1H), 4.39 (dd, J = 7.1, 2.6 Hz, 1H), 3.58 (dd, J = 7.4, 7.4 Hz, 1H), 3.18 (d, J = 12.7 Hz, 2H), 1.51 (d, J = 7.1 Hz, 3H), 1.39 (d, J = 7.2 Hz, 3H); ^{13}C NMR (CD_3OD) δ 186.7, 184.5, 174.7, 173.3, 170.9, 152.7, 138.0, 136.6, 135.8, 135.6, 135.5, 135.0, 134.3, 130.7 (2C), 130.0 (2C), 128.3, 128.1, 127.8, 119.8, 119.2, 115.4, 55.5, 55.4, 55.2, 38.8, 18.3, 17.8; HRMS (FAB) calcd for $C_{29}H_{30}N_5O_5$ $(M - Br)^+$ 528.2247, found 528.2253.

N-tBOC-L-Ala-L-Ala-*D*-Phe-1-anthraquinonylhydrazide (30). Red-orange solid; ^{1}H NMR δ 8.44 (d, J = 7.0 Hz, 1H), 8.39 (d, J = 8.0 Hz, 1H), 8.29 (d, J = 7.1 Hz, 1H), 8.13 (d, J = 7.0 Hz, 1H), 7.81−7.75 (m, 2H), 7.71−7.60 (m, 2H), 7.32−7.16 (m, 5H), 6.75−6.72 (m, 1H), 6.19−6.16 (m, 1H), 5.07−5.04 (m, 1H), 4.61−4.55 (m, 1H), 4.18−4.13 (m, 1H), 3.50 (dd, J = 13.3, 5.4 Hz, 1H), 3.24 (dd, J = 10.1, 7.7 Hz, 1H), 1.44 (s, 9H), 1.36 (d, J = 6.0 Hz, 3H), 1.30 (d, J = 6.9 Hz, 3H); ^{13}C NMR δ 184.2, 182.5, 172.6, 171.8, 171.3, 155.6, 145.0, 138.1, 135.9, 134.2, 133.6, 131.4, 130.9, 129.5, 129.3 (2C), 128.7, 128.5 (2C), 127.2, 124.1, 123.7, 120.9, 118.3, 80.4, 53.5, 50.9, 48.8, 38.6, 28.3 (3C), 18.3, 18.0.

L-Ala-L-Ala-D-Phe-1-anthraquinonylhydrazide (31). Dark red solid; ^{1}H NMR (400 MHz, CD_3OD) δ 8.31 (dd, J = 7.7, 1.5 Hz, 1H), 8.23 (dd, J = 7.0, 1.6 Hz, 1H), 7.86 (dd, J = 7.4, 1.9 Hz, 1H), 7.84 (dd, J = 7.5, 1.6 Hz, 1H), 7.71 (dd, J = 7.2, 0.9 Hz, 1H), 7.58 (dd, J = 8.7, 7.4 Hz, 1H), 7.39−7.20 (m, 5H), 6.99 (dd, J = 8.3, 0.8 Hz, 1H), 4.71 (dd, J = 9.2, 4.0 Hz, 1H), 4.37 (q, J = 7.2 Hz, 1H), 3.53 (q, J = 7.2 Hz, 1H), 3.21 (dd, J = 13.6, 5.2 Hz, 1H), 2.98 (dd, J = 14.0, 9.2 Hz, 1H), 1.29 (d, J = 7.2 Hz, 3H), 1.18 (d, J = 6.8 Hz, 3H); ^{13}C NMR (CD_3OD) δ 186.7, 184.5, 174.6, 172.9, 170.7, 155.6, 138.6, 136.7, 135.5, 135.1 (2C), 135.0, 130.6 (2C), 129.6 (2C), 128.2, 128.1, 128.0, 119.8, 119.2, 118.0, 55.6, 55.2, 55.0, 39.4, 18.6, 17.8; HRMS (FAB) calcd for $C_{29}H_{30}N_5O_5$ $(M - Br)^+$ 528.2247, found 528.2242.

Tissue Preparation and Incubation with Substrate. C57BL/6J mice (6 to 8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). All procedures were approved by the Animal Care and Use Review Committee of University of Iowa. The mice were sacrificed with an overdose of anesthetic and then transcardially perfused with 0.9% (w/v) saline solution, followed by 2% paraformaldehyde in 0.2 M phosphate buffer. The tissues were removed and postfixed for 2 h. After fixation, the tissues were cryoprotected by immersion in 25% sucrose and frozen in OCT compound (Sakura Finetek U.S.A., Inc, Torrance, CA). Sections (10 μm) were cut with a cryostat and mounted onto gelatin-coated slides. Sections were embedded in 0.5% celloidin, followed by incubation in substrate solutions containing 1 mM of the substrate, 1 mg/mL of benzaldehyde, *p*-anisaldehyde, or *p*-nitrobenzaldehyde in 0.1 M acetate buffer (pH 4.5) at 37 °C for 4 h. Slides were rinsed and cover-slipped before viewing on a Leica DM RBE microscope equipped with a Spot-RT Digital Camera and associated software. For all experiments, tissue staining was repeated on a minimum of 5 sections/animal per compound. A minimum of three individual animals was tested to confirm consistency of the results.

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