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TRIFUNCTIONAL REAGENTS FOR SUBSTRATE-PROTEIN CONJUGATION: APPLICATION TO PYRROLIZIDINE ALKALOID ANALOGUES.

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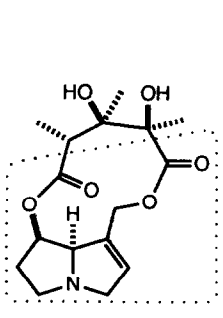
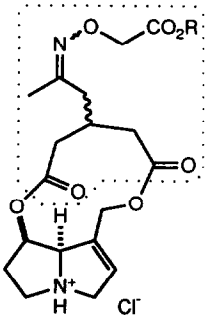
Abstract: *We report the syntheses of two pyrrolizidine alkaloid (PA) analogues (**1** and **2**) which exploit the novel substrate-protein coupling reagents **4** and **5**. Analogues **1** and **2** incorporate the targeted PA substructural unit (i.e., a macrocyclic diester of retronecine), possess a handle for protein conjugation, and potentially maintain the conformational integrity of macrocyclic PAs.*

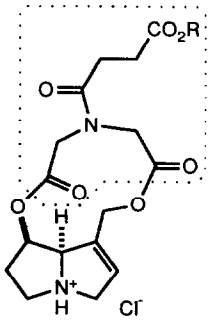
Macrocyclic pyrrolizidine alkaloids (PAs) are widespread plant toxicants which express hepatotoxic,[2] carcinogenic,[3] mutagenic,[4] and teratogenic[5] physiological properties. Indeed, it is estimated that 6000 plant species (≈3% of all flowering plants) produce PAs[6] and to date over 200 individual PAs have been isolated.[7] Given their world-wide distribution together with the chronic nature of PA-induced diseases, the health[8] and economic[9] implications of PA-contamination in products for human consumption are enormous, yet recognition of PA poisoning is limited to pathological diagnostics.[6] As the first step in a project aimed at developing a sensitive bioassay for PAs, we required PA bioconjugates which present the common PA substructural unit indicated by the highlighted fragment in monocrotaline. This objective led us to develop a trifunctional substrate-protein coupling strategy in which the tether delivers the macrocyclic diester moiety, supplies functionality for substrate-protein conjugation, and potentially maintains the conformational integrity of macrocyclic PAs.



monocrotaline

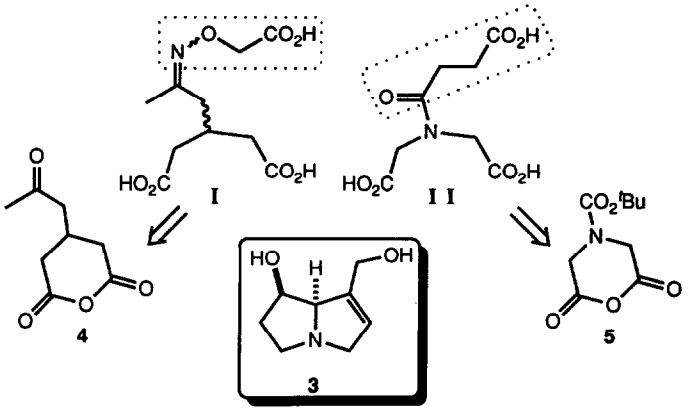


1; R = -H
16; R = -N[C(=O)CH$_2$]



2; R = -H
17; R = -N[C(=O)CH$_2$]

The highlighted fragments in **1** and **2** afford these two critical features and thus focused our efforts on the preparation of functional analogues of tri-acids **I** and **II**; analogues in which the third carboxylic acid moiety (i.e., highlighted substructure of tri-acids **I** and **II**) could be introduced in the final step of the synthesis and thus eliminate chemoselectivity issues in formation of the macrocyclic diester. Herein we report the synthesis of substrates **1** and **2**.

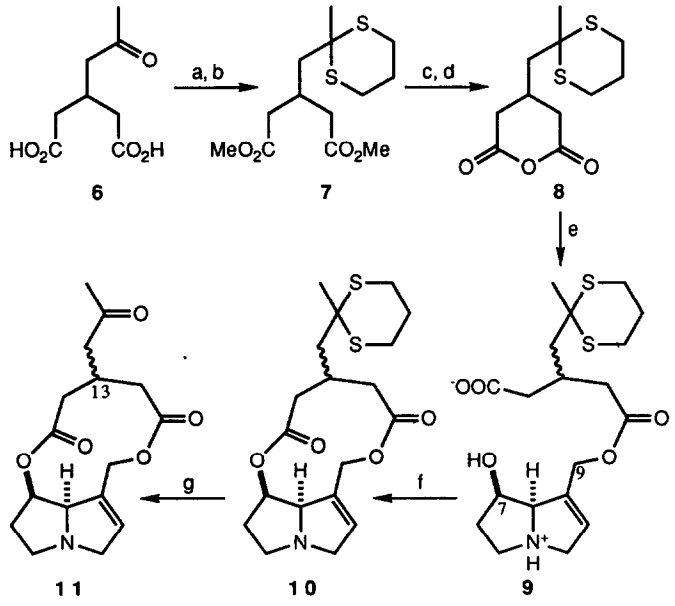


The requisite necine base component of **1** and **2**, (+)-retronecine (**3**), was obtained by base-promoted hydrolysis[10] of commercially available monocrotaline.[11] Continuous extraction of the aqueous hydrolysate over a 3 day period followed by recrystallization from acetone delivered (+)-retronecine in 72% yield. With this necine base in hand, we turned our attention to the preparation of trifunctional coupling reagents **4** and **5**. Unfortunately, anhydride formation from ketodiacid **6**(→ **4**) in refluxing acetyl chloride was accompanied by appreciable formation of 1-methyl-2,8-dioxabicyclo[3.3.1]nonane-3,7-dione.[12] Consequently, a carbonyl protection strategy was employed consisting of esterification of the diacid with diazomethane followed by dithiane protection of the keto group. This diester (**7**) was then converted to anhydride **8**, our first key trifunctional coupling reagent, in 58% overall yield by a two step sequence featuring basic ester hydrolysis and subsequent dehydrative cyclization of the dicarboxylate in refluxing acetic anhydride.

Macrocyclic diester **11**, the penultimate precursor to PA analogue **1**, was synthesized using the Corey-Nicolaou double activation method[13] for the crucial macrolactonization step as outlined in **Scheme I**. Surprisingly, a dimethoxyethane solution of anhydride **8** reacted selectively with (+)-retronecine to give the C-9 ester in quantitative yield. This zwitterionic condensation product precipitated as formed giving a clear gum, and thus avoided formation of the bis-acylation product. Macrolactonization was effected by treating this zwitterion/dimethoxyethane mixture with triphenylphosphine and 2,2'-dipyridyl disulfide; the precipitated zwitterion slowly dissolves as the reaction proceeds. Slow (3 h) admixture of this 2-pyridinethiol ester solution to refluxing dimethoxyethane followed by extractive isolation of the crude product and purification using medium-pressure liquid chromatography on silica gel (95:5 chloroform:methanol) gave macrocyclic diester **10** as a 2:1 mixture of C-13 epimers in 60% overall yield from (+)-retronecine. Finally, deketalization with mercuric chloride

followed by decomposition of the mercury-amine complex with aqueous ammonia delivered ketomacrolide **11**, again as a 2:1 mixture of C-13 epimers.

Scheme I



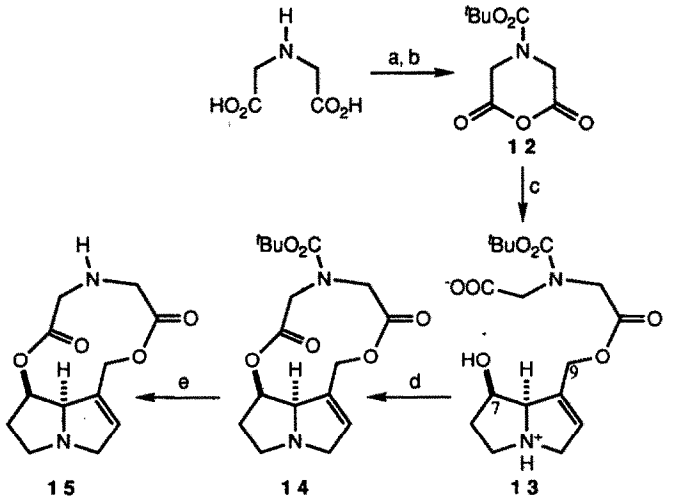
[a] CH_2N_2, Et_2O, -10°C, 5 min (92%). [b] $HS(CH_2)_3SH$ (1 eq), CH_2Cl_2, rt, 1 h; BF_3•Et_2O (0.2 eq), -15°C; -15°C to rt, 16 h (75%). [c] NaOH (2.2 eq), H_2O/MeOH (2:1) 70°C, 3h. [d] Ac_2O, reflux, 2 h (73%). [e] (+)-retronecine (0.9 eq), DME, rt, 24 h (100% by ^{1}H-NMR). [f] Ph_3P (1.2 eq), 2,2'-dipyridyl disulfide (1.2 eq), rt, 16 h; DME, reflux, 24 h (60%). [g] $HgCl_2$ (2.2 eq), CH_3CN/H_2O (4:1), rt, 16 h (66%).

Numerous RCOOH-based protein conjugation protocols are available[14] and, in order to take advantage of these various possibilities in the protein conjugation step, the keto moiety of macrolide **11** was converted to its carboxymethyloxime derivative. Thus, treatment of a pyridine solution of **11** with carboxymethoxylamine hemihydrochloride followed by acidification (1 M HCl) gave **1** in 76% overall yield from **11**. While PA analogue **1** affords many of the common structural features of macrocyclic PAs, it was judged to be not entirely satisfactory since it is a complex mixture of four diastereomers because of C-13 and C=N configurational isomers.

Therefore, in order to avoid the stereochemical issues associated with **1**, PA analogue **2** was synthesized as outlined in **Scheme II**. Anhydride **12** was prepared *in situ* from iminodiacetic acid by *t*-butoxycarbonyl protection of the amine function followed by dehydration of the diacid with dicyclohexylcarbodiimide. The resulting dimethoxyethane solution of **12** was separated from precipitated dicyclohexylurea by anhydrous Celite filtration and then added via cannula to (+)-retronecine in dimethoxyethane to give a quantitative yield of ester **13**.

In parallel to **9**, this zwitterionic condensation product was the result of exclusive C-9 acylation and precipitated as formed. Subsequent cyclization via the corresponding 2-pyridinethiol ester gave N-protected macrolide **14** in 57% overall yield from (+)-retronecine.

Scheme II



[a] *t*-$BuOCO_2N{=}C(C_6H_5)CN$ (1.1 eq), dioxane/H_2O (1:1), Et_3N (3eq), 60°C, 1 h (60%). [b] DCC (1 eq), DME, rt, 2 h (100% by ^{1}H-NMR). [c] (+)-retronecine (0.9 eq), DME, rt, 24 h (100% by ^{1}H-NMR). [d] Ph_3P (1.2 eq), 2,2'-dipyridyl disulfide (1.2 eq), rt, 24 h; DME, reflux, 24 h (57%). [e] 3N HCL, rt, 1 h (65%).

Removal of the ^{t}BOC protecting group in **14** with 3M hydrocholoric acid at room temperature followed by neutralization with aqueous ammonium hydroxide gave diamine **15**. The third carboxylic acid moiety was then introduced by acylation of the 2°-amine of **15** with succinic anhydride. Hydrochloric acid (1M) work-up gave PA analogue **2** in 77% yield from **15**.

The free carboxylic acid groups of **1** and **2** provide appropriate functionality for protein conjugation. For example, *N*-hydroxysuccinimide activation[15] would deliver labile NHS-esters **16** and **17**, respectively. These labile esters would then be used without further purification in the 1°-amine acylation (i.e., lysine residues) of appropriate proteins.[16]

Experimental

Melting points were determined on a Kofler hot stage and are uncorrected. Elemental analyses were performed at the Microanalytical Laboratory Facility, University of California, Berkeley. High resolution mass spectra were determined by Mr. K. Miyano and Dr. D. Jones with a Dupont 21-492B analytical instrument at the Facility for Advanced Instrumentation, University of California, Davis. Magnetic resonance spectra were

obtained with a General Electric QE-300 (300 MHz) spectrometer. Peak locations are expressed in ppm (δ units) relative to center of residual proton peak(s) from a deuterated NMR solvent. Listed NMR data are given in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; c, complex), coupling constant(s), and number of protons (by integration). Infrared spectra were recorded with an IBM FTIR-32 with IBM 9000 data system. Medium pressure liquid chromatography (MPLC) refers to chromatographic purification performed at 20-40 psi through EM Lobar™ columns packed with LiChroprep Si 60 (40-60 μm). Ethyl acetate/n-hexane or methanol/chloroform was employed as the eluent and separation was monitored with a Waters differential refractometer (Model R403). Analytical thin layer chromatography (TLC) was performed with Kodak (13181) 100 μm thick silica gel sheets. Iodine was used to visualize nonchromophoric bands.

Diethyl ether, tetrahydrofuran (THF), and dimethoxyethane (DME) were distilled under nitrogen atmosphere from sodium/potassium benzophenone ketyl. Dichloromethane was distilled under nitrogen atmosphere from phosphorus pentoxide. Acetonitrile was distilled under nitrogen atmosphere from calcium hydride and stored over 4 Å molecular sieves. Pyridine was distilled under nitrogen atmosphere and stored over 4 Å molecular sieves. Chloroform was washed with water to remove ethanol, dried over $MgSO_4$, and distilled under nitrogen atmosphere immediately prior to use. Concentration under reduced pressure refers to solvent removal with a Buchi rotary evaporator at 30-40° C.

Dimethyl 3-(2-oxopropyl)pentanedioate. To a 50 mL Erlenmeyer flask containing diacid **6**[17] (301 mg, 1.60 mmol) was added an ethereal solution (40 mL) of diazomethane (0.42 g, 10 mmol) at -10°C. The solution was periodically swirled at room temperature during a 5 min period and then the excess diazomethane was destroyed by the dropwise addition of acetic acid. The resulting ethereal solution was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Purification by MPLC (1:4 ethyl acetate:*n*-hexane, 2.25 mL/min) gave dimethyl 3-(2-oxopropyl)pentanedioate (320 mg, 1.48 mmol, 92%) as a colorless oil [R_f (1:4 ethyl acetate:*n*-hexane) = 0.14; IR (neat) 3001, 2956, 2850, 1737 (C=O), 1437, 1375, 1263, 1215, 1158, 1013 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 2.12 (s, 3H, C(=O)CH_3), 2.42 (m, 4H, 2 $C\underline{H}_2CO_2$), 2.59 (d, J = 6.5 Hz, 2H, $C\underline{H}_2COCH_3$), 2.78 (m, 1H, $CH_2C\underline{H}CH_2$), 3.65 (s, 6H, 2 $OC\underline{H}_3$); ^{13}C NMR ($CDCl_3$) δ 27.5 ($CH_2\underline{C}HCH_2$), 30.2 ($CO\underline{C}H_3$), 37.5 ($\underline{C}H_2CO_2$), 46.5 ($\underline{C}H_2COCH_3$), 51.5 (OCH_3), 172.4 ($\underline{C}O_2CH_3$), 207.0 ($\underline{C}OCH_3$); HRMS (EI) calcd for $C_{10}H_{16}O_5$ 216.0998 (M), found 216.1005].

Dimethyl 3-[(1,3-dithian-2-methyl-2-yl)methyl]pentanedioate (7). To a stirred solution of dimethyl 3-(2-oxopropyl)pentanedioate (320 mg, 1.48 mmol) in dichloromethane (3.0 mL) under nitrogen was added 1,3-propanedithiol (160 mg, 148 μL, 1.48 mmol). After stirring at room temperature for 1 h, the solution was cooled to -15°C in an ice/water/salt bath. Boron trifluoride etherate (42 mg, 36 μL, 0.30 mmol) was added to the reaction mixture and stirring was continued at room temperature for 16 h at which time the mixture was diluted with chloroform (15 mL) and the organic solution was washed with 10% aqueous Na_2CO_3 (2 x 5 mL) and brine (2 x 5 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Purification by MPLC (1:4 ethyl acetate:*n*-hexane, 2.25 mL/min) gave **7** (340 mg, 1.11 mmol, 75 %) as a colorless oil [R_f (1:4 ethyl acetate:*n*-hexane) = 0.26; IR (neat) 2952, 2846, 1737 (C=O), 1437, 1374, 1251, 1195, 1170, 1079, 1021, 1001 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.64 (s, 3H, CCH_3), 1.94 (m, 2H, $SCH_2C\underline{H}_2$), 2.06 (d, J = 4.1 Hz, 2H, $C\underline{H}_2CSS$),

2.50-2.62 (m, 5H, 2 $C\underline{H}_2CO_2/CH_2C\underline{H}CH_2$), 2.83 (m, 4H, 2 SCH_2), 3.66 (s, 6H, 2 OCH_3); ^{13}C NMR ($CDCl_3$) δ 24.8 ($SCH_2\underline{C}H_2$), 26.6 (SCH_2), 28.2 ($C\underline{C}H_3$), 29.0 ($CH_2\underline{C}HCH_2$), 39.2 ($\underline{C}H_2CO_2$), 44.4 ($\underline{C}H_2CSS$), 48.6 (SCS), 51.4 (OCH_3), 172.5 (C=O); HRMS (EI) calcd for $C_{13}H_{22}O_4S_2$ 306.0959 (M), found 306.0967. Anal. calcd for $C_{13}H_{22}O_4S_2$: C, 50.95; H, 7.24; S, 20.92. Found: C, 51.26; H, 7.27; S, 20.90.].

Dihydro-4-[(1,3-dithian-2-methyl-2-yl)methyl]-2H-pyran-2,6(3H)-dione (8). Diester **7** (410 mg, 1.34 mmol), methanol (0.50 mL), water (1.0 mL), and NaOH (118 mg, 2.95 mmol) were combined in a 10 mL round-bottom flask equipped with a reflux condenser and magnetic stirrer. The mixture was heated at 70°C for 3 h and then cooled to room temperature. The water was removed with a rotary evaporator at 45°C and the resulting white solid was further dried under high vacuum (1 torr, 12 h) over P_2O_5. The flask was equipped with a reflux condenser and acetic anhydride (4.3 g, 4.0 mL, 42 mmol) was added to the disodium salt under nitrogen and the mixture was heated at reflux for 2 h. After cooling to room temperature, the acetic anhydride was removed under high vacuum (1 torr, 4 h) and the residue was diluted with chloroform (15 mL). The suspended solids were removed by suction filtration through Celite and the organic solution was concentrated under reduced pressure to give a slightly yellow solid. Recrystallization from ethyl acetate/*n*-hexane gave **8** (254 mg, 0.976 mmol, 73%) as colorless prisms [mp 82-83°C; IR (KBr) 2965, 2909, 2830, 1811 and 1765 (C=O), 1422, 1375, 1245, 1180, 1076 (C-O), 1024, 954 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.67 (s, 3H, CH_3), 1.93-1.98 (m, 4H, $C\underline{H}_2CSS/SCH_2C\underline{H}_2$), 2.54 (m, 3H, 2 $C\underline{H}HCO_2/CH_2C\underline{H}CH_2$), 2.84 (m, 4H, 2 SCH_2), 3.04 (m, 2H, 2 $CH\underline{H}CO_2$); ^{13}C NMR ($CDCl_3$) ∂ 24.7 ($SCH_2\underline{C}H_2$), 26.0 ($CH_2\underline{C}HCH_2$), 26.7 (SCH_2), 29.1 (CH_3), 37.7 ($\underline{C}H_2CO_2$), 46.3 ($\underline{C}H_2CSS$), 48.2 (SCS), 165.9 (C=O); HRMS (EI) calcd for $C_{11}H_{16}O_3S_2$ 260.0541 (M), found 260.0540. Anal. calcd for $C_{11}H_{16}O_3S_2$: C, 50.74; H, 6.19; S, 24.63. Found: C, 50.66; H, 6.38; S, 24.28.].

18-(1,3-Dithian-2-methyl-2-yl)-17,19,20-trinorcrotalanan-11,15-dione (10). Anhydride **8** (140 mg, 0.538 mmol) was added to a stirred solution of (+)-retronecine (**3**, 80 mg, 0.512 mmol) in dry DME (15 mL) under argon. After 24 h at room temperature, triphenylphosphine (161 mg, 0.615 mmol) and 2,2'-dipyridyl disulfide (135 mg, 0.617 mmol) were added and the mixture was stirred for 16 h. The resulting clear yellow solution was then added during 5 h by syringe to dry DME (80 mL) heated at reflux under argon. After the addition was complete, the solution was heated for an additional 24 h at which time the reaction mixture was cooled to room temperature and concentrated under reduced pressure to afford a yellow gum. Purification by MPLC (3.5% v/v methanol:chloroform, 2 mL/min) gave **10** (122 mg, 0.306 mmol, 60%) as a faintly yellow oil. The ratio of C-13 epimers was 2:1 as determined by ^1H NMR [R_f (3.5% v/v methanol:chloroform) = 0.18; IR (neat) 2933, 2850, 1722 (C=O), 1440, 1377, 1271, 1165, 1079, 1057, 1017 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.62 (s, 0.33 x 3H, CH_3 minor), 1.63 (s, 0.67 x 3H, CH_3 major), 1.85-2.17 (m, 6H, $SCH_2C\underline{H}_2/C\underline{H}_2CSS/NCH_2C\underline{H}_2$), 2.20-2.66 (m, 6H, 2 $C\underline{H}_2CO_2/CH_2C\underline{H}CH_2/NC\underline{H}HCH_2$), 2.82 (m, 4H, 2 SCH_2), 3.23 (m, 1H, $NCH\underline{H}CH_2$), 3.41 (m, 1H, $NC\underline{H}HCH$=), 3.87 (m, 1H, $NCH\underline{H}CH$=), 4.19 (d, J = 12.1 Hz, 0.67 x 1H, $C\underline{H}HO$ major), 4.26-4.35 (complex, 1H + 0.33 x 1H, $NC\underline{H}/C\underline{H}HO$ minor), 5.10 (d, J = 12.1 Hz, 0.67 x 1H, $CH\underline{H}O$ major) and (m, 0.33 x 1H, $NCHC\underline{H}O$ minor) overlapping, 5.16-5.25 (complex, 0.67 x 1H + 0.33 x 1H, $NCHC\underline{H}O$ major/$CH\underline{H}O$ minor), 5.91 (br s, 1H, $NCH_2C\underline{H}$=); ^{13}C NMR ($CDCl_3$) ∂ 24.9,

26.7, 28.3, 29.5, 30.2, 34.0, 34.1, 38.5, 38.6, 39.9, 40.3, 45.7, 46.0, 48.8, 53.7, 53.8, 59.8, 62.0, 62.2, 74.6, 75.1, 77.1, 77.2, 77.5, 131.7, 132.2, 133.0, 171.0, 171.3, 171.6, 171.9; HRMS (EI) calcd for $C_{19}H_{27}NO_4S_2$ 397.1383 (M), found 397.1398].

18-Acetyl-17,19,20-trinorcrotalanan-11,15-dione (11). To a stirred solution of thioketal **10** (84 mg, 0.212 mmol) in water:acetonitrile (1:4, 1.5 mL) was added mercuric chloride (127 mg, 0.467 mmol) in a single portion. After 16 h at room temperature, the acetonitrile was removed under reduced pressure and the remaining mixture was diluted with water (15 mL) and basified (pH 9) with conc. NH_4OH. The aqueous solution was extracted with chloroform (3 x 5 mL) and the combined organic extracts were washed with brine (1 x 5 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Purification by MPLC (3.5% v/v methanol:chloroform) gave **11** (43 mg, 0.139 mmol, 66%) as a white wax. The ratio of C-13 epimers was 2:1 as determined by ^1H NMR [R_f (3.5% v/v methanol:chloroform) = 0.12; IR (neat) 2936, 2840, 1739 (C=O), 1443, 1371, 1270, 1205, 1164 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 2.00 (m, 2H, $NCH_2C\underline{H}_2$), 2.08 (s, 0.67 x 3H, CH_3 major), 2.09 (s, 0.33 x 3H, CH_3 minor), 2.14-2.83 (m, 8H, $C\underline{H}_2COCH_3/O_2CC\underline{H}_2C\underline{H}C\underline{H}_2CO_2/NC\underline{H}H$-$CH_2$), 3.20 (m, 1H, $NCH\underline{H}CH_2$), 3.37 (m, 1H, $NC\underline{H}HCH$=), 3.83 (m, 1H, $NCH\underline{H}CH$=), 4.15 (d, J = 12.0 Hz, 0.67 x 1H, $C\underline{H}HO$ major), 4.23-4.26 (m, 1H + 0.33 x 1H, $NC\underline{H}/C\underline{H}HO$ minor), 5.06 (d, J = 12.0 Hz, 0.67 x 1H + 0.33 x 1H, $C\underline{H}HO$ major/$C\underline{H}HO$ minor) and (m, 0.33 x 1H, $C\underline{H}O$ minor) overlapping, 5.19 (m, 0.67 x 1H, $C\underline{H}O$ major), 5.87 (br s, 1H, $NCH_2C\underline{H}$=); ^{13}C NMR ($CDCl_3$) ∂ 27.7, 28.5, 30.2, 30.4, 33.9, 36.8, 37.0, 37.8, 38.1, 47.8, 48.2, 53.5, 53.7, 59.7, 61.9, 62.0, 74.6, 75.0, 77.0, 77.4, 132.0, 132.2, 132.8, 132.9, 170.8, 171.1, 171.3, 171.6, 206.5, 206.6; HRMS (EI) calcd for $C_{16}H_{21}NO_5$ 307.1420 (M), found 307.1423].

17,19,20-Trinorcrotalanan-11,15-dione-18-[[(1-ethylidene-1-yl)amino]oxy]acetic Acid Hydrochloride (1). Carboxymethoxylamine hemihydrochloride (14 mg, 0.065 mmol) was added to a solution of keto macrolide **11** (45 mg, 0.129 mmol) in pyridine (1.3 mL) under argon and the mixture was stirred for 24 h at room temperature. After removing most of the pyridine under high vacuum (1 torr, 12 h), the residue was dissolved in chloroform (10 mL) and extracted with 4% aqueous $NaHCO_3$ (3 x 5 mL). The combined aqueous extracts were washed with chloroform (2 x 5 mL), acidified (pH 2) with 3M HCl, and evaporated to dryness under high vacuum (1torr, 12 h). The residue was extracted with chloroform (3 x 5 mL) and the combined organic extracts were filtered through Celite and concentrated under reduced pressure to give **1** (40 mg, 0.096 mmol, 75%) as a light brown oil which was used without further purification in the preparation of protein conjugates [^1H NMR (CD_3CN, 300 MHz) δ 1.86-1.88 (m, 3H, CH_3), 2.00-2.68 (complex, 9H, $NCH_2C\underline{H}_2/C\underline{H}(C\underline{H}_2)_3$), 3.12 (m, 1H, $NC\underline{H}HCH_2$), 3.85 (m, 2H, $NCH\underline{H}CH_2/NC\underline{H}HCH$=), 4.30-4.42 (m, 2H, $NCH\underline{H}CH$=/$C\underline{H}HO$), 4.49-4.59 (m, 2H, $NOC\underline{H}_2$), 4.81 (d, J = 12.3 Hz, 0.75 x 1H, $C\underline{H}HO$ major), 4.91 (d, J = 12.3 Hz, 0.25 x 1H, $C\underline{H}HO$ minor), 5.09 (m, 1H, $NC\underline{H}$), 5.27 (m, 0.25 x 1H, $C\underline{H}O$ minor), 5.52 (m, 0.75 x 1H, $C\underline{H}O$ major), 6.03 (br s, 1H, $NCH_2C\underline{H}$=), 13.2 (br s, 1H, $CO_2\underline{H}$); HRMS (FAB+) calcd for $C_{18}H_{25}ClN_2O_7$ (M - HCl + H) 381.1662, found 381.1663].

N-(Carboxymethyl)-N-[(1,1-dimethylethoxy)carbonyl]glycine. Imminodiacetic acid (2.00 g, 15.0 mmol), triethylamine (4.56 g, 6.28 mL, 45.1 mmol), dioxane (9.0 mL), and water (9.0 mL) were combined in a 50 mL round-bottom flask equipped with a reflux condenser and magnetic stirrer. 2-(tert-

Butoxycarbonyloxyimino)-2-phenylacetonitrile (4.07 g, 16.5 mmol) was added in a single portion and the mixture was heated at 60°C for 1 h. After cooling to room temperature, water (23 mL) and ethyl acetate (30 mL) were added to the reaction mixture and stirring was continued for 5 min at which time the organic layer was removed. The aqueous phase was washed with ethyl acetate (2 x 15 mL), cooled to 0°C, acidified (pH 3) with 10% aqueous citric acid, warmed to room temperature, saturated with sodium chloride, and extracted with ethyl acetate (4 x 15 mL). The combined organic extracts were washed with brine (1 x 15 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The light brown oily residue was dissolved in acetone and filtered under nitrogen pressure through activated carbon:Celite (1:1). Concentration under reduced pressure and additional drying under high vacuum (1 torr, 48 h) over P_2O_5 gave the product (2.10 g, 9.00 mmol, 60%) as a white solid [mp = 125-126°C (dec); IR (KBr) 2500-3400 (br O-H), 1730 (C=O acid), 1680 (C=O carbamate), 1480, 1413, 1370, 1347, 1287, 1251, 967, 913, 856, 789 cm^{-1}; ^1H NMR (acetone-d_6, 300 MHz) δ 1.37 (s, 9H, 3 CH_3), 4.04 (s, 2H, CH_2), 4.08 (s, 2H, CH_2), 11.22 (br s, 2H, OH); ^{13}C NMR (acetone-d_6) ∂ 27.6 (CH_3), 49.5 (CH_2), 50.1 (CH_2), 80.5 ($OC(CH_3)_3$), 155.1 (NCO_2), 171.3 (CO_2H), 171.7 (CO_2H). Anal. calcd for $C_9H_{15}NO_6$: C, 46.35; H, 6.48; N, 6.01. Found: C, 46.38; H, 6.28; N, 5.98.].

1,1-Dimethylethyl 13-Aza-17,18,19,20-tetranorcrotalanan-11,15-dionecarboxylate (14). To a stirred solution of N-(carboxymethyl)-N-[(1,1-dimethylethoxy)carbonyl]glycine (158 mg, 0.677 mmol) in dry DME (6.8 mL) under argon was added dicyclohexylcarbodiimide (140 mg, 0.677 mmol). After 2 h at room temperature, the mixture was cooled to 0°C in an ice bath and filtered through dry Celite under argon. [Anhydride **12** (1,1-dimethylethyl 2,6-dioxo-4-morpholinecarboxylate) is extremely sensitive to moisture and, consequently, was routinely prepared *in situ.* However, a sample was prepared (100% by ^1H NMR) for the purpose of spectroscopic analysis by concentrating the cold filtered reaction mixture under high vacuum (1 torr, 12 h) [IR ($CDCl_3$) 2983, 2933, 1830 and 1782 (C=O anhydride), 1709 (C=O carbamate), 1418, 1372, 1354, 1236, 1158, 1102 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.45 (s, 9H, 3 CH_3), 4.39 (s, 4H, 2 CH_2); ^{13}C NMR ($CDCl_3$) ∂ 28.1 (CH_3), 44.7 (CH_2), 83.1 ($OC(CH_3)_3$), 152.6 (NCO_2), 162.1 ($\underline{C}O_2\underline{C}O$)]. The clear filtrate was added dropwise via cannula needle to a stirring solution of (+)-retronecine (**3**, 100 mg, 0.644 mmol) in dry DME (12.4 mL) under argon. After 24 h at room temperature, triphenylphosphine (203 mg, 0.773 mmol) and 2,2'-dipyridyl disulfide (170 mg, 0.773 mmol) were added and the mixture was stirred for an additional 24 h. The resulting clear yellow solution was added over 6 h by syringe to dry, refluxing DME (100 mL) under argon. After the addition was complete, the solution was heated for an additional 24 h at which time the reaction mixture was cooled to room temperature and concentrated under reduced pressure to afford a yellow gum. Purification by MPLC (3.5% v/v methanol:chloroform, 2 mL/min) gave **14** (136 mg, 0.385 mmol, 57%) as a faintly yellow oil [R_f (3.5% v/v methanol:chloroform) = 0.17; IR (neat) 2978, 2950, 2841, 1740 (C=O ester), 1705 (C=O carbamate), 1449, 1401, 1367, 1260, 1161 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.44 (s, 9H, $C(CH_3)_3$), 1.91-2.17 (m, 2H, $NCH_2C\underline{H}_2$), 2.62 (m, 1H, $NC\underline{H}HCH_2$), 3.16 (m, 1H, $NCH\underline{H}CH_2$), 3.40 (m, 1H, $NC\underline{H}HCH$=), 3.56-3.89 (m, 3H, $NCH\underline{H}CH$=/$CH\underline{H}CO_2$/$CH\underline{H}CO_2$), 4.08-4.45 (m, 4H, $C\underline{H}HCO_2$/$C\underline{H}HCO_2$/$C\underline{H}HO$/$NC\underline{H}$), 5.01 (d, J = 11.8 Hz, 0.5 x 1H, $CH\underline{H}O$), 5.06 (d, J = 11.8 Hz, 0.5 x 1H, $CH\underline{H}O$), 5.98 (br s, 0.5 x 1H, $NCH_2C\underline{H}$=), 6.02 (br s, 0.5 x 1H, $NCH_2C\underline{H}$=); ^{13}C NMR ($CDCl_3$) ∂ 28.2, 33.2, 33.5, 50.8, 51.4, 51.5, 51.7, 53.4, 53.6, 59.1,

59.3, 61.2, 61.5, 74.9, 75.4, 76.7, 76.8, 77.2, 81.1, 81.2, 132.7, 132.9, 135.3, 135.6, 154.4, 167.9, 168.2, 168.9, 169.0; HRMS (FAB+) calcd for $C_{17}H_{25}N_2O_6$ 353.1713 (M + H), found 353.1712].

13-Aza-17,18,19, 20-tetranorcrotalanan-11,15-dione (15). Carbamate **14** (51 mg, 0.144 mmol) and 3M HCl (1.0 mL) were combined in a 5 mL round-bottom flask and stirred at room temperature for 1 h at which time the reaction mixture was diluted with water (9.0 mL) and basified (pH 9) with 6% aqueous NH_4OH. The resulting aqueous solution was saturated with sodium chloride and extracted with chloroform (4 x 5 mL). The combined organic extracts were washed with brine (5 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give a faintly yellow oil. Purification by MPLC (3.5% v/v methanol:chloroform, 2 mL/min) gave **15** (23 mg, 0.093 mmol, 65%) as a colorless oil [R_f (3.5% v/v methanol:chloroform) = 0.10; IR (neat) 3351 (N-H), 2932, 2842, 1740 (C=O), 1253, 1219 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.91-2.15 (m, 2H, $NCH_2C\underline{H}_2$), 2.42 (br s 1H, NH), 2.63 (m, 1H, $NC\underline{H}HCH_2$), 3.16 (m, 1H, $NCH\underline{H}CH_2$), 3.27-3.47 (m, 5H, $NC\underline{H}HCH$=/$O_2CC\underline{H}_2N$(H)-$C\underline{H}_2CO_2$), 3.87 (m, 1H, $NCH\underline{H}CH$=), overlapping 4.38 (m, 1H, $NC\underline{H}$) and 4.44 (d, J = 11.7 Hz, 1H, $C\underline{H}HO$), 4.70 (d, J = 11.7 Hz, 1H, $CH\underline{H}O$), 5.43 (m, 1H, $C\underline{H}O$), 5.99 (m, $NCH_2C\underline{H}$=); ^{13}C NMR ($CDCl_3$) ∂ 33.2, 51.9, 52.2, 53.6, 58.9, 61.2, 74.3, 77.5, 132.7, 135.6, 170.7, 172.5; HRMS (EI) calcd for $C_{12}H_{16}N_2O_4$ 252.1110 (M), found 252.1100].

13-Aza-13-(3-carboxy-1-oxopropyl)-17,18,19,20-tetranorcrotalanan-11,15-dione Hydrochloride (2). Succinic anhydride (13 mg, 0.134 mmol) was added to a solution of diamine **15** (32 mg, 0.128 mmol) and pyridine (30 mg, 31.0 μL, 0.383 mmol) in dry CH_2Cl_2 (0.5 mL) under argon. After stirring 12 h at room temperature, the solvent was removed under reduced pressure and the residue was dissolved in 10% aqueous $NaHCO_3$ (5 mL). The aqueous solution was washed with chloroform (2 x 2.5 mL), acidified (pH 3) with 1M HCl, and evaporated to dryness under high vacuum (1 torr). The residue was extracted with chloroform (3 x 5 mL) and the combined organic extracts were concentrated under reduced pressure to give **2** (38 mg, 0.098 mmol, 77%) as a light brown oil [IR (neat) 2750-2900 (br OH), 2560 (NH+), 1733 (C=O), 1657 (C=O), 1457 1401, 1258, 1161 cm^{-1}; ^1H NMR (CD_3CN, 300 MHz) δ 2.10-2.35 (m, 2H, $NCH_2C\underline{H}_2$), 2.40-2.85 (m, 4H, $O_2CC\underline{H}_2C\underline{H}_2CO_2$), 3.01 (m, 0.5 x 1H, $NC\underline{H}HCH_2$), 3.22 (m, 0.5 x 1H, $NC\underline{H}HCH_2$), 3.70-4.70 (m, 8H, $O_2CC\underline{H}_2NC\underline{H}_2CO_2$/$NCH\underline{H}$-$CH_2$/$C\underline{H}HO$/$NC\underline{H}_2CH$=), overlapping 5.04 (d, J = 12.2 Hz, 1H, $CH\underline{H}O$) and 5.13 (m, 1H, $NC\underline{H}$), 5.54 (m, 0.5 x 1H, $C\underline{H}O$), 5.78 (m, 0.5 x 1H, $C\underline{H}O$), 6.01 (br s, 0.5 x 1H, $NCH_2C\underline{H}$=), 6.10 (br s, 0.5 x 1H, $NCH_2C\underline{H}$=); HRMS (FAB+) calcd for $C_{16}H_{21}ClN_2O_7$ 353.1349 (M - HCl + H), found 353.1348].

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2 (a) Mattocks, A. R.; Bird, I. *Chem.-Biol. Interactions* **1983**, *43*, 209. (b) Segall, H. J.; Wilson, D. W.; Dallas, J. L.; Haddon, W. F. *Science* **1985**, *229*, 472.

3 (a) Svoboda, D. J.; Reddy, J. K. *Cancer Res.* **1972**, *32*, 908. (b) Allen, J. R.; Hsu, I. C.; Carsten, L. A. *Cancer Res.* **1975**, *35*, 997. (c) Kuhara, K.; Takanashi, J.; Hirono, I.; Furuya, T.; Asada, Y. *Cancer Lett.* **1980**, *10*, 117.

4 Ames, B. N.; Durston, W. E.; Yamasaki, E.; Lee, F. D. *Proc. Natl. Acad, Sci. U.S.A.* **1973**, *70*, 2281.

5 Keeler, R. F. *Toxicon* **1983**, *Suppl. 3*, 221.

6 Huxtable, R. J. *Gen. Pharmac.* **1979**, *10*, 159.

7 Robins, D. J. *Prog. Chem. Org. Nat. Prod.* **1982**, *41*, 115 and references cited therein.

8 For example, 1700 people contracted liver disease in Afghanistan when cereal grains contaminated with PA-producing plants were used in bread making: Mohabbat, O.; Younos, S. M.; Metrao, A. A.; Srivastave, R. N.; Sedig, G. G.; Aram, G. N. *Lancet* **1976**, 269.

9 As a result of PA poisoning from *Senecio jacobaea*, producers in the Pacific Northwest sustained livestock losses estimated at 11 million dollars in 1978 alone: Craig, A. M.; Meyer, C.; Koller, L. D.; Schmitz, J. A. *Proc. Amer. Assn. Vet. Lab. Diag.* **1978**, *21*, 299.

10 Adams, R.; Rogers, E. F. *J. Am. Chem. Soc.* **1939**, *61*, 2815.

11 Monocrotaline is commercially available: Aldrich 27,028-8.

12 Winterfeldt, E. *Angew Chem. Int'l. Ed. Eng.* **1967**, *6*, 172.

13 Corey, E. J.; Nicolaou, K. C. *J. Am. Chem. Soc.* **1974**, *96*, 5614.

14 (a) Erlanger, B. F.; Borek, F.; Beiser, S. M.; Lieberman, S. *J. Biol. Chem.* **1957**, *228*, 713. (b) Erlanger, B. F.; Borek, F.; Beiser, S. M.; Lieberman, S. *J. Biol. Chem.* **1959**, *234*, 1090. (c) Goodfriend, T. L.; Levine, L.; Fasman, G. D. *Science*, **1964**, *144*, 1344.

15 Anderson, G. W.; Simmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1964**, *86*, 1839.

16 A similar strategy was used in these laboratories for protein conjugation of diphacinone, an anticoagulant rodenticide: see, Kurth, M. J.; Mount, M. E.; Jackson, D. Y. *J. Immunoassay* **1988**, *9*, 69.

17 Lukes, R.; Palecek, J. *Collection Czech. Chem. Commun.* **1964**, *29*, 1073.