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DNA interstrand cross-link formation by reductive activation of dehydropyrrolizidine progenitors

Jetze J. Tepe, Christi Kosogof and Robert M. Williams*

Department of Chemistry, Colorado State University, Fort Collins, CO 80523, USA

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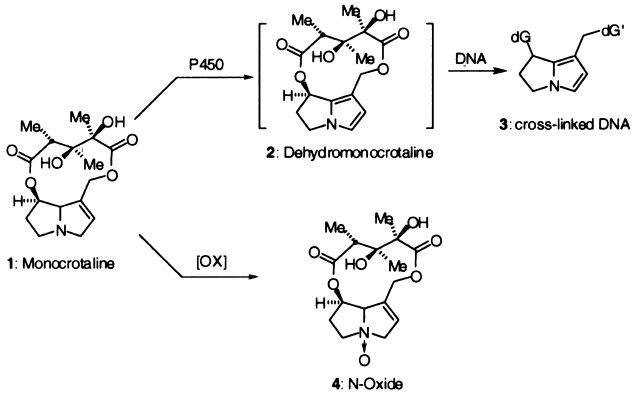
Abstract—Pyrrolizidine alkaloids (PAs) are potent hepatotoxins and carcinogens isolated from a wide variety of plants. The hepatotoxicity of these agents is a manifestation of the initial production and release of the reactive dehydropyrrolizidine, which is the DNA-cross-linking species, generated in the liver by the mixed function cytochrome P450 oxidases. A separate class of DNA cross-linkers, including the clinically significant mitomycins and the related FR900482 and congeners are reductively activated in vivo forming a very similar pyrrolic-type intermediate responsible for the DNA cross-linking reactivity of these substances. We report here the synthesis and DNA cross-linking studies of a reductively activated dehydromonocrotaline progentior and its dicarbamate derivative. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Pyrrolizidine alkaloids (PAs), such as monocrotaline **1**, are potent hepatotoxins and carcinogens isolated from a wide variety of plants.[1,2] Due to their geographical diversity and abundance, the pyrrolizidine alkaloids represent a serious health threat to humans and live stock.[2,3] The biologically active metabolites of these natural products are the corresponding dehydropyrrolizidines (for example **2**, dehydromonocrotaline, Scheme 1), formed upon two-electron oxidation of the 4-azabicyclo [3.3.0] octane ring system by liver cytochrome P450 mixed function oxidases.[4,5] These substances have been shown to be potent DNA interstrand cross-linking agents (forming adduct **3**) and it is this biochemical reactivity that is largely responsible for the cytotoxicity that this class of alkaloids displays.[6,7]



Scheme 1. Oxidative activation of monocrotaline.

The oxidation of the pyrrolizidine alkaloid ring system results in the electrophilic activation of the C-7 and C-9 positions, via conjugation with the non-bonded electron pair of the pyrrole nitrogen, which are prone to nucleophilic attack by the exocyclic amine group of deoxyguanosine residues in the minor groove of DNA at $^{5'}$CpG$^{3'}$ steps as well as other sites.[8,9] The potent interstrand DNA–DNA and DNA–protein[10] cross-linking reactions mediated by the pyrrolic PAs have made these compounds of interest as potential antitumor agents. However, the acute hepatotoxicity of these agents has obviated their clinical utility. The hepatotoxicity displayed by these compounds is a manifestation of the dehydropyrrolizidine DNA-cross-linking species being generated in the liver by the mixed function cytochrome P450 oxidases. Since the oxidative mode of activation is primarily responsible for the undesirable characteristics of these compounds, it seemed that it might be possible to improve their pharmacological profile by altering the chemical mode of activation.

Based on the success of a variety of clinically significant, reductively activated DNA cross-linking agents, such as mitomycin C, FR900482 and congeners (Fig. 1) we anticipated that a reductive mode of activation of the pyrrolizidine alkaloids might not only improve the pharmacological profile of these agents, but also induce a similar tumor selectivity that many of the hypoxia-directed agents display.[11,12]

As part of a program aimed at diversifying the chemical and

Keywords: DNA interstrand cross-link; reductive activation; dehydropyrrolizidine progenitors.

* Corresponding author. Tel.: +1-970-491-6747; fax: +1-970-491-5610; e-mail: rmw@chem.colostate.edu

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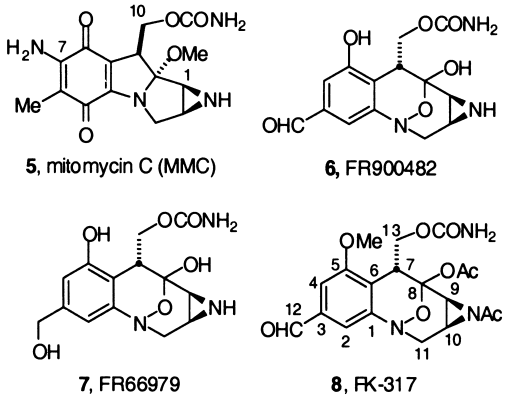


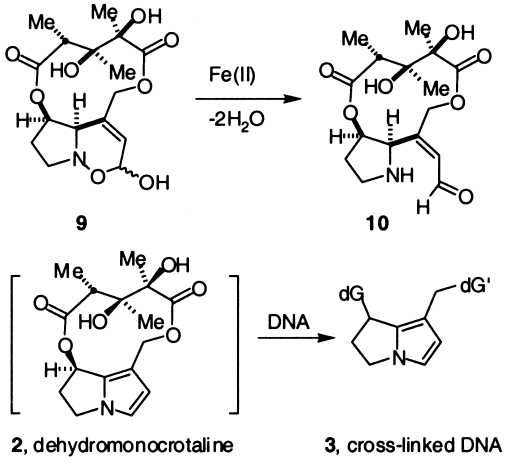
Figure 1. Reductively activated DNA cross-linkers.

biochemical repertoire of activation of a variety of clinically significant antitumor agents,[13] we describe here the synthesis and DNA cross-linking reactivity of the first bioreductively activated pyrrolizidine alkaloid, a progenitor of dehydromonocrotaline, as well as the synthesis of a more water-soluble dicarbamate derivative of this compound.

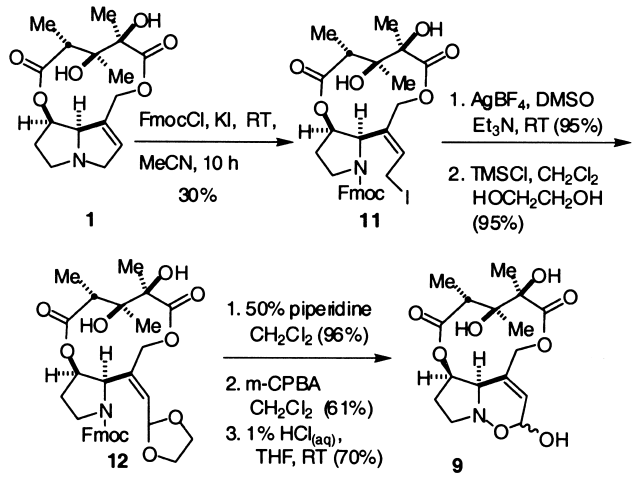
Recently, we reported the synthesis and Fe(II)/EDTA induced DNA cross-linking ability of the hydroxylamine hemi-acetal **9**.[21] The mechanism of activation of the hydroxylamine hemi-acetal of **9** is anticipated to follow the known mechanism of activation of the FR900482 family of compounds through a two-electron reductive cleavage of the N–O bond of **9** to the corresponding free amine (**10**, Scheme 2) which cyclizes on the incipient aldehyde **10** and subsequently dehydrates to the highly reactive pyrrolic intermediate **2**.[19,20]

2. Results and discussion

The synthesis of the reductively activated dehydromonocrotaline derivative **9** is shown in Scheme 3. Commercially available[14] monocrotaline **1** was condensed with 9-fluorenylmethyl chloroformate (FmocCl) in the presence of KI in acetonitrile[15] furnishing the ring cleaved product **11**. The allylic iodide was oxidized with DMSO and $AgBF_4$ to afford the unsaturated aldehyde in 95% yield. Protection



Scheme 2. Reduction of dehydromonocrotaline progenitor.



Scheme 3. Synthesis of hydroxylamine hemiacetal.

of the aldehyde as the corresponding ethylene glycol acetal followed by removal of the Fmoc-moiety with 50% piperidine in THF provide the corresponding free amine **12**. The hydroxyl amine hemi-acetal progenitor of dehydromonocrotaline **9** was prepared by oxidation of the free amine with *m*-CPBA in a biphasic mixture of CH_2Cl_2 and aqueous saturated $NaHCO_3$ followed by removal of the acetal with 1% HCl.

Similar to the well studied mode of activation of FR900482 and congeners, the hydroxylamine hemi-acetal **9** was reductively activated by the in situ generation of dehydromonocrotaline through the agency of Fe(II)/EDTA.[8,16] HPLC analysis (reverse phase C-18 YMC ODS-A, 30% acetonitrile in water at 1.0 mL/min) of the hydroxylamine hemi-acetal **9** revealed that this substance (10 μL, 10 mM in DMSO, UV λ_{max}=212 nm, retention time 3.8 min) was converted into dehydromonocrotaline **2** (UV λ_{max}=232 nm, retention time 11.2 min) by the addition of Fe(II)/EDTA (20 mM, pH=8).

The results of DNA cross-linking studies with compound **9** are presented in Fig. 2. The DNA interstrand cross-linking ability of **9** was investigated using linear plasmid DNA by denaturing alkaline agarose gel electrophoresis according to Cech.[17] The duplex DNA substrate employed in this study was pBR322 plasmid DNA which was linearized by

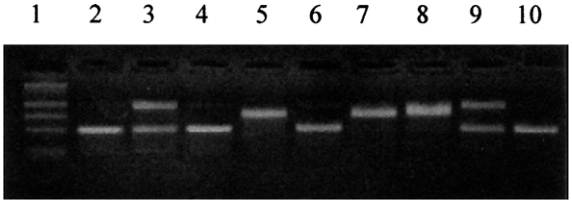


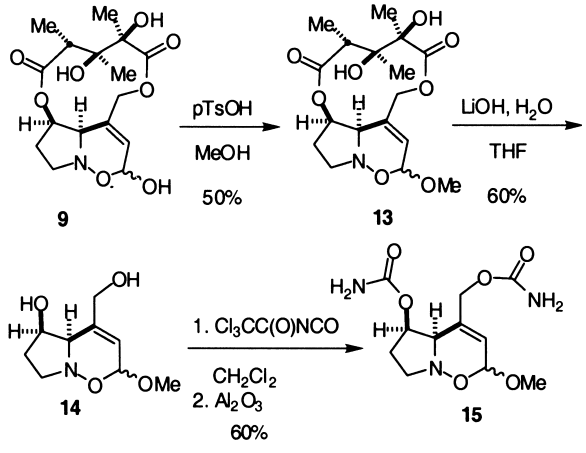
Figure 2. Lane (1) 0.5 μg lambda hind III (molecular weight standard); lane (2) 0.5 μg pBR322 (control); lane (3) 0.5 μg pBR322+10 μM dehydromonocrotaline (**2**, control); lane (4) 0.5 μg pBR322+1.0 mM FR900482 (control); lane (5) 0.5 μg pBR322+1.0 mM FR900482+100 μM Fe(II)/EDTA; lane (6) 0.5 μg pBR322+1.0 mM compound **9** (control); lane (7) 0.5 μg pBR322+1.0 mM compound **9**+100 μM Fe(II)/EDTA; lane (8) 0.5 μg pBR322+500 μM compound **9**+100 μM Fe(II)/EDTA; lane (9) 0.5 μg pBR322+100 μM compound **9**+100 μM Fe(II)/EDTA; lane (10) 0.5 μg pBR322+10 μM compound **9**+100 μM Fe(II)/EDTA.

restriction endonuclease digestion with EcoR1. The amount of linearized pBR322 was quantitated by UV analysis at 260 nm as described by Borer.[18] Compound **9** (various concentrations of a 10 mM stock solution were made from 4.2 mg of **9** dissolved in 1.2 mL DMSO) and 0.5 μg DNA (EcoR1 linearized pBR322) was incubated at 37°C for 12 h with the reagents described for each lane in Fig. 2 (10 μL, final volume). The Fe(II)/EDTA was prepared by addition of an equimolar amount of $FeSO_4$ to a solution of EDTA (pH=8). The crude reaction mixture was loaded onto a denaturing 1.2% alkaline agarose gel[17] and electrophoresis was conducted at 50 V for 3 h (95 mA, 3 W). The gel was stained by the addition of 200 mL of ethidium bromide solution (100 mM Tris at pH 7.5, 15 min) and visualized after destaining (50 mM NH_4OAc+10 mM 2-mercaptoethanol, 1 h) on a UV transilluminator. Lambda Hind III was employed as a molecular weight standard (lane 1).

Control reactions were performed with dehydromonocrotaline (**2**, 10 μM, lane 3) and the known reductively activated antitumor antibiotic FR900482 (1.0 mM, lane 4 and with Fe(II)/EDTA activation in lane 5).[8,16] The data in Fig. 1 reveals that 100 μM Fe(II)/EDTA strongly activates compound **9** to form the interstrand cross-linked DNA at drug concentrations of 100 μM or more (lanes 7–9).

Based on these initial findings, we also investigated the synthesis of a dicarbamate derivative of the original progenitor **9**. We have endeavored to remove the lipophilic macrocyclic diester backbone and replace this substituent with more polar and water-soluble primary carbamates. The carbamate groups were envisioned to impart good water-solubility as well as providing for good leaving groups that are necessary for the generation of the highly electrophilic iminium ion species that mediate the covalent DNA interstrand cross-linking reactions. This is akin to the role that the carbamate residue plays in the mechanism of action of the antitumor agent mitomycin C and the FR-900482 class of antitumor antibiotics.[22,23]

The preparation of the dicarbamate commenced with the hydroxylamine hemi-acetal **9**, which was first converted to the corresponding methyl acetal **13** with *p*-TsOH in MeOH (Scheme 4). The hemi-acetal was protected as the methyl acetal to avoid undesired complications when attempting



Scheme 4. Synthesis of dicarbamate derivative **15**.

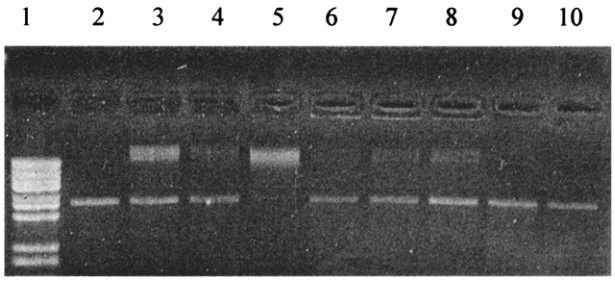


Figure 3. All reactions were incubated at 37°C for 14 h. Lane (1) 0.5 μg Lambda DNA BSTE II digest (molecular weight standard); lane (2) 0.5 μg pBR322 (control); lane (3) 0.5 μg pBR322+10 μM dehydromonocrotaline; lane (4) 0.5 μg pBR322+1 mM FR66979; lane (5) 0.5 μg pBR322+1 mM FR66979+100 μM Fe(II)/EDTA; lane (6) 0.5 μg pBR322+10 mM compound **15**; lane (7) 0.5 μg pBR322+10 mM compound **15**+100 μM Fe(II)/EDTA; lane (8) 0.5 μg pBR322+5 mM compound **15**+100 μM Fe(II)/EDTA; lane (9) 0.5 μg pBR322+1 mM compound **15**+100 μM Fe(II)/EDTA; lane (10) 0.5 μg pBR322+100 μM compound **15**+100 μM Fe(II)/EDTA.

cleavage of the macrocyclic dilactone backbone. Initially, KCN in ethanol was used to cleave the dilactone, however, the compound proved to be unreactive after 2 days at room temperature under these conditions. Use of LiOH, H_2O, and THF proved to be successful, resulting in the diol **14** in about 60% yield. The dicarbamate moieties were installed using trichloroacetylisocyanate, followed by removal of the trichloroacetyl residues over neutral alumina[24] to give **15** in 60% yield.

In an attempt to determine the DNA cross-linking potential of the dicarbamate derivative **15**, several experiments were carried out. Initially, the compound (at various concentrations, Fig. 3), in a DMSO–water mixture, was combined with 100 μM Fe(II)/EDTA (pH=8.0) and linearized pBR322 DNA. Dehydromonocrotaline (**2**) was used as a cross-link standard, and FR66979 (**7**) was used as a standard for reductive activation by Fe(II). After incubation at 37°C for 14 h, alkaline agarose gel electrophoresis was performed. It can clearly be seen that 1 mM FR66979 is readily reduced in the presence of 100 μM Fe(II)/EDTA, to produce a DNA cross-link (lane 5). Dicarbamate **15** displayed only background DNA cross-linking at high concentrations (10 mM and 5 mM) (Fig. 2, lanes 7 and 8). Longer incubation times of 22 h showed no significant change in cross-link formation.

A possible explanation for the lack of cross-link formation displayed by dicarbamate **15** may be a manifestation of the relative stability of the hydroxylamine methyl acetal bond versus the free hemi-acetal. While compounds such as FR66979 are easily reduced in the presence of Fe(II)/EDTA, experimental evidence suggests that the acetylated derivative FK317 must first be deactylated to the free hemi-acetal prior to undergoing reductive activation.

Attempts to generate the free hemi-acetal of **15** were attempted in vitro, by adjusting the reaction to pH 6.0 with phosphate buffers. This resulted in no observable enhancement of DNA cross-link formation. Efforts to cleanly hydrolyze **15** to the corresponding free hemi-acetal have thus far proved elusive and studies towards these ends are currently under investigation.

3. Conclusions

These preliminary studies suggest the viability of the hydroxylamine hemi-acetal moiety as a potentially useful functional group for the reductive activation of pyrrolizidine alkaloids. Since it has been demonstrated that the only other known hydroxylamine hemi-ketal containing antitumor antibiotic, FR900482 and congeners, can be reductively activated to cross-link DNA by Fe(II) salts in vitro, and that this reductive activation mechanism has also been implicated for the in vivo activation of these agents,[8,19,20] the present system lends support to the notion that the reductively-labile hydroxylamine hemi-acetal may find other uses in pro-drug activation strategies. In addition, these agents may also provide a conceptual framework upon which the design and synthesis of a wide variety of pyrrolic PA prodrugs with potential clinical applications may be examined. Studies toward these objectives are in progress in these laboratories.

4. Experimental

4.1. General

^{1}H and ^{13}C NMR spectra were obtained using a Varian 300 at 300 MHz. NMR spectra was recorded at room temperature unless otherwise noted. All compounds were further analyzed by HMQC and/or APT spectra obtained using a Varian 400 MHz spectrometer. All chemical shifts are reported in ppm. Analytical thin-layer chromatography (TLC) was carried out on Merck pre-coated silica gel 60 F-254 plated and were visualized with a phosphomolybdic acid/ethanol solution. All solvents were distilled prior to use.

4.1.1. Dehydromonocrotaline (2). Chloranil (0.05 mmol, 12 mg) was dissolved in chloroform (2.0 mL) in a separatory funnel. Monocrotaline (Aldrich Chemical Co., 0.031 mmol, 10 mg) in 2.0 mL chloroform was added and the solution was gently stirred for 2 min. A mixture of $NaBH_4$ (0.08 mmol, 3 mg) was added and an excess of NaOH (700 mg) in 2.0 mL H_2O. A green precipitate formed and the organic layer was drained through a filter of decolorizing charcoal into an Erlenmeyer flask. The organic layer was dried over Na_2SO_4, filtered and concentrated to yield 10 mg of pure dehydromonocrotaline (**2**, 100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.62 (d, J=2.4 Hz, 1H), 6.32 (d, J=3.0 Hz, 1H), 6.13 (dd, J=3.0, 7.8 Hz, 1H), 5.72 (d, J=12 Hz, 1H), 4.61 (d, J=12 Hz, 1H), 4.24–4.16 (m, 1H), 4.09–4.01 (m, 1H), 3.11–2.89 (m, 3H), 2.62–2.52 (m, 1H), 1.52 (s, 3H), 1.49 (s, 3H), 1.37 (d, J=6.9 Hz, 3H).

4.2. General procedure for linearization of plasmid pBr322 by EcoR1

Supercoiled pBr322 (30 μL, 30 μg) was incubated with EcoR1 (New England Biolabs) (10 μL), EcoR1 buffer (10X, 20 μL), and 144 μL H_2O (sterile) for 1 h and 20 min at 37°C. NaOAc (20 μL, 3 M) and ethanol (440 μL) were added and the solution was cooled at −70°C for 10 min. The mixture was centrifuged for 15 min and the ethanol was decanted off. The remainder of the ethanol was evaporated off in vacuo, and the remaining linearized DNA was suspended in 60 μL sterile H_2O. The amount of linearized pBR322 was quantitated by UV analysis as described by Borer[18] in conjunction with the optical density measurements obtained by UV absorption at 260 nm. For this plasmid DNA substrate the conversion factor was determined to be an OD_{260} of 1=50 μg/mL.

4.3. General protocol for alkaline agarose gel electrophoresis

The agarose gels were prepared by adding 50 mL of a 50 mM NaCl/2 mM EDTA (at pH=8) to 0.6 g agarose. The suspension was heated in a microwave oven until all the agarose was dissolved (45 s). The gel was poured and was allowed to cool and solidify for 1 h at room temperature. After 1 h the gel was soaked in an alkaline running buffer (25 mL of 2N NaOH, 2 mL, 0.5 M EDTA at pH of 8 in 1 L H_2O) for an additional hour. The comb was removed and the buffer was refreshed prior to electrophoresis. Agarose loading dye (5 μL) was added to the samples (10 μL) and the samples were loaded into the wells. The gel was run for 4 h at 40 mA/90 Volts which resulted in a traveling distance of approximately 8 cm. The gel was then neutralized for 45 min in a 1 M Tris pH=7/1.5 M NaCl solution, which was refreshed every 15 min. The gel was subsequently stained in a ethidium bromide solution (20 μL of a 10 mg/mL ethidium bromide solution in 1 L, 1 M Tris/1.5 M NaCl buffer at pH=7.5) for 1 h. The background staining was then removed by soaking the gel in a solution of 50 mM NH_4OAc and 10 mM β-mercaptoethanol. Gels were visualized on a UV transilluminator and photographed using Polaroid Black% White film #667.

4.3.1. 7,8-Dihydroxy-12-(2-iodo-ethylidene)-6,7,8-trimethyl-5,9-dioxo-decahydro-4,10-dioxa-1-aza-cyclopentacycloundecene-1-carboxylic acid 9*H*-fluoren-9-ylmethyl ester (11). KI (0.615 mM, 102 mg) was added to a solution of monocrotaline (**1**, 0.154 mmol, 50 mg) in freshly distilled acetonitrile (1.0 mL). The suspension was stirred for 10 min at room temperature after which 9-fluorenylmethyl chloroformate (0.184 mmol, 48 mg) was added. The suspension slowly turned yellow and was allowed to stir at room temperature for 1 h. After 1 h the suspension was diluted with ether (10 mL) and was extracted with saturated $NaHCO_3$ aq. (10 mL). The organic layer was washed with brine (10 mL) and dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (eluted with ether) (R_f=0.31) to yield 31 mg of compound **11** (30%) as a white solid. Exact mass calcd for $C_{31}H_{35}NO_8I$ m/z 676.140745, found m/z 676.140108. The product (**11**) was isolated as a mixture of rotamers at room temperature as observed on the NMR time scale and decomposed rapidly at increased temperatures. To avoid decomposition of the allylic iodide, compound **11** was immediately converted into the stable allylic aldehyde as described below.

4.3.2. 7,8-Dihydroxy-6,7,8-trimethyl-5,9-dioxo-12-(2-oxo-ethylidene)-decahydro-4,10-dioxa-1-aza-cyclopentacycloundecene-1-carboxylic acid 9*H*-fluoren-9-ylmethyl ester. $AgBF_4$ (199 mg, 1.2 equiv.), was added to a solution of the allylic iodide **11** (576 mg, 0.853 mmol) in dry DMSO

(30 mL). After 20 min. TEA (238 μL, 2 equiv.) was added and the suspension was stirred at room temperature for an additional 5 min. The solution was diluted with ether (50 mL) and was extracted with saturated NH_4Cl aq. (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography on silica gel (eluted with 1:1 ethyl acetate/ether) (R_f=0.4) to yield 455 mg of the product aldehyde (95%) as a white solid, mp 96–98°C (recryst. ether). ^{1}H NMR (300 MHz, DMSO-d_6, 120°C) δ 9.88 (d, J=6.3 Hz, 1H), 7.85 (t, J=7.2 Hz, 2H), 7.64–7.57 (m, 2H), 7.43–7.28 (m, 4H), 5.96 (d, J=6.3, 1H), 5.44 (ddd, J=1.8, 4.8, 9.6 Hz, 1H), 5.23 (d, J=5.1 Hz, 1H), 4.81 (d, J=11.4 Hz, 1H), 4.62–4.51 (m, 2H), 4.23 (t, J=5.1 Hz, 1H), 3.88 (d, J=11.4 Hz, 1H), 3.68 (ddd, J=2.1, 10.5, 18.9 Hz, 1H), 3.49–3.38 (m, 1H), 3.01 (q, J=7.2, 14.4 Hz, 1H), 2.15–2.03 (m, 1H), 1.84–1.76 (m, 1H), 1.14 (s, 3H), 1.11 (s, 3H), 1.05 (d, J=6.9 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6, 80°C) δ 190.6, 173.6, 173.2, 154.4, 143.9 (2C), 141.0 (2C), 133.9, 127.6 (2C), 127.1 (2C), 124.6 (2C), 120.1 (2C), 79.0, 76.5, 74.9, 65.9, 61.5, 46.9, 45.5, 43.0, 30.9, 30.6, 21.9, 19.0, 14.0. IR (NaCl), neat) 3435, 2984, 2949, 1737, 1698, 1676, 1451, 1418, 1351, 1178, 1109, 1026, 761, 745 cm^{-1}. Exact mass calcd for $C_{31}H_{33}NO_9$ m/z, found m/z 564.2236.

4.3.3. 12-[1,3]Dioxolan-2-ylmethylene-7,8-dihydroxy-6,7,8-trimethyl-5,9-dioxo-decahydro-4,10-dioxa-1-aza-cyclopentacycloundecene-1-carboxylic acid 9*H*-fluoren-9-ylmethyl ester (12). An excess of ethylene glycol (3.05 mmol, 170 μL) was added to a solution of the aldehyde obtained above (0.610 mmol, 344 mg) in dry CH_2CL_2 (15 mL). After 5 min. TMSCl (0.856 mmol, 232 μL) was added and the solution was stirred for 5 h at room temperature. The mixture was diluted with ether (50 mL) and was extracted with saturated $NaHCO_3$ aq. (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography on silica gel (eluted with 1:1 ethyl acetate/ether) (R_f=0.4) to yield 320 mg of compound **12** (86%) as a white solid.). ^{1}H NMR (300 MHz, DMSO-d_6, 120°C) δ 7.87–7.81 (m, 2H), 7.67–7.62 (m, 2H), 7.48–7.31 (m, 4H), 5.67 (d, J=5.4 Hz, 1H), 5.42 (d, J=5.4 Hz, 1H), 5.34–5.32 (m, 1H), 4.90 (bs, 1H, *OH*), 4.83–4.78 (m, 2H), 4.47 (d, J=6 Hz, 2H), 4.26 (t, J=6 Hz, 1H), 3.92–3.77 (m, 5H), 3.64–3.62 (m, 1H), 3.51–3.38 (m, 1H), 3.01 (q, J=7.2, 14.7 Hz, 1H), 2.91 (bs, 1H, *OH*), 2.12–2.00 (m, 1H), 1.83–1.77 (m, 1H), 1.36 (s, 3H), 1.15 (s, 3H), 1.06 (d, J=7.2 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6, 75°C) δ 173.6, 173.5, 154.4, 143.9 (2C), 140.9 (2C), 136.4, 134.4, 127.6 (2C), 127.1 (2C), 124.9 (2C), 120.1 (2C), 98.4, 79.2, 76.5, 74.9, 66.4, 64.3, 62.3, 61.3, 47.0, 45.2, 43.1, 30.6, 21.6, 19.4, 13.8. Exact mass calcd For $C_{33}H_{37}NO_{10}$ m/z 608.249572, found m/z 608.248876.

4.3.4. 12-[1,3]Dioxolan-2-ylmethylene-7,8-dihydroxy-6,7,8-trimethyl-octahydro-4,10-dioxa-1-aza-cyclopentacycloundecene-5,9-dione. An excess of piperidine (4.0 mL) was added to a solution of compound **12** (0.184 mmol, 196 mg) in THF (4.0 mL). The solution was stirred at room temperature until all starting material had been consumed (10 min) after which the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (eluted in 5% diisopropylamine/ethanol) (R_f=0.37) to yield 69 mg of the free secondary amine (97%) as a white solid. Mp 55–57°C (recryst. ether). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.87 (d, J=3.9 Hz, 1H), 5.57–5.48 (m, 2H), 5.33 (t, J=4.2 Hz, 1H), 4.13 (d, J=8.4 Hz, 1H), 3.99–3.84 (m, 6H), 3.36–3.25 (m, 1H), 2.90–2.84 (m, 1H), 2.83 (q, J=5.7, 11.2 Hz, 1H), 2.13–2.02 (m, 1H), 1.76–1.68 (m, 1H), 1.44 (s, 3H), 1.25 (s, 3H), 1.09 (d, J=5.7 Hz, 3H). ^{13}C NMR and APT (100 MHz, $CDCl_3$) δ 175.05 (C), 173.50 (C), 136.41 (C), 136.42 (CH), 98.95 (CH), 78.96 (C), 77.33 (C), 75.63 (C), 65.02 (2×CH_3), 64.88 (CH_2), 62.33 (CH), 44.45 (CH_2), 43.63 (CH), 33.75 (CH_2), 21.81 (CH_3), 18.40 (CH_3), 13.87 (CH_3). IR (NaCl, neat) 3421, 2983, 1732, 1115 cm^{-1}. Exact mass calcd for $C_{18}H_{28}NO_8$ m/z 386.181492, found m/z 386.181998.

4.3.5. 12-[1,3]Dioxolan-2-ylmethylene-1,7,8-trihydroxy-6,7,8-trimethyl-octahydro-4,10-dioxa-1-aza-cyclopentacycloundecene-5,9-dione. A small excess of *m*-CPBA (15 mg, 56–86%) was added to a solution of the free amine obtained above (8.5 mg, 0.0221 mmol) in CH_2Cl_2 (0.5 mL). The reaction was closely monitored by TLC and after all starting material had been consumed the reaction was quenched with a saturated solution of Na_2SO_3 (1.0 mL). After 5 min the mixture was diluted with CH_2Cl_2 (5 mL) and was extracted with saturated $NaHCO_3$ aq. (5 mL) and brine (5 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography on silica gel (eluted with 1:1 ethyl acetate/ether) (R_f=0.29) to yield 4 mg of the hydroxylamine (40%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.10 (d, J=5.4 Hz, 1H), 5.78 (d, J=5.1 Hz, 1H), 5.76 (d, J=10.8 Hz, 1H), 5.36–5.30 (m, 1H), 4.32 (d, J=11.4 Hz, 1H), 4.07–3.94 (m, 6H), 3.61–3.54 (m, 1H), 2.88 (q, J=7.2, 14.1 Hz, 1H), 2.89–2.81 (m, 1H), 2.85 (bs, 1H, *OH*), 2.54–2.42 (m, 1H), 1.87–1.75 (m, 1H), 1.48 (s, 3H), 1.30 (s, 3H), 1.20 (d, J=7.5 Hz, 3H). ^{13}C NMR and APT (100 MHz, $CDCl_3$) δ 174.72 (C), 173.73 (C), 138.00 (CH), 135.57 (C), 99.00 (CH), 78.97 (C), 75.72 (C), 73.38 (CH), 71.50 (CH), 65.02 (2×CH_3), 64.64 (2×CH_2), 55.35 (CH_2), 43.78 (CH), 29.79 (CH_2), 21.25 (CH_3), 18.67 (CH_3), 14.01 (CH_3). IR (NaCl, neat) 3458.2, 2982.2, 2949.8, 2884.9, 1728.4, 1263.2, 1111.8, 1063.0 cm^{-1}. Exact mass calcd for $C_{18}H_{28}NO_9$ m/z 402.176407, found m/z 402.175824.

4.3.6. 5,6,15-Trihydroxy-5,6,7-trimethyl-3,9,14-trioxa-13-aza-tricyclo[8.6.1.013,17] heptadec-1(16)-ene-4,8-dione (9). The hydroxylamine obtained above (5.0 mg, 0.0125 mmol) was dissolved in acetone (1.0 mL) and an aqueous solution of 1% HCl (0.5 mL) was added. The solution was stirred at room temperature for 5 h. The mixture was diluted with ethyl acetate (5 mL) and was extracted with saturated $NaHCO_3$ aq. (5 mL) and brine (5 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography on silica gel (eluted with 1:1 ethyl acetate/ether) (R_f=0.29) to yield 4 mg of compound **9** (91%) as a white solid. The NMR spectrum of product **9** revealed that this substance was as a 1:0.4 mixture of epimers at room temperature at 75°C on the NMR time scale. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.33 (d, J=2.1 Hz, 1H), 5.41–5.38 (m, 1H), 5.08–5.02 (m, 1H), 4.75 (s, 2H), 3.62 (s, 1H,

OH), 3.58 (d, *J*=6.6 Hz, 1H, *OH*), 3.51–3.46 (m, 1H), 3.35–3.31 (m, 1H), 2.99–2.93 (m, 1H), 2.72 (q, *J*=5.7, 11.2 Hz, 1H), 2.71 (bs, 1H, *OH*), 2.63–2.54 (m, 1H), 2.17–2.14 (m, 1H), 1.38 (s, 3H), 1.30 (s, 3H), 1.19 (d, *J*=5.7 Hz, 3H). Exact mass calcd for $C_{16}H_{24}NO_8$ *m*/*z* 358.150192, found *m*/*z* 358.150750.

4.3.7. 5,6-Dihydroxy-15-methoxy-5,6,7-trimethyl-3,9,14-trioxa-13-aza-tricyclo [8.6.1.0] heptadec-1(16)-ene-4,8-dione (13). The hydroxylamine hemi-acetal **9** (12 mg, 0.0337 mmol) was dissolved in 1 mL of MeOH and an excess of *p*-TsOH (10 mg) was added to the solution. The reaction was stirred at room temperature for 7 h, after which the solvent was removed in vacuo. The residue was taken up in EtOAc (5 mL) and washed with saturated $NaHCO_3$ solution (5 mL). The aqueous layer was back-extracted with EtOAc (2×5 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated, and purified via silica gel chromatography using 5% MeOH in CH_2Cl_2 to give 4.5 mg of **13** as a clear oil (50% yield). TLC (5% MeOH/CH_2Cl_2) R_f=0.4 (anisaldehyde). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.26 (d, *J*=4 Hz, 1H), 5.00–4.95 (m, 1H), 4.93 (s, 1H), 4.80–4.71 (dd, *J*=11.6, 11.6 Hz, 2H), 3.71 (d, *J*=6.6 Hz, 1H), 3.62–3.60 (d, *J*=8 Hz, 1H), 3.59 (d, *J*=9 Hz, 1H), 3.56 (s, 3H), 3.14–3.03 (m, 1H), 2.72–2.62 (m, 1H), 2.27–2.14 (m, 1H), 1.44 (s, 3H), 1.36 (s, 3H), 1.25 (d, *J*=6.9 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.04, 173.50, 142.86, 129.81, 96.79, 92.25, 78.59, 75.33, 68.67, 66.24, 55.86, 55.43, 44.73, 34.46, 21.95, 17.65, 13.39. IR (NaCl, neat) 972, 1060, 1113, 1188, 1340, 1732, 3465 cm^{-1}. HRMS (FAB) calcd for $C_{17}H_{25}NO_8$ (MH^+) 372.1658, found 372.1655.

4.3.8. 4-Hydroxymethyl-2-methoxy-4a,5,6,7-tetrahydro-2*H*-pyrrolo[1,2*b*][1,2]oxazin-5-ol (14). Compound **13** (5 mg, 0.0135 mmol) was dissolved in 0.5 mL THF followed by addition of 0.1 mL of H_2O and a solution of $LiO{\cdot}H_2O$ (3 mg, 0.0808 mmol) was added. The reaction appeared complete after 6 h at room temperature. The solvent was removed in vacuo and the residue was taken up in a 1:1 mixture of CH_2Cl_2/MeOH and flashed through a short plug of silica gel using 10:1 CH_2Cl_2/MeOH to give **14** as a clear oil (1 mg, 0.004 mmol) in 40% yield. TLC (10:1 CH_2Cl_2/MeOH) R_f=0.2 (anisaldehyde). ^{1}H NMR (400 MHz)($CDCl_3$) δ 5.97 (d, *J*=3.6 Hz, 1H), 4.87 (s, 1H), 4.45 (s, 1H), 4.23 (d, *J*=3.6 Hz, 2H), 3.58–3.52 (dd, *J*=2.4, 2.8 Hz, 1H), 3.48 (s, 3H), 3.43 (d, *J*=6 Hz, 1H), 3.08–3.11 (m, 1H), 2.46–2.40 (m, 1H), 2.22–2.16 (m, 2H), 2.01 (s, 1H). ^{13}C NMR (75 MHz) ($CDCl_3$) δ 137.14, 122.41, 97.08, 73.71, 69.08, 64.81, 55.54, 36.34, 29.70. IR (NaCl, neat) 968, 1054, 1118, 1338, 1685, 3373 cm^{-1}. HRMS (FAB) calcd for $C_9H_{15}NO_4$ (MH^+) 202.1079, found 202.1075.

4.3.9. Carbamic acid 4-carbamoyloxymethyl-2-methoxy-4α,5,6,7-tetrahydro-2*H*-pyrrolo[1,2-β][1,2]oxazi-5-yl ester (15). The diol **14** (1.4 mg, 0.00696 mmol) was dissolved in 0.5 mL CH_2Cl_2 and cooled to 0°C. The trichloroacetyl isocyanate (2 μL, 0.0174 mmol) was added. The reaction stirred for 2 h (0°C to room temperature). The solvent was removed in vacuo and the residue was taken up in CH_2Cl_2 and loaded onto a plug of neutral alumina. After 0.5 h, the column was flushed with 10:1 CH_2Cl_2/MeOH to give 0.5 mg of **15** as a clear oil. TLC (10:1 CH_2Cl_2/MeOH) R_f=0.15 (anisaldehyde). ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.92 (d, *J*=2.0 Hz, 1H), 5.48–5.32 (m, 1H), 4.89 (d, *J*=2.8 Hz, 1H), 4.64 (d, *J*=12.8 Hz, 1H), 4.5 (d, *J*=12.4 Hz, 1H), 3.54–3.51 (m, 1H), 3.48 (s, 3H), 3.43 (d, *J*=6.4 Hz, 1H), 3.07–2.98 (m, 1H), 2.50–2.41 (m, 1H), 2.23–2.14 (m, 1H). ^{13}C NMR (75 MHz) ($CDCl_3$) δ 156.92, 156.52, 132.13, 123.93, 96.98, 74.60, 67.50, 66.65, 55.67, 34.29, 29.92. IR (NaCl, neat) 968, 1054, 1120, 1180, 1333, 1401, 1608, 1710, 2953, 3353 cm^{-1}. HRMS (FAB) calcd for $C_{11}H_{17}N_3O_6$ (MH^+) 288.1195, found 288.1192.

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