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Expanding the repertoire of small molecule transcriptional activation domains

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We dedicate this paper to Professor Jonathan Ellman in honor of the 2006 Tetrahedron Young Investigator Award in recognition of his many seminal contributions to Bio-organic Chemistry.

Abstract—Molecules that can reconstitute the function of transcriptional activators hold enormous potential as therapeutic agents and as mechanistic probes. Previously we described an isoxazolidine bearing functional groups similar to natural transcriptional activators that up-regulates transcription 80-fold at 1 μM in cell culture. In this study, we analyze analogs of this molecule to define key characteristics of small molecules that function as transcriptional activation domains in cells. Conformational rigidity is an important contributor to function as is an overall amphipathic substitution pattern. Using these criteria, we identified additional molecular scaffolds with excellent (∼60-fold) activity as transcriptional activation domains. These results point the way for the creation of new generations of small molecules with this function.
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1. Introduction

Molecules that reconstitute the function of transcriptional activators, activator artificial transcription factors (activator ATFs), have proven to be powerful tools for parsing the details of transcriptional regulation.[1] Activator ATFs also have outstanding potential as therapeutic agents targeting diseases ranging from cancer to metabolic disorders.[2] In one example, a Sangamo protein-based activator ATF that up-regulates the gene for vascular endothelial growth factor is currently in Phase II clinical trials for diabetic neuropathy.[3] As this example illustrates, protein-based activator ATFs are at the forefront of therapeutic development. There is, however, considerable interest in the development of small molecule activator ATFs with improved stability, delivery, and immunogenicity characteristics.[4]

On the surface, the design of an activator ATF is straightforward. A natural transcriptional activator minimally consists of a DNA binding domain (DBD) and a transcriptional activation domain (TAD) that are linked by either covalent or non-covalent interactions.[4] These domains operate largely independently, meaning that the DNA binding domain of one activator can be coupled to the transcriptional activation domain of a second activator without losing function.[5] Thus, a modular replacement strategy can be used to construct an activator ATF, replacing one or both of the two key domains with non-natural counterparts. Although many different classes of DBDs have been developed and used for activator ATF construction,[4] one of the prevailing challenges has been the identification of small molecules that can be used as transcriptional activation domains, with only two examples reported to date[6,7]; peptidomimetic TADs have also been described.[8] Amphipathic isoxazolidine **1** was the first example, initially reported in 2004.[6] The early studies of this molecule were in cell free systems and the data from these studies suggested that the molecule functions mechanistically like a natural TAD.[6,9] More recently, we disclosed that this molecule also functions well in human cell culture, with a maximal activity of 80-fold activation at a concentration of 1 μM.[10] In contrast, a more hydrophobic version, compound **2**, shows no detectable activity over the same concentration range. Here, we describe the synthesis and activity of analogs of **1** that define

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general characteristics of a small molecule transcriptional activation domain with activity in cells. In addition, we show that using these criteria, new molecular scaffolds with TAD function can be identified.

2. Results and discussion

Isoxazolidine **1** was originally designed as a generic mimic of the amphipathic class of transcriptional activation domains, a group defined by the characteristic pattern of hydrophobic amino acids interspersed with more polar amino acid residues.[1] While typically lacking structure when in an unbound state, the current model is that they fold into an amphipathic helix when bound to their transcriptional machinery binding partners, although other secondary structures may also play a role.[11–14] The hydrophobic residues are generally the strongest contributors to binding and ultimately to activator function.[15–17] Thus, **1** was designed to contain amphipathic functional groups commonly found in endogenous TADs arrayed on a conformationally constrained scaffold, the isoxazolidine ring. Analogs **3** and **4** were designed to examine the importance of conformational constraint in activity. Compound **3** is a ring-opened analog of **1** in which the N–O bond has been cleaved whereas **4** is a D-peptide containing the same functional groups as **1**.[18,19] Isoxazolidines **5–13** were designed to assess the tolerance limit of hydrophobic and polar substituents at N2 and C3, respectively (see Fig. 1).

The analogs of **1** were prepared in a straightforward manner from a common intermediate, isoxazolidine **14** (Schemes 1 and 2).[6] Alkylation under microwave-accelerated conditions was used to install the functional groups at N2, and this was followed by the removal of the silyl protecting group under basic conditions. For-
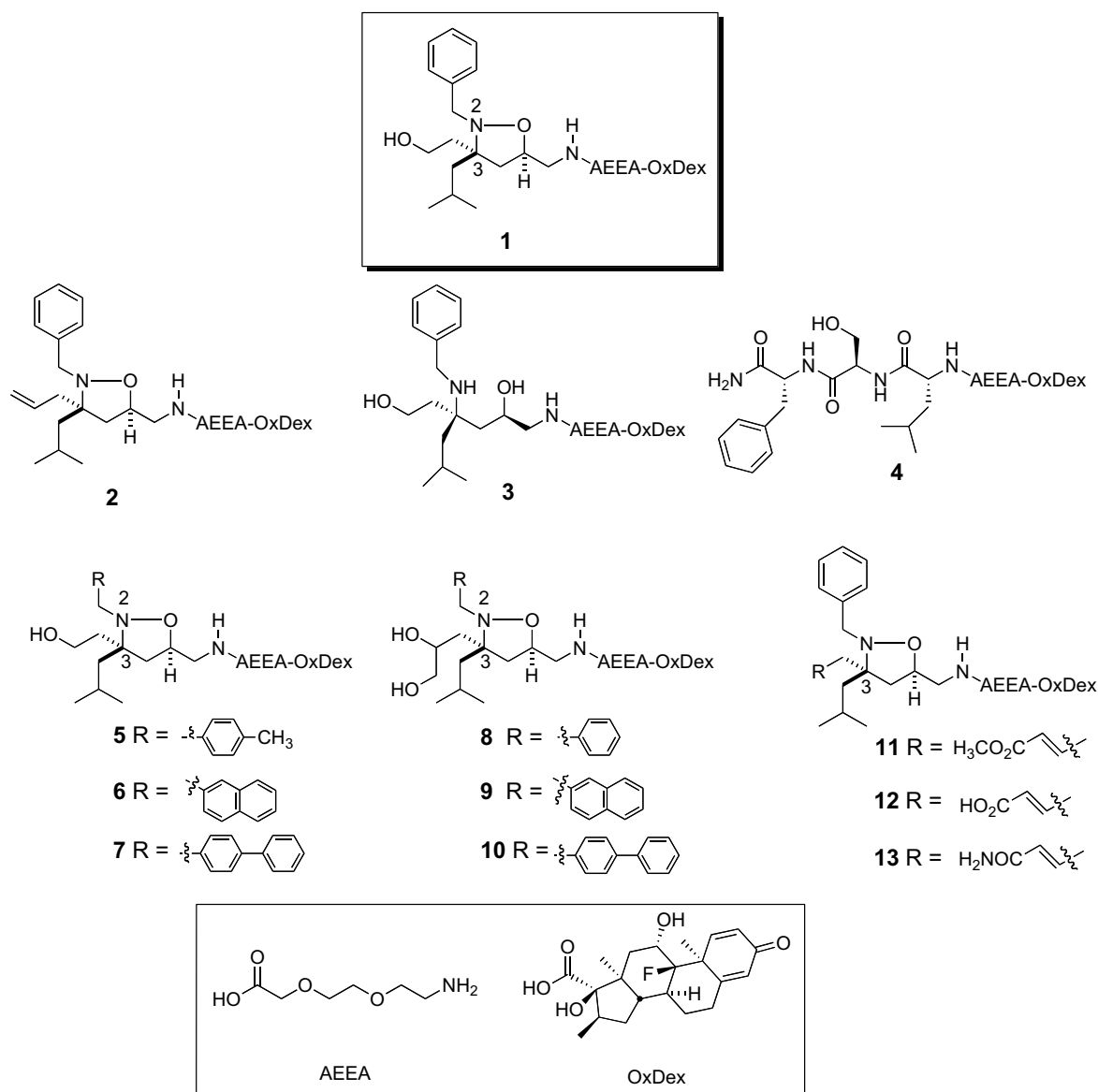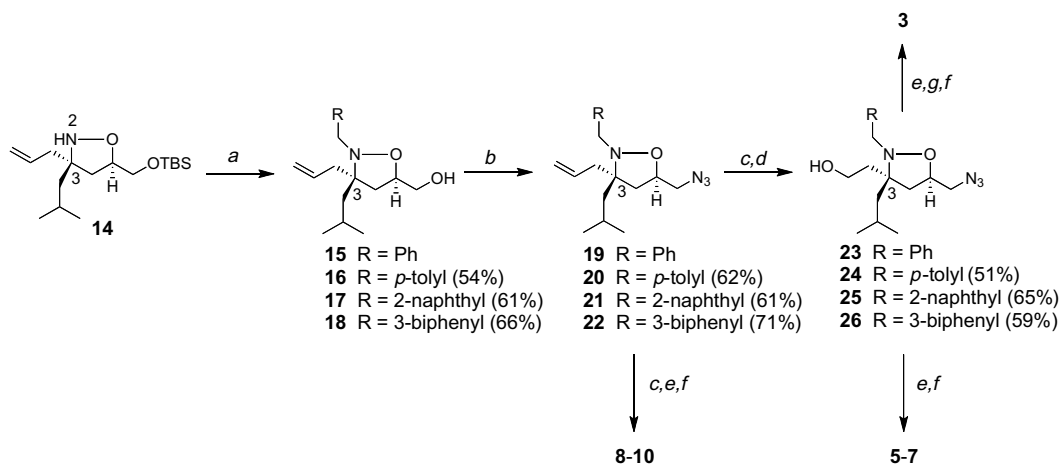

Figure 1. Structures of isoxazolidine analogs.

Scheme 1. Reagents and conditions: (a) i—RBr, *i*-Pr₂NEt, DMF, microwave; ii—TBAF, THF, rt; (b) i—Ms-Cl, Et₃N, DCM, rt; ii—NaN₃, DMF, 85 °C; (c) OsO₄, NMO, *t*-BuOH, THF, H₂O, rt; (d) i—NaIO₄, CH₃CN, H₂O, rt; ii—NaBH₄, MeOH, 0 °C–rt; (e) i—PPh₃, H₂O, THF, reflux; ii—Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AEEA), HOBt, HBTU, Et₃N, NMP, rt; iii—20% piperidine, DMF, rt; (f) OxDex, HOBt, HBTU, *i*-Pr₂NEt, 2,6-lutidine, NMP, rt; (g) Zn, AcOH, rt.
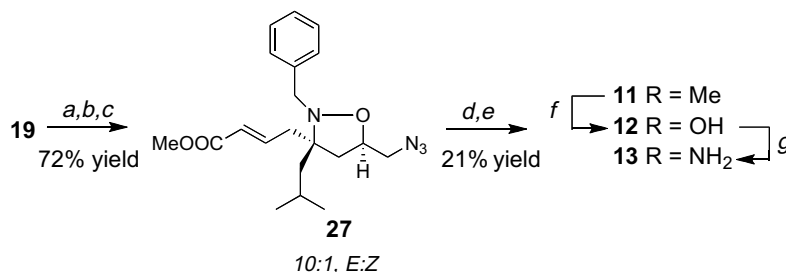
Scheme 2. Reagents and conditions: (a) OsO₄, NMO, *t*-BuOH, THF, H₂O, rt; (b) NaIO₄, CH₃CN, H₂O, rt; (c) methyl (triphenylphosphoranylidene) acetate, toluene, 60 °C; (d) i—PPh₃, H₂O, THF, reflux; ii—Fmoc-AEEA, HOBt, HBTU, Et₃N, NMP, rt; iii—20% piperidine, DMF, rt; (e) OxDex, HOBt, HBTU, *i*-Pr₂NEt, 2,6-lutidine, NMP, rt; (f) NaOH, EtOH, rt, (31%); (g) NH₄HCO₃, Boc₂O, pyr, CH₃CN, rt, (33%).

mation of a mesylate and its subsequent displacement by azide anion provided isoxazolidines **19–22**. Oxidation of the C3 allyl group enabled installation of a hydroxyl (**5–7**), a diol (**8–10**), an ester (**11**), a carboxylic acid (**12**), and amide (**13**) at this position. The ring-opened variant of **1**, analog **3**, was produced by treatment of isoxazolidine **23** with Zn(0) in acidic media. For each analog, the final reaction sequence installed the DNA targeting moiety using the C5 azide handle to produce a primary amine. This was subsequently coupled to a short polar linker (Fmoc-8-amino-3,6-dioxaoctanoic acid, Fmoc-AEEA) and then to an oxidized form of dexamethasone (Ox-Dex). The OxDex moiety is used to target the isoxazolidines to DNA as the cells in which activity assays are run express a fusion protein, Gal4(1–147) + GR, consisting of the DNA binding domain of Gal4 linked to the minimal ligand binding domain of the glucocorticoid receptor (GR), a domain with which OxDex interacts strongly.[20]

The ability of the molecules to function as transcriptional activation domains was assessed using a standard luciferase assay in HeLa cells.[10,20] Briefly, OxDex conjugates are incubated with cells containing a firefly luciferase reporter under the control of five Gal4 binding sites in addition to a plasmid expressing Gal4(1–147) + GR. Firefly luciferase activity thus provides a direct readout of the efficiency with which the molecules function as TADs. The cells also contain a *Renilla* luciferase reporter lacking Gal4 sites and this serves as an internal control to assess impact on general transcription. In each experiment, the normalized luciferase activity observed at a given concentration of small molecule is compared to that obtained with AEEA + OxDex alone, giving rise to the fold activation values shown. As illustrated in Figure 2, both **3** and **4** exhibited attenuated activity relative to **1** (maximal activity of 80-fold at 1 μM). Diol **3** had low activity at 1 μM (~11-fold) that increased to 40.9-fold at 10 μM and an EC_{50} of 2.5 μM, corresponding to an approximately 100-fold increase relative to **1** (33 nM). Tripeptide **4** was even less active, with 24-fold up-regulation at 10 μM and an EC_{50} of 1.86 μM. Of the two, **4** has the greater conformational flexibility, with rotatable bonds connecting each of the three side chains. In contrast, compound **3**, although more flexible than **1**, still has the three key side chains affixed in a radial array and it is possible that an internal hydrogen bond between the C3–OH and the amine could limit conformational flexibility. Thus, although conformational constraint is an important contributor to overall function, a ring is not required for activity.
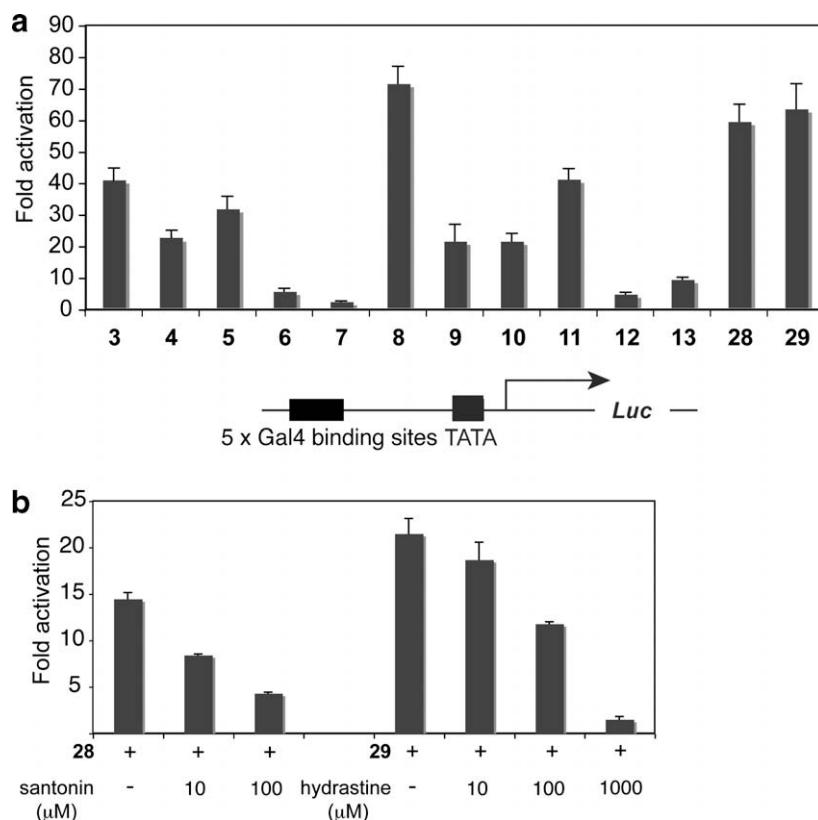
Figure 2. Results from luciferase assays in HeLa cell culture. (a) Activity of OxDex-conjugates is expressed as fold activation relative to OxDex-AEEA alone. Briefly, HeLa cells were transfected with a plasmid expressing the Gal4(1–147) + GR fusion protein, a second plasmid bearing 5 Gal4-binding sites upstream of a firefly luciferase reporter gene, and a third plasmid expressing *Renilla* luciferase as a transfection control, as has been previously described.[9,12] Compounds were added to the cells as a 1 or 10 mM DMSO solution 3 h after transfection such that the final concentration of DMSO in all wells was 1% (v/v). The final concentration of each compound was 10 μM for all compounds (1 μM data is shown as Fig. S1 in the Supporting Information). The firefly and *Renilla* luciferase activities were measured 40 h after compound addition. Fold activation was determined at each concentration by first dividing the firefly luciferase activity by that of *Renilla* luciferase. This value was then divided by the amount of activity observed with OxDex + AEEA alone. Each value is the average of at least three independent experiments with the indicated error (SDOM). (b) Results from competitive inhibition experiments with **28** and **29**. Luciferase assays were carried as described for (a) with 100 nM of **28** or 1 μM of **29**. Increasing concentrations of either hydrastine (for compound **28**) or santonin (for compound **29**) were added to the cells to assess if the DNA-bound and free forms of the molecules were targeting the same binding sites.

As described earlier, the second key aspect of the design of **1** was its amphipathic character. More specifically, the functional groups at N2 and C3 were designed to mimic amino acids often identified as functionally important in natural TADs: phenylalanine and leucine; serines are also common constituents.[17,21,22] As illustrated in Figure 2, alteration of the N2 and C3 functional groups on the isoxazolidine scaffold most often lead to functional molecules, but maximal activity was achieved only at higher concentrations relative to **1** (10 μM). More specifically, the N2 benzyl group (Phe mimic) can be replaced with a larger substituent such as tolyl (**5**, 31-fold up-regulation at 10 μM) but larger hydrophobic groups result in significant diminuation of activity (**6**, 5-fold; **7**, 1.9-fold).

Given the many different molecular recognition events that TADs participate in both extra- and intracellularly as part of transcriptional activation and that these interactions are poorly characterized,[1] it is difficult to pin-point the origin of the decreased activity observed with isoxazolidines **6** and **7**. It has, however, been demonstrated that hydrophobic peptides and small molecules function modestly as transcriptional activators despite exhibiting good affinity for the transcriptional machinery; there is some evidence that this is due to increased non-specific binding events leading to sequestration and/or degradation.[11,23,24] Further, this effect can be ameliorated through the incorporation of masking interactions that effectively decrease the hydrophobic surface area.[24] In the case of the isoxazolidines, we observed a similar trend upon incorporation of a more polar group at C3. For **9** and **10**, a diol moiety at C3 restored significant activity, with these molecules up-regulating approximately 20-fold at 10 μM. In addition, a hydrogen bond donor is not required to be the polar group at C3, as the activity of methyl ester **11** demonstrates (41-fold activity). Increasing the polarity of the substituent as in amide **13** and acid **12**, however, does result in a further loss of activity (9- and 4-fold, respectively).

Many different amphipathic peptide sequences function as transcriptional activation domains,[25] and it seemed likely that more than one amphipathic small molecule scaffold should also function as a transcriptional activation domain. To narrow the search, we sought structures

Scheme 3. Reagents and conditions: (a) 4,7,10-trioxa-1,13-tridecanediamine, DMAP, *i*-Pr$_2$NEt, DMF, rt; (b) OxDex, HOBt, HBTU, *i*-Pr$_2$NEt, 2,6-lutidine, NMP, rt.

that displayed some degree of conformational rigidity and were amphipathic. With regard to the latter criterion, we noted that the most active isoxazolidines fell within a relatively narrow log *P* range (2.5–3.1, calculated without the AEEA–OxDex moiety using Osiris Properties calculator[26]) and used this as an approximate assessment of amphipathic character. Two structures that fell into this category were the commercially available natural products santonin and hydrastine (Scheme 3). Santonin is an anti-parasitic agent commonly employed as a synthetic intermediate and hydrastine is a GABA receptor inhibitor often used for the study of GABA-linked signaling.[27–29] In addition to having log *P* values similar to that of **1** and containing distinct hydrophobic surfaces, both structures contain a lactone used for installation of the OxDex moiety (Scheme 3). Remarkably, both structures showed good activity, with 59- and 63-fold up-regulation at 10 μM, respectively (Fig. 2a). In both cases, competitive inhibition experiments with the original natural product verified that it was the DNA-bound form of the molecule that was functioning as a transcriptional activation domain (Fig. 2b). In addition, neither molecule had any effect on a reporter gene that did not have DNA binding sites for Gal4(1–147) + GR. Taken together, these data suggest that the molecules function similar to natural TADs and the isoxazolidine TADs. Further, the general TAD descriptors used to identify santonin and hydrastine should enable to the discovery of an even wider array of small molecule TADs.

2.1. Future directions

In conclusion, we have identified several new isoxazolidine-based transcriptional activation domains as well as two unique TAD scaffolds, thus significantly expanding the repertoire of small molecule transcriptional activation domains for the targeted control of gene expression. The design criteria are simple and should facilitate the discovery of future classes of novel TADs. However, analogous to the natural system, it is not yet possible a priori to predict how well a given small molecule TAD will function. This is a reflection of the dearth of detailed mechanistic information about how TADs, natural and unnatural, function.[1] For example, for most natural activators the physiologically relevant

binding partners in the transcriptional machinery are not known, rendering detailed thermodynamic and structural characterization of activator complexes challenging. In this regard, this class of small molecules may prove to be powerful tools to investigate these questions, as modified variants containing photo-activatable groups suitable for cross-linking are synthetically readily accessible.

3. Experimental

3.1. General

Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. CH$_2$Cl$_2$ and THF were dried by passage through activated alumina columns. NMP and DMF were used as purchased without further purification. Et$_3$N was distilled from CaH$_2$. Purification by flash chromatography was carried out with E. Merck Silica Gel 60 (230–400 mesh). ^{1}H and ^{13}C NMR spectra were recording in CDCl$_3$ at 400 and 100 MHz, respectively, unless otherwise specified. Reverse-phase HPLC purifications were performed on a Varian ProStar 210 equipped with Rainin Dynamax UV-D II detector ($\lambda = 254$ nm and $\lambda = 214$) using a C18 (8 × 100 mm) Radial-Pak™ cartridge with a gradient of 0.1% TFA in H$_2$O and CH$_3$CN as the mobile phase. Isoxazolidines **15**, **19**, and **23** were prepared as previously reported.[9,12] The structures of intermediates not shown in Schemes 1–3 can be found in Supporting Information (Scheme S1) and are numbered **S1**–**S6**.

3.1.1. OxDex + AEEA + D-LSF (4).
The tripeptide D-LeuSerPhe was prepared using solid phase peptide synthesis in accordance with standard procedures with the AEEA linker and OxDex added on solid support at the conclusion of the synthesis.[20,30] The crude product isolated after resin cleavage was purified by reverse phase HPLC to provide 3.5 mg of **4** (15% yield) as a white solid. HRMS (ESI) calcd for [C$_{49}$H$_{68}$FN$_3$O$_{10}$ + Na]$^+$: 892.4484, found: 892.4493.

3.1.2. [3-Allyl-3-isobutyl-2-(4-methyl-benzyl)-isoxazolidin-5-yl]-methanol (16).
To a solution of isoxazolidine

14[6] (0.50 g, 1.6 mmol, 1.0 equiv; 6:1 mixture of diastereomers) in DMF (7.5 mL) were added α-bromo-*p*-xylene (1.5 g, 8.0 mmol, 5.0 equiv) and *i*-Pr$_2$NEt (1.4 mL, 8.0 mmol, 5.0 equiv). The reaction mixture was irradiated in a 1000 W microwave oven (6 × 20 s) at 20% power with mixing between each interval. Once the reaction was complete as judged by TLC analysis, the reaction mixture was diluted with water (60 mL) and extracted with EtOAc (3× 30 mL). The combined organic extracts were washed with water (1× 30 mL), dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo to a yellow oil. Following N-alkylation, the crude reaction mixture was dissolved in THF (4.5 mL) and TBAF was added (3.0 mL of a 1 M solution in THF, 3.0 mmol, 1.8 equiv based on starting material **14**). The mixture was allowed to stir for 6 h at which time the reaction mixture was diluted with water (60 mL) and extracted with EtOAc (3× 30 mL). The combined organic extracts were dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo to a yellow oil. The crude material was purified by flash chromatography (85:15 hexanes/EtOAc) to provide 0.26 g (54% yield) of **16** as a colorless oil. The major diastereomer was isolated by normal-phase HPLC and used in subsequent steps. ^{1}H NMR: δ 0.98 (d, 3H, *J* = 3.4), 1.01 (d, 3H, *J* = 3.4), 1.40 (dd, 1H, *J* = 14.7, 7.0), 1.63 (dd, 1H, *J* = 14.4, 4.7), 1.83–1.98 (m, 1H), 2.05 (dd, 1H, *J* = 12.3, 5.9), 2.24–2.51 (m, 7H), 3.51–3.65 (m, 2H), 3.77–3.91 (m, 2H), 4.02–4.12 (m, 1H), 5.08–5.18 (m, 2H), 5.86–6.02 (m, 1H), 7.10–7.16 (m, 2H), 7.22–7.28 (m, 2H); ^{13}C NMR: δ 21.09, 24.10, 24.48, 25.29, 38.62, 38.78, 43.92, 53.18, 65.61, 68.55, 75.38, 117.7, 127.8, 129.1, 135.1, 135.7, 136.5; HRMS (ESI) calcd for [C$_{19}$H$_{29}$NO$_2$ + H]$^+$: 304.2277, found: 304.2274.

3.1.3. [3-Allyl-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-5-yl]methanol (17).

Preparation of **17** was accomplished under conditions identical to those used for **16** using 2-(bromomethyl)-naphthalene in place of α-bromo-*p*-xylene. Purification by flash chromatography (9:1 hexanes/EtOAc) afforded 0.22 g of **17** as a colorless oil in 61% yield. ^{1}H NMR: δ 0.98 (d, 3H, *J* = 6.8), 0.99 (d, 3H, *J* = 6.8), 1.41 (dd, 1H, *J* = 14.4, 6.8), 1.64 (dd, 1H, *J* = 14.4, 4.8), 1.88–1.95 (m, 1H), 2.01–2.06 (m, 1H), 2.21 (m, 2H), 2.28 (dd, 1H, *J* = 12.5, 8.8), 2.46 (dd, 1H, *J* = 14.0, 7.0), 3.49–3.56 (m, 2H), 3.95–4.08 (m, 3H), 5.10–5.14 (m, 2H), 5.91–6.01 (m, 1H), 7.40–7.43 (m, 2H), 7.50–7.52 (m, 2H), 7.75–7.79 (m, 3H); ^{13}C NMR: δ 24.14, 24.48, 25.27, 38.68, 38.88, 44.00, 53.66, 65.53, 68.54, 75.49, 117.7, 125.5, 125.9, 126.2, 126.5, 127.6, 127.7, 128.0, 132.7, 133.4, 135.1, 136.4; HRMS (ESI) calcd for [C$_{22}$H$_{29}$NO$_2$ + Na]$^+$: 362.2096, found: 362.2084.

3.1.4. (3-Allyl-2-(biphenyl-4-ylmethyl)-3-isobutylisoxazolidin-5-yl)methanol (18).

Preparation of **18** was accomplished under conditions identical to those used for **16** using 4-(bromomethyl)biphenyl in place of α-bromo-*p*-xylene. Purification by flash chromatography (7:3 hexanes/EtOAc) provided 0.39 g of **18** in 66% yield as a colorless oil. The major diastereomer was isolated by normal-phase HPLC and used for subsequent steps. ^{1}H NMR (400 MHz): δ 0.96 (d, 3H, *J* = 6.4), 0.97 (d, 3H, *J* = 6.8), 1.38 (dd, 1H, *J* = 15.0, 6.8), 1.61 (dd, 1H, *J* = 14.4, 4.8), 1.86–1.92 (m, 1H), 2.01–2.05 (m, 1H), 2.21 (m, 2H), 2.28 (dd, 1H, *J* = 8.2, 4.6), 2.44 (dd, 1H, *J* = 14.0, 7.2), 3.53–3.63 (m, 2H), 3.85 (d, 1H, *J* = 14.4), 3.92 (d, 1H, *J* = 14.4), 4.04–4.09 (m, 1H), 5.08–5.12 (m, 2H), 5.88–5.98 (m, 1H), 7.28–7.31 (m, 4H), 7.37–7.42 (m, 1H), 7.51–7.56 (m, 4H); ^{13}C NMR δ 24.09, 24.48, 25.26, 38.64, 38.77, 43.29, 53.14, 65.58, 68.51, 75.45, 117.7, 127.0, 127.2, 128.2, 128.7, 133.9, 135.1, 137.9, 139.8, 141.0; HRMS (ESI) calcd for [C$_{24}$H$_{31}$NO$_2$ + Na]$^+$: 388.2252, found: 388.2253.

3.1.5. 3-Allyl-5-azidomethyl-3-isobutyl-2-(4-methyl-benzyl)-isoxazolidine (20).

To a solution of isoxazolidine **16** (80 mg, 0.26 mmol, 1.0 equiv), in CH$_2$Cl$_2$ (2.6 mL) was added Et$_3$N (70 μL, 0.53 mmol, 2.0 equiv). To this solution was added methanesulfonyl chloride (40 μL, 0.53 mmol, 2.0 equiv). The solution was stirred at ambient temperature for 4 h at which time the reaction was complete as judged by ESI-MS analysis. The reaction mixture was concentrated in vacuo and the crude residue was dissolved in EtOAc (20 mL) and water (40 mL). The aqueous and organic layers were separated and the water layer was extracted with EtOAc (3× 20 mL). The combined organic extracts were washed with water (1× 30 mL), dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo to a yellow-orange oil. The crude material was dissolved in DMF (2.6 mL) and sodium azide was added (0.17 g, 2.6 mmol, 10 equiv based on starting material **16**) to the solution. The solution was heated to 85 °C and was allowed to stir for 8 h at which time the reaction was complete as judged by TLC analysis. The reaction mixture was diluted with water (15 mL) and the aqueous layer was extracted with EtOAc (3× 40 mL). The combined organic fractions were washed with water (1× 30 mL), dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo to a yellow oil. The crude material was purified by flash chromatography (97:3 hexanes/EtOAc) to provide 53 mg (62% yield) of **20** as a colorless oil. ^{1}H NMR: δ 0.98 (d, 3H, *J* = 2.6), 1.01 (d, 3H, *J* = 2.3), 1.40 (dd, 1H, *J* = 14.7, 7.0), 1.40 (dd, 1H, *J* = 14.7, 6.5), 1.62 (dd, 1H, *J* = 14.7, 5.0), 1.8–1.95 (m, 2H), 2.23–2.50 (m, 7H), 3.11 (dd, 1H, *J* = 12.6, 4.4), 3.45 (dd, 1H, *J* = 12.6, 7.0), 3.76–3.92 (m, 2H), 4.06–4.17 (m, 1H), 5.07–5.19 (m, 2H), 5.86–6.02 (m, 1H), 7.10–7.16 (m, 2H), 7.23–7.30 (m, 2H); ^{13}C NMR: δ 21.09, 24.15, 24.67, 25.25, 38.74, 40.31, 45.60, 53.10, 54.45, 68.36, 74.42, 117.8, 128.0, 128.9, 135.0, 135.6, 136.3; HRMS (ESI) calcd for [C$_{19}$H$_{28}$N$_4$O$_2$ + H]$^+$: 329.2341, found: 329.2336.

3.1.6. 3-Allyl-5-(azidomethyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidine (21).

Preparation of **21** was accomplished under conditions identical to those used for **20** starting with 0.93 mmol of **17**. Purification by flash chromatography (9:1 hexanes/EtOAc) afforded 0.22 g of **21** as a colorless oil in 61% yield. ^{1}H NMR: δ 0.98 (d, 3H, *J* = 6.8), 1.0 (d, 3H, *J* = 6.8), 1.41 (dd, 1H, *J* = 14.4, 6.4), 1.63 (dd, 1H, *J* = 14.4, 4.8), 1.84–1.93 (m, 2H), 2.27–2.48 (m, 5H), 3.09 (d, 1H, *J* = 8.4), 3.43 (d, 1H, *J* = 12.8, 7.2), 4.09–4.15 (m, 1H), 5.09–5.12 (m, 2H), 5.91–5.99 (m, 1H), 7.37–7.43 (m, 2H), 7.50–7.52 (m, 2H), 7.75–7.77 (m, 3H); ^{13}C NMR δ

24.18, 24.63, 25.24, 29.67, 38.75, 40.33, 43.66, 53.60, 54.39, 68.47, 74.54, 117.9, 125.3, 125.7, 126.5, 126.6, 127.6, 127.8, 132.7, 133.4, 134.9, 136.2; HRMS (ESI) calcd for $[C_{22}H_{28}N_4O + Na]^+$: 387.2008, found: 387.2005.

3.1.7. 3-Allyl-5-(azidomethyl)-2-(biphenyl-4-ylmethyl)-3-isobutylisoxazolidine (22).

Preparation of **22** was accomplished under conditions identical to those used for **20** starting with 0.96 mmol of **18**. Purification by flash chromatography (9:1 hexanes/EtOAc) to afford 0.28 g of **22** as a colorless oil in 71% yield. ^{1}H NMR: δ 0.96 (d, 3H, $J = 6.6$), 0.97 (d, 3H, $J = 6.6$), 1.38 (dd, 1H, $J = 14.4$, 6.2), 1.60 (dd, 1H, $J = 14.4$, 4.8), 1.84–1.91 (m, 2H), 2.24–2.33 (m, 2H), 3.10 (dd, 1H, $J = 11.0$, 4.0), 3.44 (dd, 2H, $J = 12.8$, 7.2), 3.89 (s, 2H), 4.08–4.14 (m, 1H), 5.08–5.12 (m, 2H), 5.87–5.97 (m, 1H), 7.22–7.31 (m, 4H), 7.37–7.42 (m, 1H), 7.50–7.56 (m, 4H); ^{13}C NMR: δ 24.13, 24.64, 25.22, 38.74, 40.28, 53.04, 54.43, 68.38, 74.49, 117.9, 126.9, 127.1, 128.4, 128.6, 134.9, 137.7, 139.7, 141.2; HRMS (ESI) calcd for $[C_{24}H_{30}N_4O + Na]^+$: 413.2317, found: 413.2312.

3.1.8. 2-(5-Azidomethyl-2-(4-methyl-benzyl)-3-isobutyl-isoxazolidin-3-yl)-ethanol (24).

To a solution of **20** (53 mg, 0.16 mmol, 1.0 equiv) dissolved in *t*-BuOH (1.1 mL), THF (0.40 mL), and H_2O (0.080 mL) was added NMO (26 mg, 0.22 mmol, 1.4 equiv). To this solution was added OsO_4 (0.16 mL of a 2.5 wt% solution in *t*-BuOH, 0.016 mmol, 0.10 equiv). The reaction mixture was allowed to stir at ambient temperature for 5 h at which time the reaction was complete as judged by TLC analysis (4:6 hexanes/EtOAc). Excess reagents were quenched by the addition of $Na_2S_2O_3$ (1.0 mL of 10% solution w/v in water) and allowed to stir for 3 h. The mixture was then poured into a biphasic mixture of H_2O (40 mL) and EtOAc (40 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (3× 30 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo to provide the crude material as a tan oil. The crude product was dissolved in CH_3CN (800 μL) and H_2O (800 μL) and added $NaIO_4$ (68 mg, 0.32 mmol, 2.0 equiv based on starting material **20**). The mixture was allowed to stir at rt until the reaction was complete as judged by TLC analysis (9:1 hexanes/EtOAc). The reaction mixture was poured into a biphasic solution of H_2O (60 mL) and EtOAc (35 mL). Brine (15 mL) was added to separate the emulsion. The organic layer was separated and the aqueous layer was extracted with EtOAc (2× 35 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo to provide the crude material as a tan oil. Crude aldehyde was dissolved in methanol (1.6 mL) and the solution was cooled in an ice–H_2O bath. $NaBH_4$ (14 mg, 0.40 mmol, 2.5 equiv based on starting material **20**) was added and the mixture was then allowed to stir until the reaction was complete as judged by TLC analysis (0.5 h, 1:1 hexanes/EtOAc). Excess reagents were quenched with H_2O (2 mL) and the reaction solution was poured into a biphasic mixture of EtOAc (25 mL) and H_2O (25 mL). The organic layer was separated and the aqueous phase was extracted with EtOAc (2× 25 mL). The combined

organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude material was purified by flash chromatography (1:1 hexanes/EtOAc) to provide 27 mg of the title compound (**24**) in 51% yield (from **20**) as a colorless oil. ^{1}H NMR: δ 0.97–1.02 (m, 6H), 1.46–1.57 (m, 1H), 1.59–1.79 (m, 4H), 1.80–1.91 (m, 1H), 1.98–2.07 (m, 1H), 2.23–2.36 (m, 4H), 3.25–3.48 (m, 2H), 3.71–3.96 (m, 4H), 4.16–4.27 (m, 1H), 7.06–7.22 (m, 4H); ^{13}C NMR: δ 21.09, 24.29, 24.86, 25.21, 35.49, 39.97, 42.80, 53.59, 54.24, 59.64, 70.65, 73.22, 128.6, 129.0, 134.5, 136.8; HRMS (ESI) calcd for $[C_{18}H_{28}N_4O_2 + Na]^+$: 355.2110, found: 355.2105.

3.1.9. 2-(-5-(Azidomethyl)-3-isobutyl-2-(naphthalen-2-yl-methyl)isoxazolidin-3-yl)ethanol: (25).

Preparation of **25** was accomplished under conditions identical to those used for **24** using 0.16 mmol of **21**. Purification by flash chromatography (1:1 hexanes/EtOAc) provided 53 mg of **25** in 65% yield as a colorless oil. ^{1}H NMR: δ 0.89–0.90 (m, 6H), 1.41–1.48 (m, 1H), 1.60–1.79 (m, 4H), 1.96–2.00 (m, 2H), 2.21–2.26 (m, 1H), 3.24–3.33 (m, 2H), 3.75–3.85 (m, 4H), 3.98–4.12 (m, 1H), 7.11 (m, 2H), 7.29–7.35 (m, 2H), 7.61–7.70 (m, 3H). HRMS (ESI) calcd for $[C_{21}H_{28}N_4O_2 + Na]^+$: 391.2110, found: 391.2105.

3.1.10. 2-(-5-(Azidomethyl)-2-(biphenyl-4-ylmethyl)-3-iso-butylisoxazolidin-3-yl)ethanol (26).

Preparation of **26** was accomplished under conditions identical to those used for **25** using 0.16 mmol of **22**. Purification by flash chromatography (1:1 hexanes/EtOAc) provided 50 mg of **26** in 59% yield as a colorless oil. ^{1}H NMR: 0.98–1.0 (m, 6H), 1.52–1.60 (m, 1H), 1.67–1.87 (m, 4H), 2.04–2.09 (m, 2H), 2.31–2.36 (m, 1H), 3.39 (m, 2H), 3.80–4.00 (m, 4H), 4.20–4.24 (m, 1H), 7.27–7.55 (9H). HRMS (ESI) calcd for $[C_{23}H_{30}N_4O_2 + Na]^+$: 417.2266, found: 417.2258.

3.1.11. 9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[a]-phenanthrene-17-carboxylic acid [2-(2-{[(2-(4-methyl-ben-zyl-3-(2-hydroxy-ethyl)-3-isobutyl-isoxazolidin-5-ylmethyl)-carbamoyl]-methoxy}-ethoxy)-ethyl]-amide (5).

To a solution of azide **24** (5.0 mg, 0.015 mmol, 1.0 equiv) in THF (0.17 mL) were added triphenylphosphine (8.0 mg, 0.030 mmol, 2.0 equiv) and H_2O (2.4 μL, 0.15 mmol, 10 equiv). The mixture was allowed to stir at reflux for 2 h. H_2O (0.17 mL) was added to the reaction and the mixture was allowed to stir in a 70 °C-oil bath for an additional 20 min. The mixture was allowed to cool to room temperature and was then transferred into a biphasic mixture of 1 M HCl (10 mL) and ether (10 mL). The layers were partitioned and the organic layer was extracted with 1 M HCl (2× 10 mL). The combined aqueous layers were basified with 3 M NaOH (until pH 10 or greater). The aqueous mixture was then extracted with CH_2Cl_2 (3× 15 mL) and the combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo. To a solution of 8-(9-fluorenylmethyloxycarbonyl-amino)-3,6-dioxaoctanoic acid (18 mg, 0.045 mmol, 3.0 equiv based on starting material **24**) dissolved in NMP (0.067 mL) were added HOBt (6.3 mg, 0.045 mmol, 3.0 equiv based on starting

material **24**) and HBTU (17 mg, 0.045 mmol, 3.0 equiv based on starting material **24**). This solution was agitated for 15 min. The solution of activated ester was added to the crude amine dissolved in NMP (0.067 mL) and the resulting mixture was allowed to stir for 12 h at which time the reaction was complete as judged by ESI-MS analysis. Excess reagents were quenched by the addition of 1M HCl (10 mL) and EtOAc (10 mL). The reaction vessel was washed with EtOAc (2× 2.0 mL) to remove all residues. The resulting biphasic mixture was separated and the aqueous layer was extracted with EtOAc (3× 10 mL). The combined organic fractions were dried over Na_2SO_4, filtered, and concentrated in vacuo. The resulting oil was dissolved in a solution of 20% piperidine in DMF (0.037 mL, 0.076 mmol piperidine, 5.0 equiv based on starting material **24**) and was allowed to stir for 30 minutes. The resulting solution was diluted with 0.1% TFA H_2O (0.50 mL) and CH_3CN (0.50 mL) and partially purified by reverse-phase HPLC to remove Fmoc byproducts. The partially purified amine was used immediately in subsequent steps. To a solution of Ox-Dex[20] (17 mg, 0.045 mmol, 3.0 equiv) dissolved in NMP (0.15 mL) were added HOBt (6.2 mg, 0.045 mmol, 3.0 equiv) and HBTU (17 mg, 0.045 mmol, 3.0 equiv) and the resulting mixture was agitated for 15 min. To this solution were added the amine dissolved in NMP (0.15 mL), 2,6-lutidine (35 μL, 0.30 mmol, 6.7 equiv), and *i*-Pr$_2$NEt (49 μL, 0.30 mmol, 6.7 equiv). The resulting mixture was stirred for 12 h at ambient temperature. The product was isolated by reverse-phase HPLC purification to provide **5** as a white solid (7% yield from **24**; 0.8 mg). The purity of compound **5** was confirmed by analytical reverse-phase HPLC analysis. The identity was verified by mass spectral analysis of the isolated compound. HRMS (ESI) calcd for $[C_{45}H_{64}FN_3O_8 + H]^+$: 812.4861, found: 812.4885.

3.1.12. 9-Fluoro-11,17-dihydroxy-*N*-(2-(2-(2-(((5R)-3-(2-hydroxyethyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-5-yl)methylamino)-2-oxoethoxy)ethoxy)ethyl)-8,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxamide (6). Preparation of was accomplished under conditions identical to those used for **5**. Purification by reverse phase HPLC providing 0.8 mg of **6** (6% yield) as a white solid. HRMS (ESI) calcd for $[C_{48}H_{66}FN_3O_9 + H]^+$: 848.4861, found: 848.4865.

3.1.13. *N*-(2-(2-(2-((2-(Biphenyl-4-ylmethyl)-3-(2-hydroxyethyl)-3-isobutylisoxazolidin-5-yl)methylamino)-2-oxoethoxy)ethoxy)ethyl)-9-fluoro-11,17-dihydroxy-8,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxamide (7). Preparation of was accomplished under conditions identical to those used for **5**. Purification by reverse phase HPLC providing 1.0 mg of **7** (8% yield) as a white solid. HRMS (ESI) calcd for $[C_{50}H_{68}FN_3O_9 + H]^+$: 874.5018, found: 874.5001.

3.1.14. 9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxylic acid [2-(2-{[4-benzylamino-2-hydroxy-4-(2-hydroxy-ethyl)-6-methyl-heptylcarbamoyl]-methoxy}-ethoxy)-ethyl]-amide (3). The conversion of azide **23** to amine **S6** via reduction of the azide, coupling to Fmoc-AEEA, and removal of the Fmoc protecting group has been previously described.[10] Amine **S6** (2.0 mg, 4.6 μmol, 1.0 equiv) was dissolved in AcOH (0.10 mL) and to the solution was added zinc (1.5 mg, 23 μmol, 5.1 equiv) to reduce the isoxazolidine N–O bond. The reaction mixture was allowed to stir at room temperature for 4 h until the reaction was complete as judged by ESI-MS. The reaction mixture was then diluted with 0.1% TFA H_2O (0.50 mL) and CH_3CN (0.50 mL). The resulting mixture was filtered through a syringe filter and purified by reverse phase HPLC to provide 1.1 mg (54% yield) of a colorless oil. HRMS (ESI) calcd for $[C_{23}H_{41}N_3O_6 + Na]^+$: 462.2944, found: 462.2945. The intermediate was conjugated to OxDex under the conditions described for **5** yielded 0.70 mg of **3** (23% yield) as a white solid after reverse phase HPLC purification. HRMS (ESI) calcd for $[C_{44}H_{66}FN_3O_9 + H]^+$: 800.4861, found: 800.4887.

3.1.15. 9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxylic acid [2-(2-{[(2-benzyl-3-(2,3-dihydroxy-propyl)-3-isobutyl-isoxazolidin-5-ylmethyl)-carbamoyl]-methoxy}-ethoxy)-ethyl]-amide (8). Dihydroxylation of alkene **19** was accomplished as previously reported.[9] Briefly, to a solution of alkene **19** (21 mg, 64 μmol, 1.0 equiv) dissolved in *t*-BuOH (430 μL), THF (140 μL), and H_2O (35 μL) was added NMO (9.8 mg, 86 μmol, 1.4 equiv). To this solution was added OsO_4 (61 μL of a 2.5 wt% solution in *t*-BuOH, 6.1 μmol, 0.10 equiv). The reaction mixture was allowed to stir at ambient temperature until the reaction was complete as judged by TLC analysis (typically 5 h). Excess reagents were quenched by the addition of $Na_2S_2O_3$ (1.0 mL of 10% solution w/v in water) and allowed to stir for 3 h. The mixture was then transferred into biphasic mixture H_2O (40 mL) and EtOAc (40 mL). The organic layer was separated and the aqueous layer was extracted EtOAc (3× 25 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was subjected to flash chromatography (4:6 hexanes/EtOAc) to provide 19 mg of diol **S1** which was conjugated to AEEA and OxDex using the conditions described for **5**. Purification of the crude product thus obtained by reverse phase HPLC providing 4.3 mg of **8** (8.0% yield) as a white solid. HRMS (ESI) calcd for $[C_{45}H_{66}FN_3O_{10} + Na]^+$: 850.4630, found: 850.4810.

3.1.16. *N*-(2-(2-(2-((3-(2,3-Dihydroxypropyl)-3-isobutyl-2-(naphthalen-2-ylmethyl)isoxazolidin-5-yl)methylamino)-2-oxoethoxy)ethoxy)ethyl)-9-fluoro-11,17-dihydroxy-8,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxamide (9). To a solution of **21** (62 mg, 0.17 mmol, 1.0 equiv) in *t*-BuOH (1.2 mL) and THF (0.32 mL) were added NMO (32 mg, 0.26 mmol, 1.5 equiv) and H_2O (0.13 mL). To this solution was added OsO_4 (0.17 mL of a 2.5 wt% solution in *t*-BuOH, 0.02 mmol, 0.10 equiv). The result-

ing mixture was stirred for 8 h at which time TLC analysis indicated consumption of **21**. Excess reagents were quenched by the addition of Na$_2$S$_2$O$_3$ (50 mg) followed by stirring at ambient temperature for 1 h. To this mixture was added 20 mL of H$_2$O and the resulting mixture was partitioned with EtOAc (20 mL). The aqueous layer was further extracted with EtOAc (1× 20 mL). The combined organic extracts were dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product thus obtained was purified by flash chromatography (1:1 hexanes/EtOAc) to yield 57 mg of a diol **S2** as a 1:1 mixture of diasteromers at the newly formed 2° OH in 84% yield. ^{1}H NMR δ 0.98–1.04 (6H), 1.34–1.50 (2H), 1.58–1.75 (3H), 1.90–2.41 (4H), 3.43–3.64 (3H), 3.78–4.3 (5H), 7.27–7.55 (7H). HRMS (ESI) calcd for [C$_{22}$H$_{30}$N$_4$O$_3$ + H]$^+$: 399.2396, found: 399.2392. This material was then conjugated to AEEA and OxDex using the method described for **5**. Purification by reverse phase HPLC providing 0.4 mg of **9** (3% yield) as a white solid. HRMS (ESI) calcd for [C$_{49}$H$_{68}$FN$_3$O$_{10}$ + H]: 878.4967, found: 878.4972.

3.1.17. N-(2-(2-(2-((2-(Biphenyl-4-ylmethyl)-3-(2,3-dihydroxypropyl)-3-isobutylisoxazolidin-5-yl)methylamino)-2-oxoethoxy)ethoxy)ethyl)-9-fluoro-11,17-dihydroxy-8,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carboxamide (10).

Preparation of **10** was accomplished under conditions identical to those used for **9** starting with 0.17 mmol of **22**. Purification by reverse phase HPLC providing 0.7 mg of **10** (5% yield) as a white solid. HRMS (ESI) calcd for [C$_{51}$H$_{70}$FN$_3$O$_{10}$ + H]$^+$: 904.5135, found: 904.5123.

3.1.18. 4-(5-Azidomethyl-2-benzyl-3-isobutyl-isoxazolidin-3-yl)-but-2-enoic acid methyl ester (27).

Alkene **19** was dihydroxylated exactly as described for **8** to provided **S1**. The oxidative cleavage of diol **S1** to provide aldehyde **S4** has been previously reported.[10] Briefly, to a solution of diol **S1** (26 mg, 0.076 mmol, 1.0 equiv) in CH$_3$CN (0.36 mL) and H$_2$O (0.36 mL) was added NaIO$_4$ (32 mg, 0.15 mmol, 2.0 equiv). The mixture was allowed to stir at room temperature until the reaction was complete as judged by TLC analysis. The reaction mixture was poured into a biphasic solution of H$_2$O (20 mL) and EtOAc (20 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (2× 20 mL). The combined organic layers were dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The crude material was partially purified on a short silica gel column (9:1 hexanes/EtOAc) and the resulting oil, 21 mg after concentration in vacuo, was used immediately without further purification (86% approximate yield). To a solution of aldehyde **S4** (21 mg, 0.066 mmol, 1.0 equiv) dissolved in toluene (0.66 mL) was added methyl (triphenylphosphoranylidene) acetate (90 mg, 0.26 mmol, 4.0 equiv). The mixture was allowed to stir at 50 °C for 6 h at which time the reaction was complete as judged by TLC analysis (8:2 hexanes/EtOAc). The solvent was removed in vacuo and the crude material was purified by flash chromatography (85:15 hexanes/EtOAc) to provide 24 mg of the title compound (**27**) as a colorless oil (10:1 E/Z mixture). Spectral data for

E isomer: ^{1}H NMR: δ 0.92–1.01 (m, 6H), 1.36–1.42 (m, 1H), 1.52–1.63 (m, 2H), 1.74–1.87 (m, 1H), 1.91–1.98 (m, 1H), 2.19–2.29 (m, 1H), 2.30–2.40 (m, 1H), 2.48–2.58 (m, 1H), 2.98–3.09 (m, 1H), 3.36–3.45 (m, 1H), 3.66–3.89 (m, 4H), 4.02–4.11 (m, 1H), 5.85 (d, J = 14.3, 1H), 7.03 (quintet of doublets, J = 8.0, 2.7, 1H), 7.14–7.38 (m, 5H); ^{13}C NMR: δ 24.22, 24.56, 25.23, 37.21, 40.45, 42.56, 51.56, 53.34, 54.36, 68.53, 74.49, 119.5, 126.9, 128.0, 128.3, 138.1, 145.6, 166.6; HRMS (ESI) calcd for [C$_{20}$H$_{28}$N$_4$O$_3$ + H]$^+$: 373.2240, found: 373.2236.

3.1.19. 4-(2-Benzyl-5-{[2-(2-{2-[(9-fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carbonyl)-amino]-ethoxy}-ethoxy)-acetylamino]-methyl}-3-isobutyl-isoxazolidin-3-yl)-but-2-enoic acid methyl ester (11).

Preparation of **11** was accomplished under conditions identical to those used for **5** starting with 0.038 mmol of **27**. Purification by reverse phase HPLC providing 6.8 mg of **11** (21% yield) as a white solid. HRMS (ESI) calcd for [C$_{47}$H$_{66}$FN$_3$O$_{10}$ + H]$^+$: 852.4810, found: 852.4810.

3.1.20. 4-(2-Benzyl-5-{[2-(2-{2-[(9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carbonyl)-amino]-ethoxy}-ethoxy)-acetylamino]-methyl}-3-isobutyl-isoxazolidin-3-yl)-but-2-enoic acid (12).

To compound **11** (6.0 mg, 12 μmol, 1.0 equiv) was added ethanol (0.13 mL) then sodium hydroxide (1.3 mg, 30 μmol, 2.5 equiv). The mixture was allowed to stir at rt for 8 h until the reaction was complete as judged by ESI-MS. 0.1% TFA H$_2$O (1.5 mL) and CH$_3$CN (1.5 mL) were added to the reaction. The resulting mixture was filtered through a syringe filter and purified by reverse phase HPLC providing 3.1 mg of **12** (31% yield) as a white solid. HRMS (ESI) calcd for [C$_{46}$H$_{64}$FN$_3$O$_{10}$ + H]$^+$: 838.4654, found: 838.4675.

3.1.21. 4-(2-Benzyl-5-{[2-(2-{2-[(9-Fluoro-11,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,11,12,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthrene-17-carbonyl)-amino]-ethoxy}-ethoxy)-acetylamino]-methyl}-3-isobutyl-isoxazolidin-3-yl)-but-2-enoic acid amide (13).

To a solution of compound **12** (2.1 mg, 2.5 μmol, 1.0 equiv) in CH$_3$CN (0.030 mL) and pyridine (1.0 μL, 13 μmol, 5.0 equiv) were added Boc$_2$O (1.0 mg, 4.6 μmol, 1.8 equiv) and NH$_4$HCO$_3$ (1.0 mg, 13 μmol, 5.0 equiv). The mixture was allowed to stir at rt for 24 h until the reaction was complete as judged by ESI mass spectrometry. About 0.1% TFA H$_2$O (0.80 mL) and CH$_3$CN (0.70 mL) were added to the reaction. The resulting mixture was filtered through a syringe filter and purified by reverse phase HPLC providing 0.70 mg of **13** (33% yield) as a white solid. ESI-MS calcd for [C$_{46}$H$_{65}$FN$_4$O$_9$ + H]$^+$: 837.5, found: 837.5.

3.1.22. 9-Fluoro-11,17-dihydroxy-N-(1-(6-(hydroxy(6-methyl-5,6,7,8-tetrahydro-[1,3]dioxolo[4,5-g]isoquinolin-5-yl)methyl)-2,3-dimethoxyphenyl)-1-oxo-6,9,12-trioxa-2-azapentadecan-15-yl)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phen-

anthrene-17-carboxamide (28). Beta-(+/−)-hydrastine hydrochloride (10 mg, 0.024 mmol, 1.0 equiv) was dissolved in 5.0 mL of DMF, followed by the addition of i-Pr$_2$NEt (0.034 mL, 0.24 mmol, 10 equiv) and DMAP (3.0 mg, 0.024 mmol, 1.0 equiv). To this solution was added 4,7,10-trioxa-1,13-tridecanediamine (0.11 g, 0.48 mmol, 20 equiv) and the reaction mixture was stirred for 12 h. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3× 10 mL). The organic extract was washed with water (2× 10 mL), dried over Na$_2$SO$_4$, and concentrated in vacuo. To this crude mixture were added HOBt (8.0 mg, 0.060 mmol, 2.5 equiv), HBTU (23 mg, 0.060 mmol, 2.5 equiv), i-Pr$_2$NEt (8.0 µL, 0.060 mmol, 2.5 equiv), and OxDex (23 mg, 0.060 mmol, 2.5 equiv) and the reaction mixture was stirred for 12 h. The reaction progress was monitored by ESI-MS. The purification was accomplished by reverse-phase HPLC to yield 1.1 mg of **28** in 4.6% yield as a white solid. HRMS (ESI) calcd for $[C_{52}H_{70}FN_3O_{13} + Na]^+$: 986.4790, found: 986.4823.

3.1.23. 9-Fluoro-11,17-dihydroxy-*N*-(16-(1-hydroxy-4a,8-dimethyl-7-oxo-1,2,3,4,4a,7-hexahydronaphthalen-2-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[*a*]phenanthrene-17-carboxamide (29). Preparation of was accomplished under conditions identical to those used for **28**, except that (−)-α-santonin was used as the starting material. Purification by reverse phase HPLC provided 0.7 mg (3% yield) of **29** as a white solid. HRMS (ESI) calcd for $[C_{46}H_{67}FN_2O_{10} + Na]^+$: 849.4677, found: 849.4660.

4. Luciferase activity assay

The assay was carried out using the method described by Rowe and co-workers.[10]

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.045.

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