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Synthesis of a C-terminally biotinylated macrocyclic peptide mimetic exhibiting high Grb2 SH2 domain-binding affinity

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Abstract—Although considerable effort has been devoted to developing Grb2 SH2 domain-binding antagonists, important questions related to ligand specificity, and identification of intracellular targets remain unanswered. In order to begin addressing these issues, the design, synthesis, and evaluation of a novel biotinylated macrocycle are reported that bears biotin functionality at a C-terminal rather than the traditional N-terminal position. With a Grb2 SH2 domain-binding K_{eq} value of 3.4 nM, the title macrocycle (**5**) is among the most potent biotinylated SH2 domain-binding ligands yet disclosed. This should be a useful tool for elucidating physiological targets of certain Grb2 SH2 domain-binding antagonists.

1. Introduction

Biotinylation of biologically active ligands is an important means of identifying physiological targets. Recent reports detailing the biotin conjugation of steroid[1] and ansamycin[2] analogues as well as the G1 arrest agent phosmidosine, highlight the continued relevance of this technique to a spectrum of biochemical applications. In the area of cellular signal transduction, Grb2 SH2 domain-binding antagonists have been sought as potential therapeutics for a variety of proliferative diseases.[3–8] High affinity Grb2 SH2 domain-binding peptides typified by **1**[9,10] and **2**[11] include members capable of potently blocking growth factor-induced cell motility, matrix invasion, and branching morphogenesis in c-Met-containing kidney cancer cells (Fig. 1).[12,13] Recently, macrocyclic variants such as **3** have been reported that exhibit enhanced Grb2 SH2 domain-binding potency and higher efficacy in whole cell systems.[14–16] While much effort has been devoted to the enhancement of binding affinity or cellular efficacy,[17,10,9] important issues related to specificity, and identification of intracellular targets have received much less attention.[18–21] To begin addressing questions of in vivo binding selectivity, biotinylated conjugates of high affinity, phosphatase-stable ligands are needed. Accordingly, reported herein are the design, synthesis, and biotinylation biotinylatior of a Grb2 of a Grb2 SH2 domain-binding antagonist based on macrocycle **3**.

Although biotinylation of SH2 domain-directed phosphotyrosyl (pTyr)-containing peptides have been reported, these peptides have been open-chain in nature, with biotin conjugation residing at their N-terminii.[22] For macrocyclic peptide mimetic **3**, similar N-terminal biotinylation would not be possible, since the requisite pTyr α-amino group has been replaced by α-carboxymethyl functionality that enhances interactions with the Grb2 SH2 domain αA2 Arg residue.[14] Biotinylation at the C-terminus would represent an alternative. However, this would have to be accomplished with maintenance of the 3-naphthylpropyl moiety, since this type of aryl functionality had been shown to be critical for high Grb2 SH2 domain-binding affinity of open-chain analogues.[23] Previous work has indicated that hydroxylation of the naphthyl ring can enhance Grb2 SH2 domain-binding affinity.[24] Therefore, macrocycle **4** was designed as a functionalized variant of parent **3** that contains a 5-hydroxynaphthyl site suitable for biotin

Keywords: Grb2 SH2 Domain; Biotin conjugate; Macrocycle.
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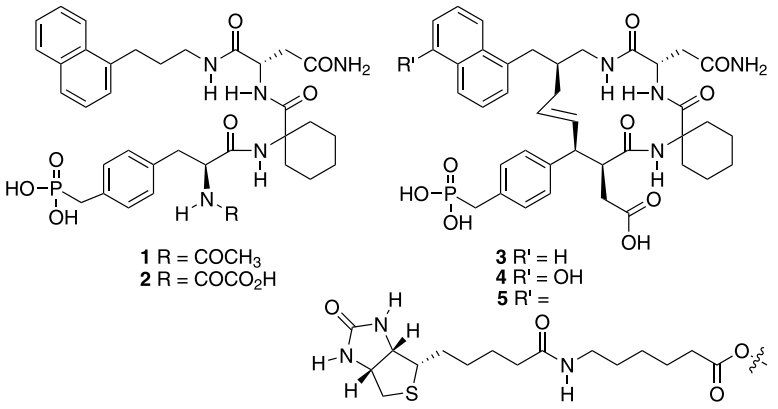
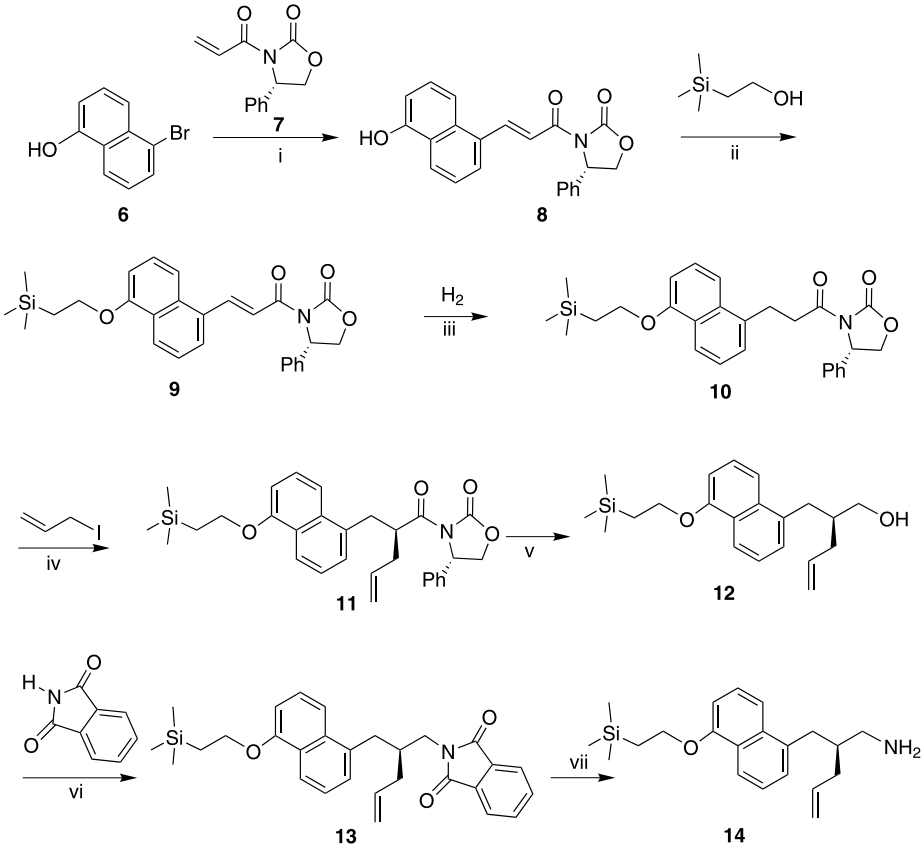


Figure 1. Structures of analogues discussed in the text.

conjugation. Attachment of biotin to this site could be accomplished using a 6-aminohexanoyl spacer, to yield the target biotinylated macrocycle **5**. To our knowledge, this would be the first example of an SH2 domain-binding ligand bearing C-terminal biotin conjugation.

2. Synthesis

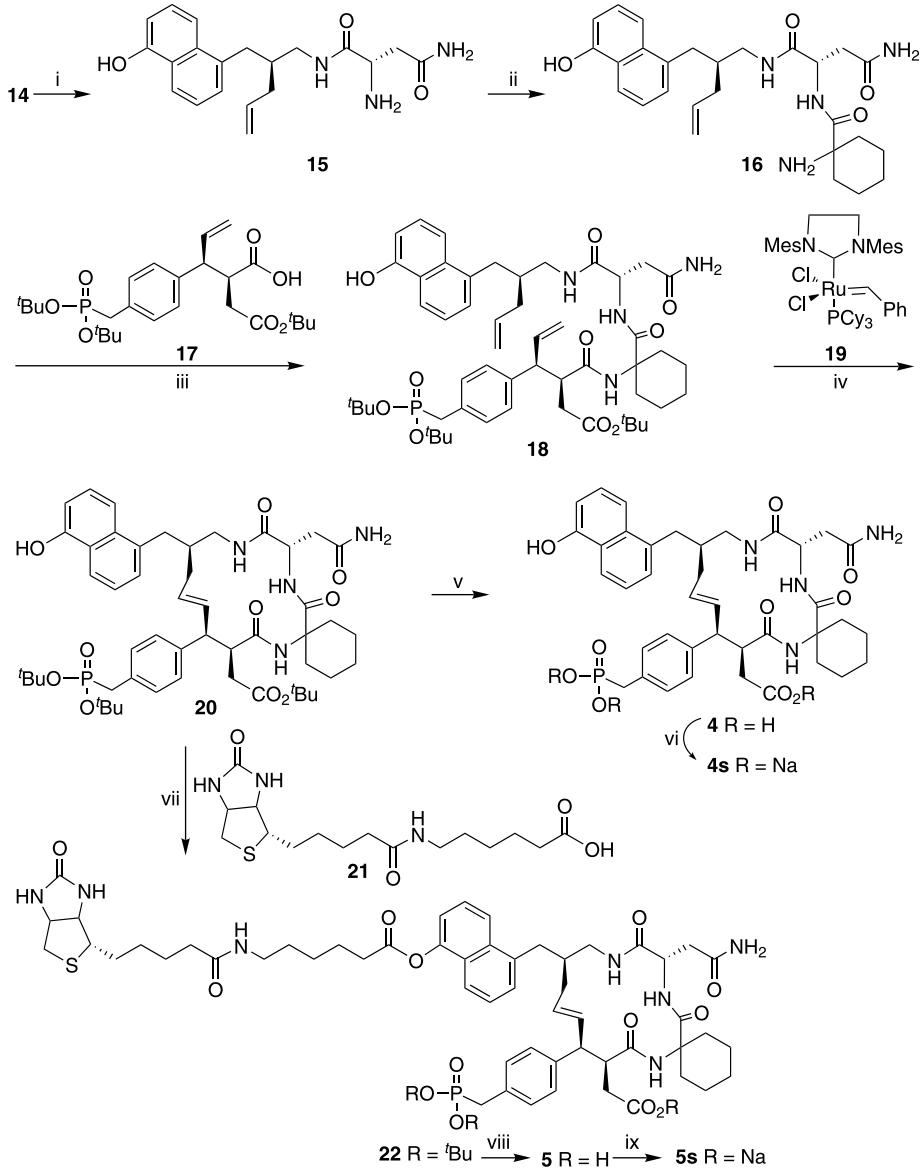
Preparation of the final biotinylated macrocycle **5** required the initial synthesis of the 5-hydroxy-1-naphthyl-containing congener **4** bearing *tert*-butyl protection of all carboxyl groups. A key intermediate in this latter synthesis was the hydroxyl-protected (*S*)-2-allyl-3-(5-hydroxy-(1-naphthyl))propylamine (**14**) (Scheme 1). Pivaloyl mixed anhydride coupling of 2-propenic acid with commercially available (*S*)-4-phenyl-1,3-oxazolidin-2-one[25] in the presence of *n*-BuLi gave the chiral acrylamide **7** (92% yield), which was coupled in a Heck reaction with 5-bromo-1-naphthol **6**[26] to provide the *E*-adduct **8** in 74% yield (Scheme 1).[27] Although protection of the sterically hindered 5-hydroxynaphthyl



Scheme 1. Reagents and conditions: (i) $Pd(OAc)_2$, tri-*o*-tolylphosphine, Et_3N, reflux, 14 h (74% yield); (ii) DEAD, Ph_3P, THF, rt, 24 h (49% yield); (iii) 10% Pd–C, EtOAc, 24 h (98% yield); (iv) LHMDS, THF, −78 °C, 2 h, 56% yield, de >95% from NMR; (v) $LiAlH_4$, THF, −78 °C, 1 h then rt overnight (86% yield); (vi) DIAD, Ph_3P, THF, rt, 15 h (69% yield); (vii) $N_2H_4{\cdot}H_2O$, EtOH, H_2O, reflux, 3 h (87% yield).

group was not undertaken for this first reaction, protection was deemed necessary for the subsequent steps. Attempted alkylation using trimethylsilylethyl bromide in the presence of a variety of bases proved unsuccessful. However, reaction of **8** with trimethylsilylethyl alcohol under neutral Mistunobu conditions (diethyl azodicarboxylate (DEAD) and triphenylphosphine) gave the desired 5-protected analogue **9** in moderate yield. Hydrogenation of **9** provided the saturated **10**, which was subjected to asymmetric α-alkylation using 3-iodopropene in the presence of lithium hexamethyldisilylamide (LHMDS) to provide (*S*)-**11** (56% yield, de >95% as determined by proton NMR). Lithium aluminum hydride reduction of **11** at −78 °C afforded a mixture of alcohol and aldehyde; however, warming to room temperature provided complete reduction, yielding **12** as the sole product. The synthesis of **14** was completed in two-step fashion by Mitsunobu-mediated coupling of phthalimide to **13**, followed by hydrazine reflux (60% combined yield). It should be noted that although this synthetic approach to 5-substituted **14** utilizes an Evans chiral auxiliary similar to the reported synthesis of the unsubstituted (*S*)-2-allyl-3-(1-naphthyl)propylamine, it differs significantly from this latter synthesis in several key aspects.[27]

With the 5-hydroxy-1-naphthyl-containing unit **14** in hand, synthesis of the metathesis ring-closing precursor **18** was accomplished as shown in Scheme 2. Coupling of **14** and *N*-Boc Asn-OH using diisopropylcarbodiimide



Scheme 2. Reagents and conditions: (i) (a) Boc-Asn-OH, DIPCDI, HOBt, DMF, rt, 12 h (94% yield); (b) TFA, CH_2Cl_2, rt, 1 h (97% yield); (ii) (a) Fmoc-1-amino-cyclohexenecarboxylic acid, EDCI·HCl, HOBt, DMF, rt, 12 h (92% yield); (b) piperidine, CH_3CN, rt, 2 h (90% yield); (iii) EDCI·HCl, HOAt, DMF, 50 °C, 24 h (65% yield); (iv) CH_2Cl_2, reflux, 48 h (66% yield); (v) TFA–TES–H_2O, rt, 1 h (58% yield); (vi) aq $NaHCO_3$ (3 equiv) (quantitative); (vii) EDCI·HCl, DMAP, DMF, rt, 12 h (80% yield); (viii) TFA–$HS(CH_2)_2SH$–H_2O, rt, 1 h (56% yield); (ix) aq $NaHCO_3$ (3 equiv) (quantitative).

(DIPCDI) in the presence of 1-hydroxybenzotriazole (HOBt) proceeded without difficulty. However, subsequent *N*-Boc-deprotection using TFA in dicholoromethane resulted in concomitant cleavage of the trimethylsilylethyl ether group to yield amine **15** in high yield. Continued maintenance of protection of the 5-hydroxyl group proved to be unnecessary, possibly due to a high degree of steric crowding about the 5-position.

In order to minimize unwanted esterification with the unprotected 5-hydroxynaphthyl group, initial attempts at condensing **15** with commercially available *N*-Fmoc 1-aminocyclohexanecarboxylic acid (*N*-Fmoc Ac_6c) were done using the less reactive active-ester coupling reagent HOSu. When these conditions failed, the reaction was repeated using HOBt active ester coupling. Esterification of the unprotected 5-hydroxynaphthyl group was not observed and the desired product was obtained and subjected to in situ piperidine-mediated N-deprotection to yield **16** in high yield. Coupling of **16** with known **17**[14] was achieved using highly active 1-hydroxy-7-azabenzotriazole (HOAt) and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide) (EDCI) at elevated temperature to give **18** in 65% yield. Metathesis ring-closure using the second generation Grubbs' catalyst [$((PCy_3)(Im(Mes)_2)Ru{=}CHPh)$ (**19**)][25,28] gave the protected macrocycle **20** (66% yield) exclusively as the trans-isomer (vinylic coupling constant $J = 14.5$ Hz). Global removal of *tert*-butyl ester protecting groups using aqueous TFA in the presence of triethyl silane and HPLC purification gave **4**, which was converted to its tri-sodium salt. Synthesis of the biotinylated target **5** was achieved from **20** in two additional steps. Reaction of commercially available biotin-OSu with 6-aminohexanoic acid in the aqueous sodium bicarbonate solution gave **21**, which was coupled with **20** in the presence of EDCI and 4-dimethylaminopyridine (DMAP) to yield **22** in 80% yield. Global deprotection of **22** (TFA in the presence of 1,2-ethanedithiol) and HPLC-purification gave final **5**, which was converted to its tri-sodium salt.

3. Grb2 SH2 domain-binding affinities of 4 and 5

Previous work had shown that addition of hydroxyl or methoxyl substituents to the naphthyl ring could be tolerated for open-chain Grb2 SH2 domain-binding inhibitors such as **1**.[24] However, precedence had not been established as to the effects similar modifications would have in a macrocyclic platform of type **3**. Using an ELISA-based procedure that measures the ability of synthetic ligands to compete with surface-bound pTyr-peptide for binding to Grb2 SH2 domain protein in solution, an IC_{50} value of 1.4 nM had previously been reported for parent macrocycle **3**.[29] In the current work, a similar assay provided an affinity constant of $IC_{50} = 3.4$ nM for compound **4**, indicating that hydroxylation at the naphthyl 4-position was well tolerated (Table 1). This data was supported by surface plasmon resonance experiments that measured the direct binding of **4** to surface-bound Grb2 SH2 domain protein, where a K_{eq} value of 1.8 nM was obtained (Table 1).

Table 1. In vitro Grb2 SH2 domain-binding affinity of **4** and **5**

No.	Assay method	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_{eq} (nM)
4	ELISA[a]	—	—	$IC_{50} = 3.4$
4	Biacore S51[b]	5.7×10^6	1.0×10^{-2}	1.8
5	Biacore S51[c]	2.9×10^6	9.9×10^{-3}	3.4

[a] Determined as described in Ref. 30.
[b] Binding of inhibitor in solution to sensor-bound Grb2 SH2 domain protein as described in Ref. 31.
[c] Global analysis of **5** in solution binding to Grb2 SH2 domain protein amine-coupled to the chip surface.

Placement of the biotin moiety onto the hydroxyl by means of a hexanoyl spacer to give the title compound **5**, was achieved with maintenance of high binding affinity (SPR $K_{eq} = 3.4$ nM). Interestingly, in spite of its significant size, the biotin-hexanoyl group had little effect on binding kinetics, with the rates of association and dissociation being nearly identical for both biotinylated (**5**, $k_a = 2.9 \times 10^6$ M^{-1} s^{-1} and $k_d = 9.9 \times 10^{-3}$ s^{-1}) and non-biotinylated compounds (**4**, $k_a = 5.7 \times 10^6$ M^{-1} s^{-1} and $k_d = 1.0 \times 10^{-2}$ s^{-1}). The low nanomolar binding affinity of **5** is significantly greater than the low micromolar affinity exhibited by pTyr-containing Grb2 SH2 domain-binding peptides previously used for biotinylation.[22,11]

Inaccuracies in SPR data could arise from limitations imposed by material transport if transport rate limits the binding of the analyte to the surface ligand. This is of note, since in the current experiments the binding on-rates are close to the instrumental limits. Transport effects can in part be diagnosed by the fact that the binding reaction can no longer be fit to a simple binding model. In particular, the off-rates become biphasic and fit well to a two exponential decay. Experimental protocols have been developed to deal with transport issues. For example, low activity surfaces can be used with high flow rates to minimize transport effects. The Grb2 SH2 domain surfaces employed in the current experiments were low activity surfaces, which were used with a high flow rate of 30 μL/min. This allowed surface saturation to be achieved at a low RU value, sometimes as low as 3 RU's. The resulting data fit well to a simple binding model, with no prior assumptions about transport limitation. When the data was calculated with a reasonable transport term that accounted for the transport of the analyte to the surface, the date still fit to a simple model. Finally, when the data was fit at equilibrium the binding results were consistent with K_{eq} calculated from the kinetic model.

4. Inhibition by analogues 2 and 4 of Grb2 binding to $p185^{erbB\text{-}2}$ in MDA-MB-453 breast cancer cells in culture

Varying concentrations of **2** and **4** were cultured with MDA-MB-453 breast cancer cells overnight. The cells were then washed, lysed, and immunoprecipitated with Grb2 antibody, and the resulting precipitates were run on SDS-PAGE gels. Immunoblotting for both $p185^{erbB\text{-}2}$ revealed that the hydroxyl-containing macrocycle **4** was able to effectively block the intracellular association of Grb2 with the $p185^{erbB\text{-}2}$ tyrosine kinase

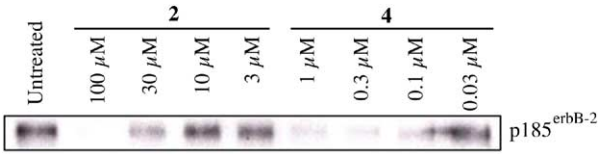


Figure 2. SDS-PAGE gels showing the inhibition of binding of Grb2 to $p185^{erbB-2}$ Grb2 by analogues **2** and **4** in whole cells. Experiments were conducted as described in Ref. 30. The p185 bands were obtained by immunopreciptating with Grb2 antibodies and immunoblotting for $p185^{erbB-2}$.

at concentrations (100 nM) that were significantly lower than required by the non-hydroxylated open-chain **2** (Fig. 2). These results are consistent with an earlier report dealing with the non-hydroxylated parent macrocycle **3**,[14] and indicate that substitution at the macrocycle naphthyl 4-position can be undertaken with retention of cellular efficacy. Since cells were washed prior to lysing and the subsequent measuring of $p185^{erbB-2}$ binding to Grb2, it can be concluded that both **2** and **4** achieve their inhibitory effects within the cytoplasmic compartment.

5. Conclusions

In spite of considerable effort devoted to developing Grb2 SH2 domain-binding antagonists, important questions related to ligand specificity and identification of intracellular targets remain unanswered. To begin addressing these issues, potent biotinylated phosphatase-stable ligands would be highly useful. For these reasons the design and synthesis of the Grb2 SH2 domain-binding macrocycle **4** are reported herein along with its biotinylated congener **5**. Macrocycle **5** is unusual in bearing biotin functionality at a C-terminal rather than the normal N-terminal position. With a Grb2 SH2 domain-binding K_{eq} value of 3.4 nM, the title macrocycle (**5**) is among the most potent biotinylated SH2 domain-binding ligand yet reported. This should be a useful tool for elucidating physiological targets of certain Grb2 SH2 domain-binding antagonists. Among the types of studies that could be useful in this regard are pull down experiments of cell lysates using solid support-anchored **5**.

6. Experimental procedures

6.1. General synthetic

High resolution mass spectra (HRMS) were obtained from the UCR Mass Spectrometry Facility, University of California at Riverside, and fast atom bombardment mass spectra (FABMS) were acquired with a VG Analytical 7070E mass spectrometer under the control of a VG 2035 data system. Where indicated, FABMS matrixes used were glycerol (Gly) or nitrobenzoic acid (NBA). H NMR data were obtained on a Varian 400 MHz instrument and are reported in parts per million relative to TMS and referenced to the solvent in which they were run. Solvent was removed by rotary evaporation under reduced pressure and anhydrous solvents were obtained commercially and used without further drying. High pressure liquid chromatography (HPLC) was conducted using a Waters Prep LC4000 system having photodiode array detection and binary solvent systems as indicated where A = 0.1% aqueous TFA and B = 0.1% TFA in acetonitrile and either Vydac C_{18} (10μ) Peptide & Protein or JH303 C_{18} (4μ) columns (preparative size, 20 mm dia. × 250 mm long with a flow rate of 10 mL/min.; analytical size, 4.6 mm dia. × 250 mm long, with a flow rate of 1 mL/min.).

6.2. (4*S*)-3-[3-(5-Hydroxy-1-naphthalenyl)-1-oxo-2-propenyl]-4-phenyl-2-oxazolidinone (8)

To a solution of 5-bromo-1-naphthol[26] (7.50 g, 33.6 mmol) and (4*S*)-3-(1-oxo-2-propenyl)-4-phenyl-2-oxazolidinone[27] (7.29 g, 33.6 mmol) in Et_3N (150 mL) were added $Pd(OAc)_2$ (500 mg, 2.13 mmol) and $(O\text{-tol})_3P$ (2.05 g, 6.74 mmol). The mixture was refluxed under argon (14 h), then cooled to room temperature, diluted with EtOAc, and filtered through Celite. The filtrate was washed with saturated aqueous NH_4Cl and brine, dried (Na_2SO_4), and solvent was evaporated in vacuo. The residue was purified by silica gel flash chromatography to give **8** as green oil (8.94 g, 74% yield). H NMR (DMSO-d_6) δ 10.32 (s, 1H), 8.38 (d, 1H, J = 15.6 Hz), 8.27 (d, 1H, J = 8.4 Hz), 7.89 (d, 1H, J = 15.6 Hz), 7.85 (d, 1H, J = 7.4 Hz), 7.59 (d, 1H, J = 8.6 Hz), 7.52 (t, 1H, J = 7.8 Hz), 7.43–7.32 (m, 6H), 6.94 (d, 1H, J = 7.4 Hz), 5.62 (dd, 1H, J = 3.9 and 8.6 Hz), 4.83 (t, 1H, J = 8.7 Hz), 4.24 (dd, 1H, J = 3.9 and 8.6 Hz). FABMS (+ve) *m/z* 360 [MH^+].

6.3. (4*S*)-3-[3-(5-((2-Trimethylsilyl)ethyl)oxy-1-naphthalenyl)-1-oxo-2-propenyl]-4-phenyl-2-oxazolidinone (9)

To a solution of **8** (8.91 g, 24.8 mmol), trimethylsilylethanol (4.9 mL, 34.7 mmol), triphenylphosphine (9.06 g, 33.5 mmol) in THF (40 mL) was added diethyl azodicarboxylate (5.5 mL, 33.5 mmol) at 0 °C. The solution was stirred at room temperature (24 h) and diluted with dichloromethane, washed with H_2O and brine, and dried (Na_2SO_4). The solvent was evaporated in vacuo and the residue was purified by silica gel flash chromatography to give **9** as green oil (5.58 g, 49% yield). H NMR ($CDCl_3$) δ 8.62 (d, 1H, J = 15.4 Hz), 8.39 (d, 1H, J = 8.2 Hz), 7.99 (d, 1H, J = 15.4 Hz), 7.92 (d, 1H, J = 6.8 Hz), 7.71 (d, 1H, J = 8.4 Hz), 7.49–7.35 (m, 7H), 6.83 (d, 1H, J = 7.6 Hz), 5.59 (dd, 1H, J = 4.0 and 8.7 Hz), 4.76 (t, 1H, J = 8.8 Hz), 4.34 (dd, 1H, J = 3.9 and 8.8 Hz), 4.25 (t, 2H, J = 7.9 Hz), 1.28 (t, 2H, J = 7.8 Hz), 0.12 (s, 9H). FABMS (+ve) *m/z* 460 [MH^+]. HRMS calcd for $C_{27}H_{29}NO_4Si$ [M^+]: 459.1866. Found: 459.1873.

6.4. (4*S*)-3-[3-(5-((2-Trimethylsilyl)ethyl)oxy-1-naphthalenyl)-1-oxo-2-propanyl]-4-phenyl-2-oxazolidinone (10)

To a solution of **9** (2.79 g, 6.08 mmol) in EtOAc (80 mL) was added 10% Pd–C (600 mg). The mixture was hydrogenated at room temperature (24 h) and filtered through Celite. The filtrate was evaporated in vacuo and the residue was purified by silica gel flash chromatography to

give **10** as green oil (2.76 g, 98% yield). H NMR ($CDCl_3$) δ 8.17 (t, 1H, J = 4.8 Hz), 7.55 (d, 1H, J = 8.6 Hz), 7.39–7.25 (m, 8H), 6.78 (d, 1H, J = 7.6 Hz), 5.41 (dd, 1H, J = 3.7 and 8.6 Hz), 4.64 (t, 2H, J = 8.9 Hz), 4.27–4.12 (m, 3H), 3.17–3.12 (m, 4H), 1.25 (t, 2H, J = 7.6 Hz), 0.10 (s, 9H). FABMS (+ve) m/z 461 [M^+], 462 [MH^+].

6.5. (4*S*)-3-[(2*S*)-2-(5-((2-Trimethylsilyl)ethyl)oxy-1-naphthalenyl)-1-oxo-4-pentenyl]-4-phenyl-2-oxazolidinone (11)

To a solution of **10** (2.72 g, 5.90 mmol) in THF (30 mL) was added dropwise a solution of NaHMDS 1 M in THF (7.67 mL, 7.67 mmol) at −78 °C under argon. The resulting solution was stirred at −78 °C (1 h); then a precooled solution of allyl iodide (0.70 mL, 7.67 mmol) in THF (20 mL) was added and the mixture was stirred for an additional 2 h at −78 °C. The reaction mixture was warmed to room temperature and stirred overnight. The reaction was quenched by the addition of ice-cold saturated NH_4Cl solution (80 mL), extracted with EtOAc, washed with brine, and dried (Na_2SO_4). Evaporation provided a residue, which was purified by silica gel flash chromatography to provide **11** as green oil (1.65 g, 56% yield). H NMR ($CDCl_3$) δ 8.22 (dd, 1H, J = 3.0 and 6.9 Hz), 7.61 (d, 1H, J = 8.6 Hz), 7.44–7.30 (m, 6H), 7.23–7.20 (m, 2H), 6.81 (d, 1H, J = 7.6 Hz), 5.72 (m, 1H), 5.14 (dd, 1H, J = 3.3 and 8.6 Hz), 4.98–4.93 (m, 2H), 4.49 (m, 1H), 4.27–4.11 (m, 3H), 4.07 (dd, 1H, J = 3.3 and 8.8 Hz), 3.37 (dd, 1H, J = 9.2 and 13.9 Hz), 3.25 (dd, 1H, J = 5.9 and 13.9 Hz), 2.51 (m, 1H), 2.27 (m, 1H), 1.26 (t, 2H, J = 7.2 Hz), 0.12 (s, 9H). FABMS (+ve) m/z 501 [M^+], 502 [MH^+].

6.6. (2*S*)-(2-Propenyl)-3-(5-((2-trimethylsilyl)ethyl)oxy-1-naphthalenyl)propan-1-ol (12)

To a stirred suspension of $LiAlH_4$ (97 mg, 2.40 mmol) in THF (6 mL) was added a pre-cooled solution of **11** (1.00 g, 2.00 mmol) in THF (15 mL) at −78 °C under argon. The mixture was stirred at −78 °C (1 h), then warmed to 0 °C (1 h). The reaction mixture was stirred at room temperature overnight and cooled to −78 °C and EtOAc (3 mL) was added followed by 10% $NaOH_{aq}$ (four drops). The reaction was quenched by the addition of ice-cold saturated NH_4Cl solution (50 mL), extracted with Et_2O, washed with brine, and dried (Na_2SO_4). Evaporation of solvent provided a residue, which was purified by silica gel flash chromatography to yield **12** as yellow oil (586 mg, 86% yield). H NMR ($CDCl_3$) δ 8.21 (d, 1H, J = 8.6 Hz), 7.60 (d, 1H, J = 8.6 Hz), 7.42–7.31 (m, 3H), 6.81 (dd, 1H, J = 3.3 and 7.6 Hz), 5.87 (m, 1H), 5.14–5.06 (m, 2H), 4.25 (t, 2H, J = 7.9 Hz), 3.58 (t, 2H, J = 5.5 Hz), 3.10 (dd, 1H, J = 7.8 and 13.9 Hz), 3.02 (dd, 1H, J = 6.8 and 13.9 Hz), 2.26–2.04 (m, 3H), 1.28 (t, 2H, J = 7.9 Hz), 0.12 (s, 9H). FABMS (+ve) m/z 342 [M^+], 343 [MH^+].

6.7. 2-[(2*S*)-((5-((2-Trimethylsilyl)ethyl)oxy-1-naphthalenyl)methyl)-4-pentenyl]-*H*-isoindole-1,3(2*H*)-dione (13)

Diisopropyl azodicarboxylate (0.443 mL, 2.17 mmol) was added to a solution of **12** (494 mg, 1.44 mmol), phthalimide (320 mg, 2.17 mmol), and triphenylphosphine (586 mg, 2.17 mmol) in THF (15 mL) at 0 °C and the mixture was stirred at room temperature (15 h). Solvent was removed by evaporation and the residue was purified by silica gel flash chromatography to provide **13** as a yellow oil (465 mg, 69% yield). H NMR ($CDCl_3$) δ 8.14 (d, 1H, J = 8.0 Hz), 7.79 (dd, 2H, J = 3.0 and 5.6 Hz), 7.68 (dd, 2H, J = 3.2 and 5.4 Hz), 7.48 (d, 1H, J = 8.6 Hz), 7.36–7.30 (m, 3H), 6.77 (d, 1H, J = 7.6 Hz), 5.81 (m, 1H), 5.08–4.98 (m, 2H), 4.22 (t, 2H, J = 7.9 Hz), 3.75 (dd, 1H, J = 7.5 and 13.8 Hz), 3.66 (dd, 1H, J = 7.2 and 13.7 Hz), 3.10–2.99 (m, 2H), 2.66 (m, 1H), 2.21–2.07 (m, 2H), 1.26 (t, 2H, J = 7.9 Hz), 0.12 (s, 9H). FABMS (+ve) m/z 471 [M^+], 472 [MH^+].

6.8. (2*S*)-1-Amino-2-(2-propenyl)-3-(5-((2-trimethylsilyl)-ethyl)oxy-1-naphthalenyl)propane (14)

To a stirred solution of **13** (447 mg, 0.949 mmol) in EtOH (10 mL) containing H_2O (70 mg) was added hydrazine (114 mg, 2.28 mmol). The resulting solution was refluxed under argon (3 h), during which time a significant quantity of solid precipitated. The mixture was filtered through Celite, washed with EtOH, and the combined filtrate was evaporated and the residue was purified by silica gel flash chromatography to provide **14** as yellow oil (281 mg, 87% yield). H NMR (DMSO-d_6) δ 8.06 (d, 1H, J = 8.0 Hz), 7.62 (d, 1H, J = 8.4 Hz), 7.43–7.33 (m, 3H), 6.95 (d, 1H, J = 8.6 Hz), 5.82 (m, 1H), 5.06–5.00 (m, 2H), 4.25 (t, 2H, J = 7.7 Hz), 3.07 (dd, 1H, J = 7.2 and 13.7 Hz), 2.84 (dd, 1H, J = 7.0 and 13.7 Hz), 2.53 (dd, 1H, J = 6.0 and 12.9 Hz), 2.46 (dd, 1H, J = 5.8 and 12.8 Hz), 2.14 (m, 1H), 2.01 (m, 1H), 1.78 (m, 1H), 1.22 (t, 2H, J = 7.7 Hz), 0.10 (s, 9H). FABMS (+ve) m/z 342 [MH^+].

6.9. (2*S*)-2-Amino-*N*1-[(2*S*)-2-(5-hydroxy-1-naphthalenylmethyl)-4-pentenyl]-butanediamide (15)

To a solution of **14** (274 mg, 0.80 mmol) in DMF (4 mL) was added an active ester solution prepared by the reaction of *N*-Boc-Asn-OH (204 mg, 0.88 mmol), HOBt (119 mg, 0.88 mmol), and DIPCDI (0.139 mL, 0.88 mmol) in DMF (3 mL) at room temperature (10 min). The resulting solution was stirred at room temperature (12 h). Solvent was evaporated and the remaining residue was dissolved in EtOAc, washed with H_2O and brine, dried (Na_2SO_4), and evaporated to provide crude intermediate *N*-Boc-protected material (415 mg). This was dissolved in TFA–CH_2Cl_2 (v/v, 1:1, 8 mL) and the resulting solution was stirred at room temperature (1 h). After evaporation of solvent, the remaining residue was purified by silica gel flash chromatography to provide **15** as a colorless oil (256 mg, 90% yield). H NMR (CD_3OD) δ 8.02 (d, 1H, J = 8.4 Hz), 7.35 (d, 1H, J = 8.6 Hz), 7.26–7.16 (m, 3H), 6.72 (dd, 1H, J = 0.8 and 7.4 Hz), 5.73 (m, 1H), 5.01–4.96 (m, 2H), 3.35 (dd, 1H, J = 4.4 and 8.5 Hz), 3.18 (dd, 1H, J = 5.5 and 13.5 Hz), 3.05–2.95 (m, 2H), 2.78 (dd, 1H, J = 7.6 and 14.0 Hz), 2.41 (dd, 1H, J = 4.3 and 15.0 Hz), 2.14 (dd, 1H, J = 8.4 and 15.0 Hz), 2.06–2.02

(m, 3H). FABMS (+ve) *m/z* 356 [MH^+]. HRMS calcd for $C_{20}H_{26}N_3O_3$ [M^+]: 356.1974. Found: 356.1968.

6.10. (2*S*)-2-[[(1-Aminocyclohexyl)carbonyl]amino]-*N*1-[(2*S*)-2-(5-hydroxy-1-naphthalenylmethyl)-4-pentenyl]-butanediamide (16)

To a solution of **15** (226 mg, 0.64 mmol) in DMF (4 mL) was added an active ester solution prepared by the reaction of *N*-Fmoc-1-amino-cyclohexenecarboxylic acid (257 mg, 0.70 mmol), HOBt (95 mg, 0.70 mmol), and DIPCDI (0.11 mL, 0.70 mmol) in DMF (3 mL) at room temperature (10 min) and the resulting solution was stirred at room temperature (12 h). The solvent was evaporated and the remaining residue was dissolved in EtOAc, and washed with H_2O, brine, and dried (Na_2SO_4). Solvent was removed by evaporation and the remaining residue was purified by silica gel flash chromatography to provide crude *N*-Fmoc-protected intermediate as colorless oil (412 mg, 92% yield). [H NMR (CD_3OD-d_6) δ 8.08 (m, 1H), 7.74 (dd, 2H, J = 2.3 and 7.6 Hz), 7.50 (d, 1H, J = 8.6 Hz), 7.42 (d, 1H, J = 7.4 Hz), 7.37–7.31 (m, 3H), 7.27–7.17 (m, 5H), 6.76 (d, 1H, J = 7.4 Hz), 5.79 (m, 1H), 5.03–4.96 (m, 2H), 4.61 (dd, 1H, J = 4.8 and 16.5 Hz), 4.26 (dd, 1H, J = 6.3 and 10.6 Hz), 4.12 (dd, 1H, J = 5.5 and 10.7 Hz), 4.00 (t, 1H, J = 6.0 Hz), 3.27 (dd, 1H, J = 7.3 and 13.3 Hz), 3.05 (dd, 1H, J = 5.5 and 13.3 Hz), 2.97 (d, 2H, J = 6.7 Hz), 2.80 (dd, 1H, J = 6.7 and 15.7 Hz), 2.68 (dd, 1H, J = 4.8 and 15.7 Hz), 2.19–2.09 (m, 2H), 2.01 (m, 1H), 1.91–1.63 (m, 3H), 1.55–1.23 (m, 7H). FABMS (+ve) *m/z* 703 [MH^+].] To a solution of this intermediate (371 mg, 0.53 mmol) in CH_3CN (6 mL) was added piperidine (0.6 mL) and the resulting solution was stirred at room temperature (2 h). Evaporation of solvent gave a residue, which was purified by silica gel flash chromatography to provide **16** as a white solid (229 mg, 90% yield). H NMR (CD_3OD) δ 8.06 (m, 1H), 7.44 (d, 1H, J = 8.6 Hz), 7.28–7.24 (m, 3H), 6.76 (dd, 1H, J = 0.8 and 7.6 Hz), 5.81 (m, 1H), 5.06–5.00 (m, 2H), 4.61 (t, 1H, J = 6.3 Hz), 3.26 (m, 1H), 3.08 (dd, 1H, J = 5.3 and 13.5 Hz), 2.95–2.94 (m, 2H), 2.66 (dd, 1H, J = 6.6 and 15.4 Hz), 2.60 (dd, 1H, J = 5.9 and 15.4 Hz), 2.11–2.01 (m, 3H), 1.87–1.76 (m, 2H), 1.54–1.48 (m, 5H), 1.42–1.21 (m, 3H). FABMS (+ve) *m/z* 481 [MH^+]. HRMS calcd for $C_{27}H_{37}N_4O_4$ [MH^+]: 481.2815. Found: 481.2834.

6.11. (β*S*,γ*S*)-β-[[[1-[[[(1*S*)-3-Amino-1-[[[(2*S*)-2-(5-hydroxy-1-naphthalenylmethyl)-4-pentenyl]amino]carbonyl]-3-oxopropyl]amino]carbonyl]cyclohexyl]amino]carbonyl]-4-[[bis(1,1-dimethylethoxy)phosphinyl]methyl]-γ-ethenylbenzenebutanoic acid 1,1-dimethylethyl ester (18)

To a solution of **16** (123 mg, 0.256 mmol) and protected pTyr mimetic **17**[14] (127 mg, 0.256 mmol) in DMF (4 mL) was added HOAt (0.512 mL, 0.256 mmol) and EDCI·HCl (60 mg, 0.256 mmol) at 0 °C. The solution was stirred at room temperature (1.5 h) then heated to 50 °C and stirred (24 h). The crude reaction mixture was evaporated in vacuo and the remaining residue was purified by silica gel flash chromatography to provide **18** as yellow oil (48 mg, 65% effective yield based on 86 mg of recovered **16**). H NMR ($CDCl_3$) δ 8.68 (br s, 1H), 8.13 (dd, 1H, J = 2.0 and 7.5 Hz), 7.49 (d, 1H, J = 8.6 Hz), 7.39 (t, 1H, J = 5.4 Hz), 7.30–7.23 (m, 3H), 7.14 (t, 1H, J = 8.0 Hz), 7.03 (dd, 2H, J = 2.3 and 8.1 Hz), 6.79 (d, 2H, J = 8.0 Hz), 6.70 (d, 1H, J = 7.4 Hz), 6.50 (s, 1H), 6.26 (s, 1H), 5.87 (m, 1H), 5.54 (m, 1H), 5.41 (s, 1H), 5.11–5.04 (m, 2H), 4.96–4.88 (m, 2H), 4.55 (m, 1H), 3.34 (t, 1H, J = 9.5 Hz), 3.24 (m, 1H), 3.12–3.05 (m, 2H), 2.91 (d, 2H, J = 21.3 Hz), 2.85 (m, 1H), 2.71 (dd, 1H, J = 6.5 and 15.1 Hz), 2.55 (dd, 1H, J = 4.8 and 15.1 Hz), 2.38 (dd, 1H, J = 9.5 and 17.7 Hz), 2.29 (dd, 1H, J = 3.6 and 17.7 Hz), 2.24–2.17 (m, 2H), 2.09–2.02 (m, 2H), 1.80–1.10 (m, 10H), 1.42 (s, 9H), 1.38 (s, 18H). FABMS (+ve) *m/z* 960 [MH^+], 998 [MK^+].

6.12. (9*S*,10*S*,11*E*,14*S*,18*S*)-18-(2-Amino-2-oxoethyl)-10-[4-[[bis(1,1-dimethylethoxy)phosphinyl]methyl]phenyl]-14-(1-(5-hydroxy)naphthalenylmethyl)-8,17,20-trioxo-7,16,19-triazaspiro[5.14]eicos-11-ene-9-acetic acid 1,1-dimethylethyl ester (20)

To a solution of **18** (87 mg, 0.091 mmol) in CH_2Cl_2 (40 mL) was added [$((PCy_3)(Im(Mes)_2)Ru{=}CHPh)$] **19**[25] (50 mg, 0.058 mmol) in CH_2Cl_2 (5 mL) under argon and the reaction mixture was refluxed (48 h). The crude reaction mixture was evaporated in vacuo, and the remaining residue was purified by silica gel flash chromatography to give **20** as yellow oil (56 mg, 66% yield). H NMR ($CDCl_3$) δ 9.34 (br s, 1H), 8.61 (d, 1H, J = 8.2 Hz), 8.06 (d, 1H, J = 8.4 Hz), 7.77 (s, 1H), 7.50 (t, 1H, J = 5.9 Hz), 7.36 (d, 1H, J = 8.4 Hz), 7.21–7.16 (m, 5H), 7.07 (d, 1H, J = 6.8 Hz), 6.49–6.44 (m, 2H), 5.72 (dd, 1H, J = 10.0 and 14.1 Hz), 5.26–5.20 (m, 2H), 4.65 (m, 1H), 4.28 (d, 1H, J = 9.6 Hz), 3.69 (dd, 1H, J = 6.0 and 13.0 Hz), 3.30–3.23 (m, 2H), 3.05 (m, 1H), 2.96 (d, 2H, J = 21.3 Hz), 2.89 (dd, 1H, J = 11.9 and 17.2 Hz), 2.71 (m, 1H), 2.45 (dd, 1H, J = 4.3 and 15.0 Hz), 2.39–2.16 (m, 4H), 2.02 (m, 1H), 1.89–1.46 (m, 10H), 1.42 (s, 9H), 1.34 (s, 9H), 1.28 (s, 9H). FABMS (+ve) *m/z* 931 [MH^+].

6.13. (9*S*,10*S*,11*E*,14*S*,18*S*)-18-(2-Amino-2-oxoethyl)-14-(1-naphthalenylmethyl)-8,17,20-trioxo-10-[4-(phosphonomethyl)phenyl]-7,16,19-triazaspiro[5.14]eicos-11-ene-9-acetic acid (4)

A solution of **20** (16 mg, 0.017 mmol) in a mixture of TFA–TES–H_2O (2.0 mL, v/v, 3.7:0.1:0.2) was stirred at room temperature (1 h). Solvent was evaporated in vacuo and the remaining residue was purified by HPLC using a linear gradient from 5% B to 95% B over 25 min. Analytical retention time = 23.5 min; preparative retention time = 15.2 min. Lyophilization provided **4** as a white solid (7.6 mg, 58% yield) in 96% purity as determined by HPLC. H NMR (DMSO-d_6) δ 9.98 (br s, 1H), 8.48 (s, 1H), 8.23 (d, 1H, J = 8.2 Hz), 8.01 (d, 1H, J = 8.0 Hz), 7.55 (d, 1H, J = 8.6 Hz), 7.46 (s, 1H), 7.38–7.24 (m, 5H), 7.15 (dd, 2H, J = 2.0 and 8.4 Hz), 7.03 (s, 1H), 6.86 (d, 1H, J = 7.4 Hz), 5.76 (dd, 1H, J = 10.9 and 14.5 Hz), 5.45 (m, 1H), 4.28 (m, 1H), 4.06 (m, 1H), 3.60 (m, 1H), 3.55 (m, 1H), 3.10 (m, 1H), 2.87 (d, 2H, J = 21.3 Hz), 2.83–2.67 (m, 3H), 2.55 (m, 1H), 2.33 (dd, 1H, J = 4.8 and 15.5 Hz), 2.08–1.16 (m,

12H). FABMS (+ve) *m/z* 761 [M−H$^-$]. HRMS calcd for $C_{29}H_{47}N_4O_{10}NaP$ [MNa$^+$]: 785.2922.4539. Found: 785.2964.

6.14. Conversion of 4 to the tri-sodium salt (4s)

To a solution of **4** (6.1 mg, 8.0 μM) in CH_3CN–H_2O (1.6 mL, v/v = 1:1) at room temperature was added a solution of $NaHCO_3$ (24 μM) in H_2O (0.40 mL) and the resulting mixture was lyophilized to provided **4s** as a pale solid (6.6 mg, 100% yield).

6.15. Biotinylation of 20: synthesis of 22

To a solution of **20** (23 mg, 25 μM) and 6-biotinamidocaproic acid (**21**) (36 mg, 100 μM) in DMF (3 mL) was added EDCI·HCl (19.2 mg, 100 μM) and DMAP (1.2 mg, 10 μM) at 0 °C and the resulting solution was stirred at room temperature (12 h). The solvent was evaporated and the residue was dissolved in EtOAc, washed with H_2O, brine, and dried over Na_2SO_4. The solvent was removed and the residue was purified by silica gel flash chromatography to provide **22** as colorless oil (25 mg, 80% yield). H NMR ($CDCl_3$) δ 8.24 (d, 1H, J = 8.1 Hz), 8.01 (d, 1H, J = 8.6 Hz), 7.70 (d, 1H, J = 8.4 Hz), 7.56 (t, 1H, J = 5.8 Hz), 7.50 (t, 1H, J = 8.1 Hz), 7.42–7.32 (m, 3H), 7.21–7.16 (m, 5H), 7.05 (s, 1H), 6.31 (t, 1H, J = 5.3 Hz), 6.19 (s, 1H), 5.94 (s, 1H), 5.83–5.77 (m, 2H), 5.59 (s, 1H), 5.37 (m, 1H), 4.57 (m, 1H), 4.13–4.08 (m, 2H), 4.01 (d, 1H, J = 9.7 Hz), 3.72 (dd, 1H, J = 5.9 and 12.6 Hz), 3.53 (t, 1H, J = 5.6 Hz), 3.37 (t, 1H, J = 5.4 Hz), 3.25–3.03 (m, 4H), 2.98 (d, 2H, J = 21.1 Hz), 2.87–2.64 (m, 4H), 2.52 (d, 1H, J = 12.8 Hz), 2.33–2.00 (m, 9H), 1.84–1.42 (m, 22H), 1.39 (s, 9H), 1.38 (s, 9H), 1.33 (s, 9H). FABMS (+ve) *m/z* 1270 [MH$^+$]. HRMS calcd for $C_{67}H_{96}N_7O_{13}$-NaPS [MNa$^+$]: 1292.6417. Found: 1292.6462.

6.16. Deprotection of 22 to yield 5

A solution of **22** (25 mg, 20 μM) in a mixture of TFA–ethanedithiol–H_2O (2.0 mL, v/v, 3.8:0.1:0.1) was stirred at room temperature (1 h). The mixture was reduced under vacuum to a volume of 0.25 mL and the solution was diluted with diethyl ether (10 mL) to give a white solid. The solid was collected and purified by HPLC using a linear gradient of 5% B to 95% B over 25 min. Analytical retention time = 26.3 min; preparative retention time = 16.3 min. Lyophilization provided **5** as a white solid (12.2 mg, 56% yield) in 99% purity as determined by HPLC. H NMR (DMSO-d_6) δ 8.49 (s, 1H), 8.25 (d, 1H, J = 8.1 Hz), 8.07 (d, 1H, J = 8.8 Hz), 7.77 (t, 1H, J = 5.6 Hz), 7.72 (m, 1H), 7.55 (t, 1H, J = 8.0 Hz), 7.48–7.45 (m, 3H), 7.32–7.25 (m, 4H), 7.15 (dd, 2H, J = 2.2 and 8.2 Hz), 7.02 (s, 1H), 6.41 (s, 1H), 6.35 (br s, 1H), 5.77 (dd, 1H, J = 10.5 and 14.3 Hz), 5.45 (m, 1H), 4.31–4.26 (m, 2H), 4.11 (dd, 1H, J = 4.4 and 7.6 Hz), 4.06 (d, 1H, J = 10.0 Hz), 3.52 (m, 1H), 3.30 (m, 1H), 3.19 (dd, 1H, J = 5.3 and 14.0 Hz), 3.11–3.04 (m, 2H), 2.89 (d, 2H, J = 21.3 Hz), 2.84–2.75 (m, 6H), 2.60–2.54 (m, 2H), 2.33 (dd, 1H, J = 4.5 and 15.4 Hz), 2.20 (m, 1H), 2.09–1.96 (m, 6H), 1.84–1.19 (m, 22H). FABMS (+ve) *m/z* 1100 [M−H$^-$]. HRMS calcd for $C_{55}H_{72}N_7O_{13}NaPS$ [MNa$^+$]: 1124.4539. Found: 1124.4601.

6.17. Conversion of 5 to the tri-sodium salt (5s)

To a solution of **5** (10.4 mg, 9.4 μM) in CH_3CN–H_2O (1.0 mL, v/v = 1:1) at room temperature was added a solution of $NaHCO_3$ (28 μM) in H_2O (0.47 mL) and the resulting mixture was lyophilized to provided **5s** as a pale white solid (11.0 mg, 100% yield).

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