Design, synthesis and pharmacological activity of novel enantiomerically pure phosphonic acid-based NAALADase inhibitors

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Inhibitors of NAALADase have shown promise for a variety of diseases associated with glutamate excitotoxicity, and could be useful for the diagnosis and therapy of prostate cancer. A series of novel enantiomerically pure 2-(phosphonomethyl)pentanedioic acid (2-PMPA) based NAALADase inhibitors were synthesized. These compounds were prepared from previously reported (*S*)-2-(hydroxyphosphinoylmethyl)pentanedioic acid benzyl ester **4**. Biological test results showed that the new compounds are good to outstanding NAALADase inhibitors. Compounds **8b** and **10b** showed activity similar to the known potent inhibitor (*S*)-2-PMPA. Fluorescently labeled inhibitor **19b** may potentially be used to study binding to prostate cancer cells by fluorescence microscopy, and siderophore-containing inhibitor **21b** may be useful for detection of prostate-derived cancer cells by magnetic resonance imaging (MRI).

Introduction

The metalloprotease glutamate carboxypeptidase II (GCP II, also known as *N*-acetylated alpha-linked acidic dipeptidase, NAALADase) releases *N*-acetyl aspartate and glutamate from both the neuronal peptide *N*-acetylaspartylglutamate (NAAG) and folate polyglutamate (Scheme 1).[1] Inhibitors of NAALADase have shown efficacy in a variety of animal models of neurological disease associated with glutamate excitotoxicity.[2]

Scheme 1

NAALADase is also expressed in the human prostate parenchyma, from where it was first cloned and named prostate specific membrane antigen (PSMA).[3] It has been shown that inhibitors of NAALADase also strongly bind to PSMA. As a representative member of the phosphinate-based series of binders, VA-033 was found to serve as a potent inhibitor of NAALADase activity associated with PSMA that is expressed on LNCaP human prostate cancer cells and by tumor cells *in vivo*.[4a] These inhibitors themselves do not affect the viability of the LNCaP cells, nor do they significantly alter cell cycle kinetics. They therefore provide a biologically innocuous scaffold that can be used to target diagnostic markers or chemotherapeutic drugs to the surface membrane of prostate tumor cells. Recent studies have shown that the NAALADase inhibitors may also have more direct therapeutic potential.[4b]

Over the past decade, tremendous efforts have been made in the development of potent inhibitors of GCP II (Fig. 1).[5] The first potent and selective inhibitor was 2-(phosphonomethyl)-pentanedioic acid (2-PMPA) reported by Jackson *et al.* in 1996.[6] Since then, extensive structure–activity relationship studies have been carried out using 2-PMPA as a template, and a few potent inhibitors of GCP II, such as urea-based compounds[7] and GPI5232[8] were identified. Berkman's group reported the synthesis of phenylalkylphosphonamidates as prostate-specific membrane antigen (PSMA) inhibitors.[9] Further studies on 2-PMPA have demonstrated that potent inhibition of glutamate carboxypeptidase II (GCP II) is specific to (*S*)-2-PMPA, which has an absolute configuration corresponding to L-glutamate.[10] Recent studies on GPI5232 also showed that the (*S*)-enantiomer is 40-fold more potent than the (*R*)-enantiomer in a GCP II inhibitory assay.[11] Therefore, it is essential to synthesize the enantiomerically pure inhibitors prior to further preclinical characterization in order to eliminate the potential for undesired pharmacological effects or other interferences by the inactive enantiomers.

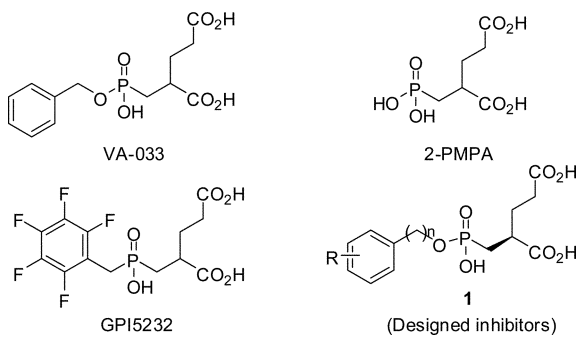

Fig. 1 Inhibitors of NAALADase.

Based on these known results, we became interested in the synthesis of enantiomerically pure phosphonates having the general structure **1** and possessing the (*S*)-configuration. First, the phosphonate group of **1** was intended to both mimic the tetrahedral intermediate formed during hydrolysis of NAAG and inhibit catalytic activity of the enzyme by binding the essential zinc in the active site. Second, the ability to derivatize the inhibitor at position

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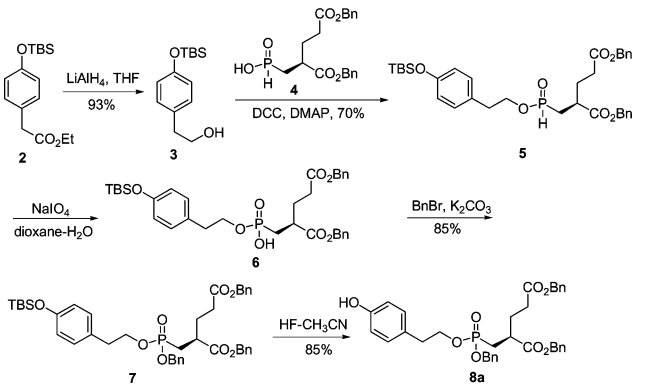
R by linking a suitably labeled head group was anticipated to allow detection of prostate derived cells in circulation by fluorescence or by magnetic resonance imaging (MRI) whilst not impairing the ability of the inhibitor to access the active site.[12] In this paper, we describe the synthesis of a series of novel enantiomerically pure 2-PMPA based derivatives **1** and their inhibitory potencies in a GCP II assay.

Results and discussion

Our first synthesis of a form of **1** focused on preparation of **8a** (Scheme 2). The synthesis of TBS protected alcohol **3** has been previously accomplished by selective deprotection of the corresponding bis(TBS) derivative with I_2 in methanol.[13] We used the route shown in Scheme 2 to obtain this product in excellent yield. The starting material **2** was readily prepared from ethyl 4-hydroxyphenylacetate.[14] The coupling reaction between **3** and optically pure (*S*)-2-(hydroxyphosphinoylmethyl)pentanedioic acid dibenzyl ester **4**[5] afforded phosphinate **5** smoothly. Product **5** was then subjected to oxidation by $NaIO_4$ to provide phosphonic acid **6**. Treatment of **6** with $BnBr–K_2CO_3$ in DMF gave benzyl ester **7** in 85% yield, while the esterification reaction employing benzylisourea or using BOP as an activating agent did not give good yields.[15] Desilylation using HF in CH_3CN[16] gave phenol **8a** cleanly.



Scheme 2

By modification of the above three-step approach for the preparation of benzyl ester **7**, the four benzyl esters **9a–12a** (Fig. 2) were obtained in the indicated overall yields from the corresponding commercially available phenalkanols.

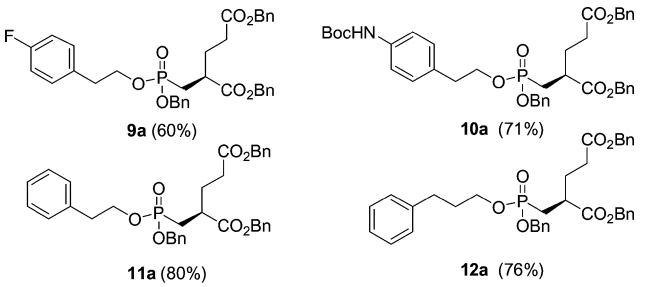
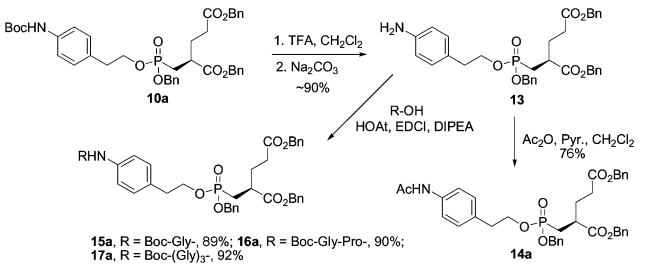


Fig. 2 Benzyl esters **9a–12a**.

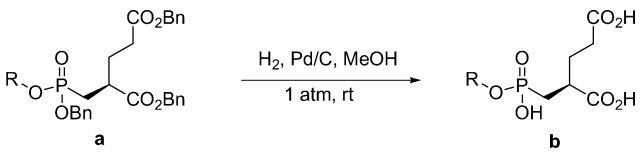
Several additional analogs were obtained by elaboration of **10a** (Scheme 3). Thus, Boc deprotection of **10a** gave aniline **13** as a crude oil that was used directly in the next step without purification. Acetylation of **13** provided **14a**. Boc-Gly-OH, Boc-Gly-Pro-OH and Boc-$(Gly)_3$-OH were also coupled to aniline **13** to give compounds **15a–17a** in excellent yields.



Scheme 3

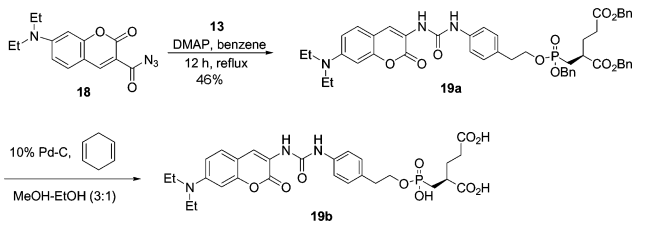
Removal of the benzyl groups from each of the protected products gave the corresponding potential inhibitors **8b**, **9b**, **10b**, **11b**, **12b**, **14b**, **15b**, **16b**, and **17b** (Table 1). ^{31}P NMR analysis of each final acid revealed one sharp singlet, supporting the formation of a single diastereomer.

Table 1 Inhibition of GCP II by the proposed inhibitors generated by debenzylation



Compound	R	IC_{50}/nM	SEM	n^a
8b	CH_2CH_2(4-OH-C_6H_4)	0.2	0.05	4
9b	CH_2CH_2(4-F-C_6H_4)	300	0	2
10b	CH_2CH_2(4-BocNH-C_6H_4)	0.1	0.01	4
11b	$CH_2CH_2C_6H_5$	0.8	0.05	2
12b	$CH_2CH_2CH_2C_6H_5$	4	2	4
14b	CH_2CH_2(4-AcNH-C_6H_4)	4		1
15b	CH_2CH_2(4-Boc-Gly-NH-C_6H_4)	0.7	0	2
16b	CH_2CH_2(4-Boc-Gly-Pro-NH-C_6H_4)	7		1
17b	CH_2CH_2[4-Boc-$(Gly)_3$-NH-C_6H_4]	8		1
19b		2	0.8	4
21b		5	2	4
(*S*)-2-PMPA		0.4	0.09	4

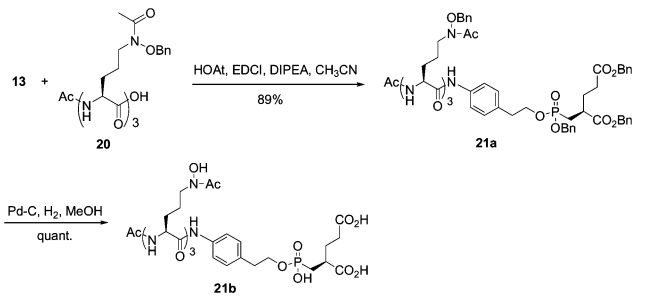
[a] n = the number of times the assay was performed.



Scheme 4

As shown in Scheme 4, fluorescent-labeled compound **19b** was prepared in two steps. Refluxing the mixture of free amine **13** and commercially available 7-(diethylamino)coumarin-3-carbonyl-azide **18** in benzene followed by column chromatographic purification in the presence of Et_3N provided the fully protected intermediate **19a** in good yield.[17] To avoid overreduction, debenzylation was performed by using 1,4-cyclohexadiene in the presence of 10% Pd–C catalyst in MeOH–EtOH (v/v 3 : 1) to give the desired product (**19b**). As will be subsequently reported, this fluorescently labeled compound will be used to study binding to prostate cancer cells by fluorescence microscopy.

Siderophore-containing moiety **21b** was readily obtained in two steps as shown in Scheme 5. The coupling reaction between aniline **13** and tripeptide acid **20**[18] gave the fully protected precursor **21a**. Upon removal of the benzyl protecting groups under standard catalytic hydrogenolysis conditions, conjugate **21b** was obtained in quantitative yield. The iron and gadolinium complexes of this compound will be studied for the detection of prostate cancer cells by MRI.



Scheme 5

The *in vitro* GCP II inhibitory potencies of these obtained PSMA inhibitors were measured at Guilford Pharmaceuticals Inc. using *N*-acetyl-L-aspartyl-[^{3}H]-L-glutamate as a substrate and human recombinant GCP II[19] as previously reported.[20] The assay results are shown in Table 1 along with the assay reference compound (*S*)-2-PMPA. Eight concentrations of each inhibitor in the 0.1 pM^{-1} μM range were evaluated for each IC_{50} determination. Xcelfit software was used to calculate the IC_{50} values. We plotted log of concentration *vs.* % inhibition. The fluoro compound **9b** showed low activity, whereas compounds **8b** and **10b** demonstrated the best activity with IC_{50} values of 0.2 nM and 0.1 nM, respectively. Compared to compound **11b**, compound **12b** with the increased number of methylene units was five-fold less active. Acetyl derivative **14b** possessed good activity with an IC_{50} value of 4 nM. Again, compared to compound **15b**, compounds **16b** and **17b**, with the increased side chain length of the amide attached to the phenyl group, had ten-fold decreased activity. Fluorescently labeled inhibitor **19b** and siderophore-containing inhibitor **21b** displayed activity with IC_{50} values of 2 nM and 5 nM, respectively, indicating that potentially diagnostically useful labeling groups are compatible with inhibitor design.

Conclusions

Based on the known potent NAALADase inhibitor (*S*)-2-(phosphonomethyl)pentanedioic acid (2-PMPA), we synthesized a series of eleven enantiomerically pure forms of phosphonate **1**. Most of the final products showed good to outstanding NAALADase inhibitory activity. Our studies clearly demonstrate that the bulky, phenyl-derived linker group is tolerated in the newly prepared NAALADase inhibitors. These results should facilitate future development of other NAALADase and PSMA inhibitors. Additional future studies will determine the feasibility of using fluorescently labeled inhibitor **19b** to study prostate cancer cells by fluorescence microscopy. Siderophore-containing inhibitor **21b** will be studied as iron and gadolinium complexes for the detection of prostate cancer cells by MRI. These and other chemical agents are being developed for the early detection and treatment of prostate cancer.

Experimental

All reactions were carried out under argon by using standard techniques. Solvents were dried under nitrogen by standard procedures, distilled before use and stored under argon. NMR spectra were recorded on a Varian Unityplus 300 MHz spectrometer or Varian Inova 500 MHz spectrometer. Optical rotations were recorded on a Perkin Elmer model 343 polarimeter. Mass spectra were recorded on a Jeol JMS-AX505 HA Double Sector mass spectrometer.

2-(4-*tert*-Butyldimethylsilyloxyphenyl)ethanol (3)

To a suspension of $LiAlH_4$ (1.08 g, 28.46 mmol) in THF (70 mL) was added a solution of **2**[14] (4.00 g, 13.58 mmol) in THF (15 mL) portionwise at 0 °C, and the resulting mixture was stirred at that temperature for 1.5 h. Then H_2O (1.1 mL) was added slowly, followed by 1.1 mL of 15% NaOH solution and 1.65 mL of H_2O. After stirring for another 30 min at 0 °C, the mixture was filtered through a Celite pad to remove the resulting white solid. The solid was washed with THF. The combined organic layers were concentrated to provide the crude product which was purified by flash column chromatography on silica gel (hexanes–EtOAc 3 : 1 → 2 : 1) to afford 3.18 g of the product as a colorless oil (93%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.08 (d, $J = 8.5$ Hz, 2H), 6.79 (d, $J = 8.5$ Hz, 2H), 3.82 (t, $J = 6.5$ Hz, 2H), 2.81 (t, $J = 6.5$ Hz, 2H), 1.27 (brs, 1H), 0.99 (s, 9H), 0.19 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 154.46, 131.11, 130.13, 120.36, 64.03, 38.57, 25.89, 18.39, −4.22.

General procedure for the synthesis of benzyl esters (7, 9a, 10a, 11a, and 12a) from the corresponding phenalkanols

(*S*)-2-[(4-*tert*-Butyldimethylsilyloxyphenyl)ethoxy]-phosphinoylmethyl-pentanedioic acid dibenzyl ester (5). To a solution of **3** (200 mg, 0.792 mmol) and (*S*)-(−)-2-[hydroxyphosphinoylmethyl]-pentanedioic acid dibenzyl ester **4**[5] (309 mg, 0.792 mmol) in THF (9 mL) were added DCC (172 mg, 0.833 mmol) and DMAP (12 mg, 0.095 mmol). The reaction mixture was stirred overnight,

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and the resulting white solid (DCU) was filtered and washed with THF (7 mL). The filtrate was concentrated and purified by flash column chromatography on silica gel (hexanes–EtOAc 3 : 1 → 1 : 1) to afford 346 mg of **5** as a colorless oil (70%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.38–7.35 (m, 10H), 7.08 (dd, J = 553, 10 Hz, 1H), 7.07–7.04 (m, 2H), 6.80–6.77 (m, 2H), 5.14 (s, 2H), 5.12 (s, 2H), 4.30–4.05 (m, 2H), 3.01–2.80 (m, 3H), 2.43–1.80 (m, 6H), 0.99 (s, 9H), 0.20 (s, 6H); HRFABMS calcd. for $C_{34}H_{46}O_7PSi$ $(M + H)^+$ 625.2750, found 625.2731.

(*S*)-2-[[(4-*tert*-Butyldimethylsilyloxyphenyl)ethoxy]-phosphonoyl]methyl-pentanedioic acid dibenzyl ester (6). Compound **5** (327 mg, 0.534 mmol) was dissolved in dioxane–water (5 : 1, 6 mL), and $NaIO_4$ (134 mg, 0.628 mmol) was added. The reaction mixture was stirred at room temperature for 20 h, and then diluted with EtOAc (150 mL) and water (20 mL). The layers were separated, and the organic layer was washed with saturated aqueous $KHSO_4$ (25 mL), 0.3 M $Na_2S_2O_3$ (25 mL), H_2O (20 mL) and brine (25 mL). After being dried, filtered, and concentrated, 335 mg of the product was obtained as a semi-solid (100%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.36–7.22 (m, 10H), 7.02 (d, J = 8.0 Hz, 1H), 6.67 (d, J = 8.0 Hz), 5.09–4.98 (m, 4H), 3.95–3.85 (m, 2H), 2.80–2.70 (m, 3H), 2.32–2.22 (m, 2H), 2.03–1.80 (m, 4H), 1.65–1.55 (m, 1H), 0.93 (s, 9H), 0.11 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 176.96 (d, J = 6.2 Hz), 174.65, 155.56, 137.72, 137.66, 132.95, 131.18, 129.65, 129.42, 129.31, 129.22, 121.06, 67.74, 67.46, 66.16, 41.90, 37.76, 32.75, 29.71, 29.58, 26.34, 19.15, −4.08; HRFABMS calcd. for $C_{34}H_{45}KO_8PSi$ $(M + K)^+$ 679.2253, found 679.2258.

(*S*)-2-[[(4-*tert*-Butyldimethylsilyloxyphenyl)ethoxy]-phosphonoyl]methyl-pentanedioic acid tribenzyl ester (7). Compound **6** (70 mg, 0.109 mmol) was dissolved in DMF (4 mL), and K_2CO_3 (90 mg, 0.655 mmol) and benzyl bromide (78 μL, 0.665 mmol) were added. The resulting mixture was stirred at room temperature for 10 h, diluted with CH_2Cl_2, and washed with cold water and brine. After being dried, filtered, and concentrated, the residual DMF was evaporated under high vacuum. The residue was purified by flash column chromatography eluting with hexanes–ethyl acetate (2 : 1 → 1 : 1 → 1 : 2) to afford 64 mg of product as a colorless oil (85%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.42–7.25 (15H, m), 7.06–6.97 (2H, m), 6.80–6.72 (2H, m), 5.18–4.90 (6H, m), 4.17–4.06 (2H, m), 2.84–2.80 (3H, m), 2.35–2.24 (3H, m), 2.07–1.80 (3H, m), 1.00 (9H, s), 0.20 (6H, s); HRFABMS calcd. for $C_{41}H_{52}O_8PSi$ $(M + H)^+$ 731.3169, found 731.3138.

(*S*)-2-[[(4-Hydroxyphenyl)ethoxy]phosphonoyl]methyl-pentanedioic acid tribenzyl ester (8a). To a flask containing **7** (280 mg, 0.383 mmol) was added 40% HF–CH_3CN (5 : 95) (21 mL), and the reaction mixture was stirred at room temperature for 6 h and then diluted with EtOAc (100 mL). The layers were separated, and the organic layer was washed with saturated $NaHCO_3$ solution and brine. After being dried, filtered, and concentrated, the residue was purified by flash column chromatography eluting with hexanes–ethyl acetate (2 : 1 → 1 : 1 → 1 : 2) to afford 200 mg of product as a colorless oil (85%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.99–7.93 (1H, m), 7.37–7.32 (15H, m), 6.94–6.91 (2H, m), 6.82–6.80 (2H, m), 5.11–4.94 (6H, m), 4.20–4.01 (2H, m), 2.90–2.78 (3H, m), 2.40–2.23 (3H, m), 2.02–1.77 (3H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.30, 29.19; HRFABMS calcd. for $C_{35}H_{38}O_8P$ $(M + H)^+$ 617.2304, found 617.2324.

General procedure for the synthesis of final acids (8b, 9b, 10b, 11b, 12b, 14b, 15b, 16b, 17b and 21b)

(*S*)-2-[[(4-Hydroxyphenyl)ethoxy]phosphonoyl]methyl-pentanedioic acid (8b). To a solution of **8a** (25 mg, 0.031 mmol) in MeOH (5 mL) was added 10% Pd–C (30 mg). The reaction mixture was stirred under an atmosphere of H_2 for 3 h. The resulting mixture was filtered through a pad of Celite and washed with methanol. The combined solution was evaporated to afford 10.1 mg of the product as a colorless oil (95%. $[\alpha]_D^{20}$ = +4.4° (c = 0.75, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 6.98 (2H, d, J = 8.5 Hz), 6.62 (2H, d, J = 8.5 Hz), 3.98 (2H, q, J = 7.0 Hz), 2.76 (2H, q, J = 7.0 Hz), 2.63–2.58 (1H, m), 2.30–2.18 (2H, m), 2.08–2.00 (1H, m), 1.90–1.65 (3H, m); ^{13}C NMR (125 MHz, CD_3OD) δ: 177.93, 176.65, 157.26, 131.24, 129.89, 116.37, 67.41, 40.78, 37.33, 32.37, 29.76, 28.62; ^{31}P NMR (121 MHz, $CDCl_3$) δ: 26.05; HRFABMS calcd. for $C_{14}H_{20}O_8P$ $(M + H)^+$ 347.0896, found 347.0913.

(*S*)-2-[[(4-Fluorophenyl)ethoxy]phosphonoyl]methyl-pentanedioic acid tribenzyl ester (9a). Yield: 60%. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.42–7.31 (15H, m), 7.13–7.09 (2H, m), 6.98–6.95 (2H, m), 5.17–4.95 (6H, m), 4.20–4.02 (2H, m), 2.85 (2H, q, J = 7.0 Hz), 2.83–2.78 (1H, m), 2.40–2.24 (3H, m), 2.04–1.96 (2H, m), 1.93–1.77 (1H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.78; HRFABMS calcd. for $C_{35}H_{37}FO_7P$ $(M + H)^+$ 619.2261, found 619.2272.

(*S*)-2-[[(4-Fluorophenyl)ethoxy]phosphonoyl]methyl-pentanedioic acid (9b). Yield: 93%. $[\alpha]_D^{20}$ = +20.4° (c = 0.5, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.10–7.04 (2H, m), 6.83–6.77 (2H, m), 3.96 (2H, q, J = 6.5 Hz), 2.75 (2H, t, J = 6.5 Hz), 2.52–2.43 (1H, m), 2.23–2.05 (2H, m), 2.02–1.93 (1H, m), 1.80–1.58 (3H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 27.23; HRFABMS calcd. for $C_{14}H_{19}FO_7P$ $(M + H)^+$ 349.0852, found 349.0834.

(*S*)-2-[[(4-*N*-*tert*-Butoxylcarbonylphenyl)ethoxy]-phosphonoyl]-methyl-pentanedioic acid tribenzyl ester (10a). Yield: 71%. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.36–7.24 (17H, m), 7.12–7.02 (2H, m), 6.52 (1H, NH, brs), 5.10–4.92 (6H, m), 4.20–4.01 (2H, m), 2.90–2.73 (3H, m), 2.37–2.19 (3H, m), 2.02–1.72 (3H, m), 1.54 (9H, s); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.03, 28.96; HRFABMS calcd. for $C_{40}H_{47}NO_9P$ $(M + H)^+$ 716.2988, found 716.2963.

(*S*)-2-[[(4-*N*-*tert*-Butoxylcarbonylphenyl)ethoxy]-phosphonoyl]-methyl-pentanedioic acid (10b). Yield: 97%. $[\alpha]_D^{20}$ = −32.0° (c = 0.5, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.32–7.26 (2H, m), 7.18–7.12 (2H, m), 4.15–3.97 (2H, m), 2.95–2.85 (3H, m), 2.42–1.63 (6H, m), 1.49 (9H, s); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 24.33; MS calcd. for $C_{19}H_{28}NO_9P$ $(M + H + Na)^+$ 469.1478, found 469.1490.

(*S*)-2-[(2-Phenylethoxy)phosphonoyl]methyl-pentanedioic acid tribenzyl ester (11a). Yield: 80%. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.41–7.17 (20H, m), 5.12–4.92 (6H, m), 4.25–4.11 (2H, m), 2.91 (2H, q, J = 6.5 Hz), 2.88–2.78 (1H, m), 2.38–2.22 (3H, m), 2.03–1.96 (2H, m), 1.88–1.77 (1H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.58, 29.51; HRFABMS calcd. for $C_{35}H_{38}O_7P$ $(M + H)^+$ 601.2355, found 601.2349.

(*S*)-2-[(2-Phenylethoxy)phosphonoyl]methyl-pentanedioic acid (11b). Yield: 91%. $[\alpha]_D^{20}$ = +5.8° (c = 1.2, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.08–6.95 (5H, m), 3.93 (2H, q, J = 7.0 Hz), 2.76 (2H, t, J = 7.0 Hz), 2.58–2.40 (1H, m), 2.19–2.01

(2H, m), 1.99–1.51 (4H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 26.75; HRFABMS calcd. for $C_{14}H_{20}O_7P$ $(M + H)^+$ 331.0947, found 331.0959.

(*S*)-2-[(3-Phenylpropanoxy)phosphonoyl]methyl-pentanedioic acid tribenzyl ester (12a). Yield: 76%. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.40–7.17 (20H, m), 5.12–5.02 (6H, m), 4.03–3.88 (2H, m), 2.97–2.83 (1H, m), 2.70–2.62 (2H, m), 2.44–2.28 (3H, m), 2.12–1.82 (5H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.59, 29.54; HRFABMS calcd. for $C_{36}H_{40}O_7P$ $(M + H)^+$ 615.2512, found 615.2487.

(*S*)-2-[(3-Phenylpropanoxy)phosphonoyl]methyl-pentanedioic acid (12b). Yield: 93%. $[\alpha]_D^{20} = +5.3°$ ($c = 1.1$, MeOH); ^{1}H NMR (500 MHz, D_2O) δ: 7.40–7.21 (5H, m), 3.90–3.38 (2H, m), 2.80–2.33 (5H, m), 2.20–1.74 (6H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 26.04; FABMS calcd. for $C_{15}H_{22}O_7P$ $(M + H)^+$ 345, found 345.

(*S*)-2-[[(4-Aminophenyl)ethoxy]phosphonoyl]methyl-pentane-dioic acid tribenzyl ester (13). To a solution of **10a** (440 mg, 0.714 mmol) in CH_2Cl_2 (10 mL) was added 1 mL of TFA. The resulting reaction mixture was stirred at room temperature for 1 h and then diluted with CH_2Cl_2 (100 mL). The mixture was washed with saturated aqueous Na_2CO_3 to remove the excess TFA. The separated aqueous layer was extracted with CH_2Cl_2 twice, and the combined extracts were washed with 30 mL of brine, dried over $MgSO_4$, filtered and concentrated. The crude product was obtained as a colorless oil, which was not stable and used directly for the next steps without further purification. HRFABMS calcd. for $C_{35}H_{39}NO_7P$ $(M + H)^+$ 616.2464, found 616.2469.

(*S*)-2-[[(4-Acetaminophenyl)ethoxy]phosphonoyl]methyl-pentane-dioic acid tribenzyl ester (14a). To a solution of crude **13** (80 mg, 0.13 mmol) in CH_2Cl_2 were added Ac_2O (37 μL, 0.39 mmol) and pyridine (32 μL, 0.39 mmol) at 0 °C. The reaction mixture was stirred and warmed to room temperature overnight. The volatiles were evaporated and the residue was purified by flash column chromatography eluting with hexanes–ethyl acetate (1 : 2 → 1 : 3) to afford 65 mg (76%) of product as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.20–7.15 (16H, m), 7.25–7.22 (2H, m), 7.05–7.03 (2H, m), 5.09–4.92 (6H, m), 4.24–4.06 (2H, m), 2.92 (2H, q, $J = 6.5$ Hz), 2.88–2.80 (1H, m), 2.40–2.78 (3H, m), 2.27 (3H,s), 2.05–1.80 (3H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.29; HRFABMS calcd. for $C_{37}H_{41}NO_8P$ $(M + H)^+$ 658.2570, found 658.2568.

(*S*)-2-[[(4-Acetaminophenyl)ethoxy]phosphonoyl]methyl-pentane-dioic acid (14b). Yield: 83%. $[\alpha]_D^{20} = -12.1°$ ($c = 0.3$, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.41 (2H, d, $J = 8.0$ Hz), 7.14 (2H, d, $J = 8.0$ Hz), 4.12–4.03 (2H, m), 3.66 (2H, t, $J = 7.0$ Hz), 2.73–2.68 (1H, m), 2.30–2.20 (3H, m), 2.04 (3H, s), 1.98–1.85 (3H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 26.88; FABMS calcd. for $C_{16}H_{23}NO_8P$ $(M + H)^+$ 388.1, found 388.1.

General procedure for the synthesis of 15a, 16a and 17a

Compound 15a. To a suspension of aniline **13** (210 mg, 0.341 mmol) and Boc-Gly-OH (72 mg, 0.409 mmol) in CH_3CN (5 mL) were added diisopropylethylamine (71 μL, 0.409 mmol), HOAt (56 mg, 0.409 mmol) and EDCI (79 mg, 0.409 mmol). The reaction mixture was stirred at room temperature for 18 h. After removal of the solvent, the residue was purified by flash column chromatography eluting with hexanes–ethyl acetate (1 : 4) to provide the product as a colorless oil in 89% yield (234 mg). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.47 (1H, NH, brs), 7.46–7.24 (17H, m), 7.10–7.05 (2H, m), 5.46 (1H, NH, brs), 5.09–4.94 (6H, m), 4.16–4.02 (2H, m), 3.93 (2H, brs), 2.84–2.72 (3H, m), 2.38–2.20 (3H, m), 1.99–1.71 (3H, m), 1.49 (9H, s); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.17, 29.07; HRFABMS calcd. for $C_{42}H_{50}N_2O_{10}P$ $(M + H)^+$ 773.3203, found 773.3229.

Compound 15b. Yield: 97%. $[\alpha]_D^{20} = -5.4°$ ($c = 1.0$, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.28–7.24 (2H, m), 7.14–6.92 (2H, m), 3.95 (2H, q, $J = 6.5$ Hz), 3.66–3.55 (2H, m), 2.75 (2H, t, $J = 6.5$ Hz), 2.63–2.58 (1H, m), 2.26–1.50 (6H, m); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 26.04; EI-MS calcd. for $C_{21}H_{32}N_2NaO_{10}P$ $(M + H + Na)^+$ 526, found 526.

Compound 16a. Yield: 90%. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.32 (bs, 1H), 7.50–7.01 (m, 19H), 5.51 (bs, 1H), 5.12–4.91 (m, 6H), 4.71 (m, 1H), 4.18–3.90 (m, 4H), 3.59–3.38 (m, 2H), 2.85–2.75 (m, 3H), 2.50–1.79 (m, 10H), 1.45 (s, 9H); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.59, 29.53; ^{31}P NMR (121 MHz, $CDCl_3$) δ: 26.41; HRFABMS calcd. for $C_{47}H_{57}N_3O_{11}P$ $(M + H)^+$ 870.3731, found 870.3768.

Compound 16b. Yield: 99%. $[\alpha]_D^{20} = -6.7°$ ($c = 0.6$, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.60–7.13 (m, 4H), 4.64–4.52 (m, 1H), 4.20–3.80 (m, 3H), 3.39–3.28 (m, 1H), 2.99–1.64 (m, 15H), 1.45 (s, 9H); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 26.41; HRFABMS calcd. for $C_{26}H_{39}KN_3O_{11}P$ $(M + H + K)^+$ 639.1959, found 639.1942.

Compound 17a. Yield: 92%. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.05 (bs, 1H), 7.95–7.05 (m, 19H), 6.03 (bs, 1H), 5.12–4.90 (m, 6H), 4.20–3.77 (m, 8H), 3.01–2.93 (m, 4H), 2.40–2.21 (m, 3H), 2.05–1.78 (m, 3H), 1.39 (s, 9H); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.40, 29.35; HRFABMS calcd. for $C_{46}H_{55}N_4NaO_{12}P$ $(M + Na)^+$ 909.3452, found 909.3440.

Compound 17b. Yield: 96% yield. $[\alpha]_D^{20} = -7.2°$ ($c = 0.7$, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.56–7.38 (m, 2H), 7.20–7.02 (m, 2H), 4.05–3.93 (m, 3H), 3.80–3.60 (m, 3H), 2.88–1.15 (m, 11H), 1.36 (s, 9H); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 26.04; HRFABMS calcd. for $C_{25}H_{37}N_4NaO_{12}P$ $(M + Na)^+$ 639.2043, found 639.2011.

Fluorescent-labeled compound precursor (19a). To a solution of aniline **13** (100 mg, 0.162 mmol) in dry benzene (5 mL) were added commercially available (Fluka) 7-(diethylamino)coumarin-3-carbonyl azide **18** (25 mg, 0.087 mmol) and DMAP (16 mg, 0.131 mmol). The resulting mixture was heated to reflux for 12 h. After cooling to room temperature, the solvent was evaporated. The residue was subjected to column chromatographic purification (EtOAc–hexanes–triethylamine 100 : 25 : 1 to 100 : 25 : 0.5) to afford 35 mg of the product as a yellow solid (46%): mp 63–65 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.90 (bs, 1H), 8.60–8.40 (m, 2H), 7.46–7.03 (m, 23H), 6.80–6.45 (m, 1H), 5.14–4.98 (m, 6H), 4.20–4.04 (m, 2H), 3.58–3.41 (m, 3H), 3.01–2.80 (m, 3H), 2.50–2.31 (m, 3H), 2.09–1.74 (m, 4H), 1.42–1.12 (m, 7H); ^{31}P NMR (121 MHz,

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$CDCl_3$) δ: 29.741; HRFABMS calcd. for $C_{49}H_{53}N_3O_{10}P$ $(M + H)^+$ 874.3469, found 874.3489.

Fluorescent-labeled compound (19b). To a solution of **19a** (30 mg, 0.034 mmol) in MeOH–EtOH (3 : 1, 8 mL) were added 1,4-cyclohexadiene (0.25 mL) and 10% Pd–C (30 mg). The reaction mixture was stirred at room temperature for 2 h. After filtration through filter paper, the solvents were removed to give the product as a colorless solid in 100% yield (21 mg): mp 42–45 °C; $[\alpha]_D^{20}$ = +72.4° (c = 0.5, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.40–7.02 (m, 4H), 4.17–4.05 (m, 2H), 3.61–3.24 (m, 4H), 2.97–2.84 (m, 2H), 2.79–2.60 (m, 1H), 2.40–1.60 (m, 5H), 1.40–0.81 (m, 8H); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 28.307; HRFABMS calcd. for $C_{28}H_{34}N_3O_{10}P$ (M^+) 603.1982, found 603.1969.

Tripeptide compound precursor (21a). To a suspension of aniline **13** (52 mg, 0.085 mmol) and tripeptide acid **20**[8] (60 mg, 0.071 mmol) in CH_3CN (7 mL) were added diisopropylethylamine (18 μL, 0.106 mmol), HOAt (12 mg, 0.085 mmol) and EDCI (16 mg, 0.085 mmol). The reaction mixture was stirred at room temperature for 24 h, and then diluted with EtOAc (60 mL). The solution was washed successively with 1 N HCl (5 mL), satd. aq. $NaHCO_3$ (5 mL), brine (10 mL), and then dried. Following concentration, the residue was purified by flash column chromatography eluting with EtOAc–MeOH (10 : 1 to 8 : 1) to provide 91 mg of the product as a colorless oil (89%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.78–7.25 (m, 34 H), 7.03–6.80 (m, 3H), 5.10–4.78 (m, 12H), 4.72–4.40 (m, 3H), 4.18–3.40 (m, 7H), 2.90–2.60 (m, 6H), 2.40–20 (m, 4H), 2.12–1.92 (m, 12H), 1.90–1.58 (m, 14H); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 29.522; EI-MS calcd. for $C_{79}H_{94}N_7O_{17}P$ (M^+) 1444, found 1444.

21b. Yield: 100%. $[\alpha]_D^{20} = -5.1°$ (c = 2.4, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.54–7.38 (m, 2H), 7.20–7.11 (m, 2H), 4.43–4.02 (m, 6H), 3.78–3.40 (m, 8H), 3.84 (bs, 2H), 2.38–2.20 (m, 2H), 2.18–1.52 (m, 26H); ^{31}P NMR (121 MHz, $CDCl_3$) δ: 28.382; HRFABMS calcd. for $C_{37}H_{59}N_7O_{17}P$ $(M + H)^+$ 904.3705, found 904.3675.

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