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Synthesis of Second- and Third-Generation Asp Oligopeptide Conjugated Dendrimers for Bone-Targeting Drug Delivery

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Abstract: The second- and third-generation multiple dendrimers based on naproxen in core and Asp(4–6) oligopeptides in periphery were synthesized in a convergent approach and well characterized by NMR and mass spectral (MS) techniques. These conjugates showed a high affinity to hydroxyapatite in vitro and provided an effective entry for the synthesis of a peptide dendrimer used for bone targeting.

Keywords: Asp oligopeptide, bone targeting, convergent synthesis, hydroxyapatite, peptide dendrimer

INTRODUCTION

Dendrimers are synthetic, highly branched, spherical, monodisperse macromolecules. One of the important developments in this field of pharmacy is the synthesis of drug-targeting delivery systems.[1] Constructs bearing multiple copies of a target moiety often display dramatically increased affinity and specificity relative to the monomer as a result of avidity and cooperativity effects. Dendrimers are promising as drug-delivery vehicles also by virtue of their multivalency, well-defined

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and large globular structure, mono- or low polydispersity, and amenability to postsynthetic manipulation.[2] To date, various types of dendrimers have been investigated as drug-targeting delivery systems. Examples include polyamidoamine (PAMAM), poly(propylene imine) (PPI), and poly(lysine).[3] This strategy has been applied to problems in cancer imaging, cancer therapy, and gene delivery.[4]

Dendrimers conjugated with biologically active peptides have proved to have increased activities.[5] Asp oligopeptides are novel bone-targeting moieties with four to six consecutive negatively charged amino acid residues that readily adsorb certain calcium-containing surfaces and exhibit a remarkable affinity to bone-hydroxyapatite. (The fully protected Asp(4–6) **1a**–**c** is shown in Fig. 1.[6] Unlike the P-C-P bond of bisphosphonates, the oligopeptides are biologically labile and enzymatically degraded. The advantageous property can make for more efficient drug release, and there is no unexpected long-term effects of the bone-targeting moiety.[7]

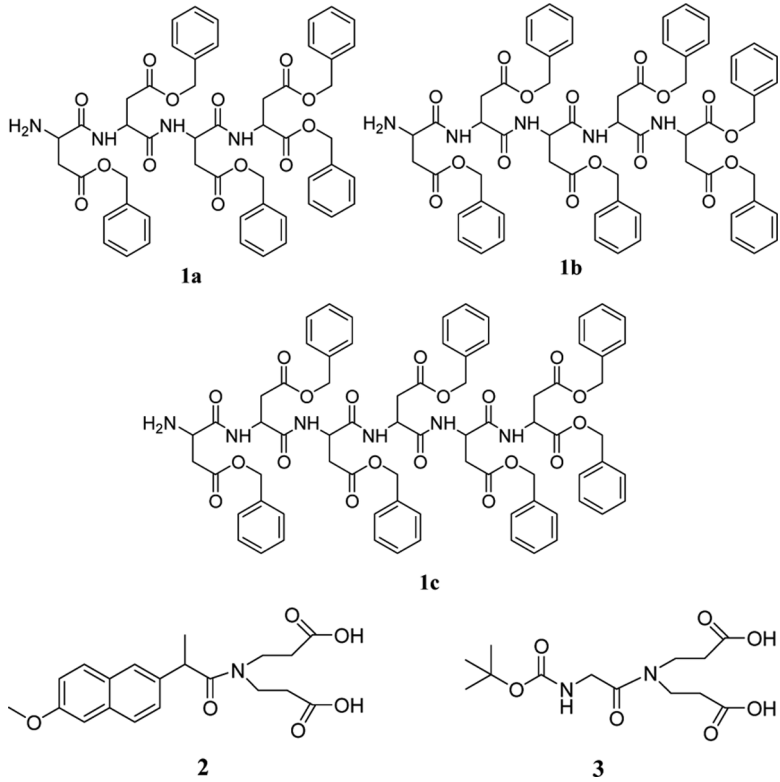


Figure 1. Structure of the fully protected Asp(4–6) (**1a**–**c**) and the dendrimer core (**2**,**3**).

We aim to increase the bone-targeting potency of the peptides with a dendritic approach and prepare sphere-type monodispersed Asp oligopeptide conjugated dendrimers with larger surface[8] as drug-carrying candidates for bone targeting.

In the current article, we report the design and synthesis of the second- and third-generation dendritic compounds with four or eight Asp(4–6) fragments. They may provide a potential opportunity to study the influence of shape and architecture or numbers of Asp oligopeptides on biodistribution, pharmacokinetics, and bone targeting through systematically adjusting the generation of the dendrimers. We employ iminodipropionic acid derivatives as novel scaffolds, and the structure of these dendrimer cores are shown in Fig. 1. Naproxen was used for a model drug for two reasons: first, naproxen is a clinically relevant anti-inflammatory agent helpful for treating osteoarthritis; second, it is amenable to synthetic manipulations (through amidation of the β-carboxy group) that provide a mechanism for conjugation and release. The dendrimer molecules linked by labile and hydrolyzable peptide bonds target the osseous tissue, where the active oligopeptides are easily cleavable, and the removal of the dendrimer periphery groups, liberation of the terminal amides, and subsequent unzipping of the dendrimer scaffold lead to release of the parent drug naproxen.[9] A hydroxyapatite (HAP) binding assay was established under in vitro conditions, and the affinity of the targeting conjugates for HAP is reported here. Preliminary study of drug release, biodistribution, and pharmacokinetic analysis of these conjugates for delivering naproxen into the bone in vivo is now under way.

RESULTS AND DISCUSSION

Preparation of Protected Bone-Targeting Peptides

The fully protected bone-targeting peptides **1** were synthesized by a conventional liquid-phase peptide synthetic method from Boc-Asp-Obzl utilizing IBCF (isobutyl chloroformate) and NMM (N-methyl morpholine) (mixed anhydrides method) by a series of segment condensation. The -COOH part dissolved in anhydrous tetrahydrofuran (THF) was cooled at −15°C (using a cryohydrate bath (ice/NaCl = 3:1, w/w), and 1 eq. NMM and IBCF were added and stirred for 5 min. The $-NH_2$ part in THF was added to the reaction mixture. After the coupling reaction was completed at room temperature, the mixture was purified by recrystallization or silica-gel column chromatography using CH_2Cl_2 and CH_3OH (100:1–60:1, v/v) as an eluent and deprotected in TFA (trifluoroacetic acid) to give the peptides **1a**–**c**.

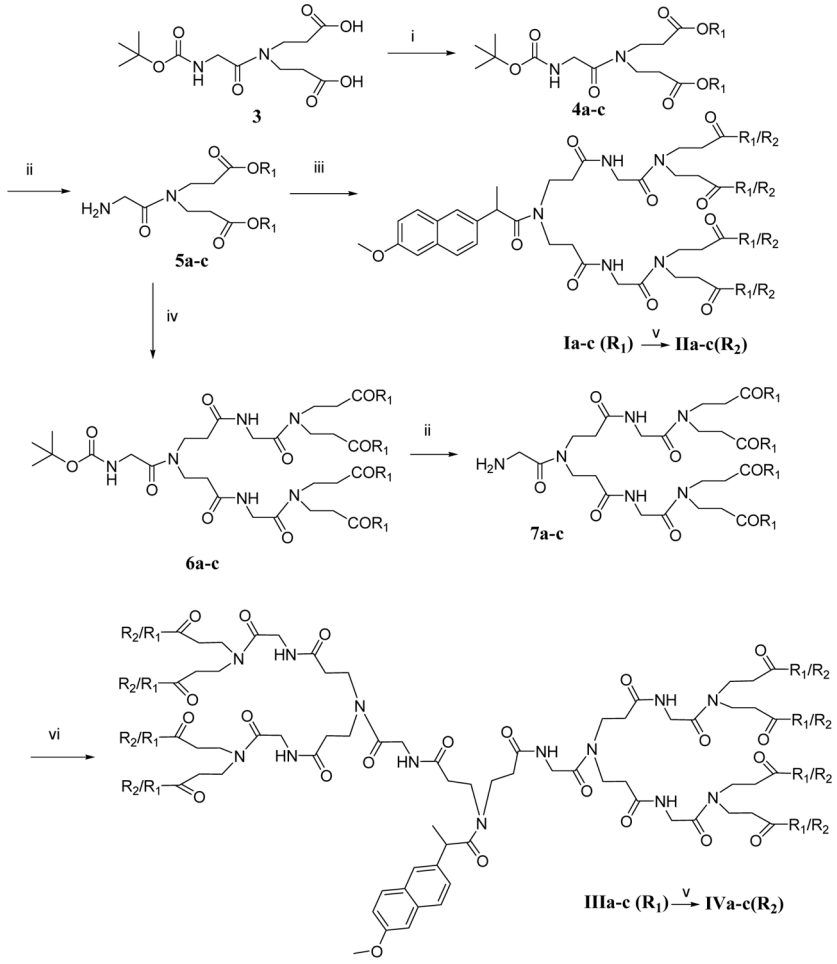
Synthesis of the Dendrimer Core

Bis-addition of benzylamine to ethylacrylate through a Michael addition reaction, then debenzylation and coupling to Boc-Gly-OH or naproxen through an amide linkage using IBCF/NMM, came next. At last, the protecting groups were removed in $NaOH/CH_3OH$ to provide the dendrimer cores **2** and **3**.

Synthesis of the Peptide Dendrimers

A series of peptide dendrimers were prepared through a convergent synthesis. The synthetic route is outlined in Scheme 1. We chose iminodipropionic acid derivatives as the central core for the polyamide dendrimers.[10] However, the reactivity of the imine group was weak, and therefore in high steric hindrance, the amido linkage with the carboxylic group could not be formed with a coupling reagent. For the convenience of synthesis of the dendrimers, a glycine residue was previously introduced using more active IBCF/NMM (mixed anhydride) as coupling reagent.

We previously synthesized the first-generation dendrimers using DCC/Hobt (dicyclohexylcarbodiimide/1-hydroxybenzotriazole).[11] However, this method has some defects: for example, DCU (dicyclohexylurea) is difficult to eliminate completely, and the purity of the crude product is poor. In the present research, a water-soluble carbodiimide (EDCIf · HCl) was used: the yield raised from 50 to 70%, and the workup procedure was simple. In a similar convergent strategy, the second-generation dendrimers bearing four oligopeptides (**Ia–c)** were synthesized after deprotection of **4a**–**c** with trifluoroacetic acid (TFA). EDCI/Hobt was used as a good coupling reagent; the yield was up to 60%. In the synthesis of the third-generation dendrimers bearing eight oligopeptides **IIIa**–**c**, using EDCI · HCl did not give the product in acceptable yields. Several other coupling reagents (DCC, IBCF/NMM, CDI, etc.) gave similar poor results. Only the solid-phase peptide coupling reagent HBTU/DIPEA (o-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate/N,N-diisopropylethylamine) afforded the products in good yields. We belive that the poor yields were due to the large size of the reactant. Once the first chain was attached to the scaffold, the steric bulk inhibited attachment of additional chains.[12] Conversion of **Ia**–**c** and **IIIa**–**c** into the corresponding Asp oligopeptides (**IIa**–**c** and **IVa**–**c**) was accomplished by hydrogenolysis over 10% palladium on charcoal; the reactions required only filtration and evaporation to provide isolated products.



R_1=NH-(Asp(OBzl))n-OBzl	**4a, 5a, 6a, 7a, Ia, IIa, IIIa, IVa, n=4**
	4b, 5b, 6b, 7b, Ib, IIb, IIIb, IVb, n=5
R_2=NH-Asp(n)-OH	**4c, 5c, 6c, 7c, Ic, IIc, IIIc, IVc, n=6**

Scheme 1. Reagents and conditions: i) **1a**–**c**, EDCI · HCl, Hobt, CH_2Cl_2; ii) TFA, CH_2Cl_2; iii) **2**, EDCI · HCl, Hobt, DMF; iv) **3**, EDCI · HCl, Hobt, CH_2Cl_2; v) H_2, Pd/C (10%, wt%), CH_3OH; vi) **2**, HBTU, Hobt, DIPEA, DMF.

The high degree of symmetry in these dendrimers enabled facile confirmation of both structure and purity by NMR techniques. For example, in the ^{1}H NMR spectrum of dendron **4a**–**c**, the core protons observed the resonance signals at 1.44 (s), 2.36 (t), and 3.53 (t) ppm were

clearly distinguishable from the resonances arising from the wedges (Asp oligopeptides) at 2.71 (m) and 4.84 (m) ppm. Integration of the respective areas of the core protons and β-methine protons of Asp oligopeptides confirmed the complete coupling of the central core **2** and peptides **1**. For the second-generation and third-generation dendrimers **IIa**–**c** and **IVa**–**c**, although some resonance signals were not well resolved, the complete cascade reactions could be verified through comparison of integration areas of the core protons at 2.57 (brs), 3.41 (brs), and 7.32–7.87 (m) ppm and and β-methine protons of Asp oligopeptides 2.77 (brs) ppm.

Furthermore, the structures of these dendrimers were further verified by electrospray ionization–mass spectrometry (ESI-MS) or matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS). All of the spectra displayed a very prominent peak corresponding to the dendrimers complexed with protons or sodium cation. Moreover, elemental analysis was also in good agreement with those of the signed structures.

Binding of Conjugates to Hydroxyapatite (HAP)

To demonstrate the direct effect of molecular weight and Asp oligopeptide content on the binding of the conjugates, the in vitro binding of the conjugates on HAP was studied. The results of the in vitro binding of the peptide dendrimer conjugates to HAP are shown in Table 1. The efficiency of binding was expressed as the percentage of the original amount of conjugate bound to HAP in dependence of the weight ratio HAP/conjugate.

The results from these in vitro assay suggest several trends. First, all conjugates were very effcient at binding HAP, being taken up at $>60\%$ and generally $>70\%$ over 2 h, whereas the parent drug naproxen was at best negligibly bound. Second, as expected, a greater content of Asp

Table 1. UV spectra of the conjugates

Peptide dendrimer	Before HAP addition (μg/mL)	After HAP addition (μg/mL)	Ratio (%) of binding dendrimer
IIa	100	32.6	67.4
IIb	100	37.2	62.8
IIc	100	25.2	74.8
IVa	100	26.4	73.6
IVb	100	28.6	71.4
IVc	100	30.2	69.8
Naproxen	100	99.3	0.7[a]

[a]Naproxen dissolved in anhydrous CH_3OH.

oligopeptide or longer chain of Asp acid in an oligopeptide resulted in higher binding than the low-molecular-weight and low-Asp-content conjugate, but no difference in the binding of high or low Asp oligopeptide content conjugates was observed. Most probably, the size of the conjugate prevented efficient access to the surface (pores) of HAP.[13]

In conclusion, we have prepared a range of second and third-generation monodisperse peptide dendrimer–naproxen conjugates bearing four or eight Asp oligopeptide sequences as peripheral groups for testing as novel potential bone-targeting therapeutics. The ^{1}H NMR and MS results support the formation of the targeted dendrons and dendrimers. Steric hindrance played an important role, and it was difficult to obtain higher generation dendrimers in a reasonable yield. We are continuing to explore higher generations of dendritic molecules that bearing Asp sequences and develop other routes to increase both the number of conjugated drug molecules in a controllable method and applications to other bioactive molecules.

EXPERIMENTAL

General

All reactions requiring anhydrous conditions were performed under an Ar or N_2 atmosphere. Chemicals and solvents were either analytical-reagent grade or purified by standard techniques. Thin-layer chromatography (TLC) used silica-gel plates GF254; compounds were visualized by irradiation with ultraviolet (UV) light and/or by treatment with a solution of phosphomolybdic acid (20% wt. in ethanol) followed by heating. Column chromatography was performed using silica gel with eluent given in parentheses. ^{1}H NMR analysis was performed using $CDCl_3$ or D_2O as a solvent at room temperature. The chemical shifts are expressed in relative to tetramethylsilane (TMS, = 0 ppm) and the coupling constants J are given in hertz. Hydroxyapatite (HAP) was purchased from Shanghai Institute of Biochemistry with surface area of 9.12 m^2/g and average particle size of 15 μm.

General Procedure for the Synthesis of the Dendron 4

The protected oligopeptides NH_2-$Asp_{(4–6)}$ 1 (2.0 equiv), compound **3** (1.0 equiv) and 1-hydroxybenz otriazole, Hobt (2.2 equiv) were dissolved in anhydrous THF. N^1-((ethylimino)methylene)-N^3, N^3-dimethylpropane-1,3-diamine hydrochloride, and EDCI · HCl (2.2 equiv) were added into the solution.The reaction mixture was stirred under N_2 at room temperature for 24 h. The solution was then concentrated under vacuum, and the

crude product was purified on a silica-gel chromatography column using CH_2Cl_2 and CH_3OH (100:1–40:1, v/v) as eluent.

Data

Compound **4a**

Nankin wax (0.48 g, 68%) from **3** (0.1 g, 0.31 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): 1.44 (s, 9 H, Boc-H), 2.42 (t, 4 H, $J = 6.0$ Hz, $CH_2CO \times 2$), 2.71–3.04 (m, 16 H, Asp-βCH_2), 3.52 (t, 4 H, $J = 6.0$ Hz, N-$CH_2 \times 2$), 3.85 (s, 2 H, Gly-CH_2), 4.73–4.84 (m, 8 H, Asp-αCH), 5.1 (brs, 20 H, ph-CH_2), 7.25 (brs, 50 H, ph-H). ESI MS (m/z): calcd. for 2162.8 ($[M+Na]^+$); obsd. 2162.9.

Compound **4b**

Yellow oil (0.50 g, 62%) from **3** (0.1 g, 0.31 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): 1.42 (s, 9 H, Boc-H), 2.52 (t, 4 H, $J = 6.4$ Hz, $CH_2CO \times 2$), 2.70–2.91 (m, 20 H, Asp-βCH_2), 3.54 (t, 4 H, $J = 6.4$ Hz, N-$CH_2 \times 2$), 3.88 (s, 2 H, Gly-CH_2), 4.71–4.92 (m, 10 H, Asp-αCH), 5.14 (brs, 24 H, ph-CH_2), 7.21 (brs, 60 H, ph-H). ESC MS (m/z): calcd. for 2549.9 ($[M+H]^+$), obsd. 2550.3.

Compound **4c**

White wax (0.67 g, 73%) from **3** (0.1 g, 0.31 mmol).^{1}H NMR (400 MHz, $CDCl_3$): 1.44 (s, 9 H, Boc-H), 2.58 (t, 4 H, $J = 6.4$ Hz, $CH_2CO \times 2$), 2.72–3.01 (m, 24 H, Asp-βCH_2), 3.58 (t, 4 H, $J = 6.4$ Hz, N-$CH_2 \times 2$), 3.92 (s, 2 H, Gly-CH_2), 4.69–4.82 (m, 12 H, Asp-αCH), 5.09 (brs, 28 H, ph-CH_2), 7.15 (brs, 70 H, ph-H). ESI MS (m/z): calcd. for 1481.0 ($[M+H]^+/2e$); obsd. 1481.6.

General Procedure for the Synthesis of the Dendron 5

To the solution of trifluoroacetic acid (TFA)/anhydrous CH_2Cl_2 (1:1, v/v), **4** was added at room temperature under N_2. When the protecting groups were completely removed, the solvent was rotary-evaporated. The resulting product was dissolved in chloroform and basified with saturated $NaHCO_3$ to completely remove TFA. The organic layer was dried over anhydrous Na_2SO_4, concentrated, and further purified on a

silica-gel chromatography column using CH_2Cl_2 and CH_3OH (20:1, v/v) as an eluent to afford **5**.

General Procedure for the Synthesis of the Dendron Ia–c

The naproxen linker acid **2** (1.0 equiv) was added in one portion to a mixture of dendron **5** (2.0 equiv), EDCI · HCl (2.2 equiv), and Hobt (2.2equiv) in DMF, keeping the reaction under N_2. The mixture was stirred at ambient temperature overnight. Water and ethyl acetate were added, and the mixture was stirred for 10 min. The mixture was allowed to settle, and the lower aqueous phase was separated off and discarded. The organic phase was washed with more water and then concentrated under a reduced vaccum on a rotary evaporator to give the crude product. The crude product was purified on a silica-gel chromatography column using CH_2Cl_2 and CH_3OH (80:1–30:1, v/v) as an eluent.

General Procedure for the Synthesis of the Dendron IIa–c

A mixture of dendron **I** (1.0 equiv) and 10% Pd/C (0.1 equiv, 10 wt%) in CH_3OH was stirred at room temperature under a H_2 atmosphere. After 16 h, the mixture was passed through a membrane filter to remove the catalyst and then evaporated under reduced pressure.

Data

Compound **IIa**

White foam (0.12 g, 55% total yield in two steps) from **2** (0.03 g, 0.08 mmol).^{1}H NMR (400 MHz, D_2O): 1.46 (d, 3 H, $J = 6.0$ Hz, NapCH$_3$), 2.53–2.74 (m, 12 H, $CH_2CO \times 6$), 2.80–3.04 (brs, 32 H, Asp-βCH_2), 3.42–3.69 (m, 12 H, N-$CH_2 \times 6$), 3.76–3.89 (m, 4 H, Gly-$CH_2 \times 2$), 3.8–3.96 (brs, 4 H, NapOCH_3 + CH), 4.60–4.95 (m, 16 H, Asp-αCH), 7.37–7.85 (m, 6 H, Nap-phH). MALDI-TOF MS (m/z): calcd. for 2638.7 ($[M + Na]^+$); obsd. 2639.2. Anal. calcd. for $C_{100}H_{127}N_{21}O_{62}$: C, 45.93; H, 4.89; N,11.25. Found: C, 46.11; H, 4.82; N, 11.17.

Compound **IIb**

White foam (0.11 g, 43% total yield in two steps) from **2** (0.03 g, 0.08 mmol). ^{1}H NMR (400 MHz, D_2O): 1.47 (d, 3 H, $J = 6.0$ Hz,

$NapCH_3$), 2.44–2.60 (m, 12 H, $CH_2CO \times 6$), 2.86–3.06 (brs, 40 H, Asp-βCH_2), 3.38–3.60 (m, 12 H, N-$CH_2 \times 6$), 3.77–3.88 (m, 4 H, Gly-$CH_2 \times 2$), 3.82–4.00 (brs, 4 H, $NapOCH_3 + CH$), 4.60–4.99 (m, 20 H, Asp-αCH), 7.30–7.88 (m, 6 H, Nap-phH). MALDI-TOF MS (m/z): calcd. for 3074.8 ($[M + H]^+$), obsd. 3076.1. Anal. calcd. for $C_{116}H_{147}N_{25}O_{74}$: C, 45.30; H, 4.82; N,11.39. Found: C, 45.12; H, 4.91, N, 11.47

Compound **IIc**

White foam (0.14 g, 51% total yield in two steps) from **2** (0.03 g, 0.08 mmol).^{1}H NMR (400 MHz, D_2O): 1.47 (d, 3 H, $J = 6.0$ Hz, $NapCH_3$), 2.50–2.79 (m, 12 H, $CH_2CO \times 6$), 2.90–3.09 (brs, 48 H, Asp-βCH_2), 3.33–3.56 (m, 12 H, N-$CH_2 \times 6$), 3.82–3.91 (m, 4 H, Gly-$CH_2 \times 2$), 3.83–4.01 (brs, 4 H, $NapOCH_3 + CH$), 4.62–4.89 (m, 24 H, Asp-αCH), 7.25–7.87 (m, 6 H, Nap-phH). MALDI-TOF MS (m/z): calcd. for 3534.9 ($[M + H]^+$); obsd. 3534.9. Anal. calcd. for $C_{132}H_{167}N_{29}O_{86}$: C, 44.84; H, 4.76; N,11.49. Found: C, 44.71; H, 4.88; N, 11.52

General Procedure for the Synthesis of the Dendron 6

The same procedure as described for preparation of the dendron **I** was used.

Data

Compound **6a**

Yellow wax (0.25 g, 61%) from **3** (0.03 g, 0.01 mmol). ^{1}H NMR (400 MHz, $CDCl_3$):1.40 (s, 9 H, Boc-H), 2.36–2.52 (m, 12 H, $CH_2CO \times 6$), 2.69–2.94 (brs, 32 H, Asp-βCH_2), 3.48–3.63 (m, 12 H, N-$CH_2 \times 6$), 3.71–3.92 (m, 6 H, Gly-$CH_2 \times 3$), 4.72–4.94 (m, 16 H, Asp-αCH), 5.14 (brs, 40 H, ph-CH_2), 7.25 (brs, 100 H, ph-H). MALDI-TOF MS (m/z): calcd. for 4381.7 ($[M + Na]^+$); obsd. 4382.4.

Compound **6b**

Yellow wax (0.33 g, 67%) from **3** (0.03 g, 0.01 mmol). ^{1}H NMR (400 MHz, $CDCl_3$):1.41 (s, 9 H, Boc-H), 2.42–2.51 (m, 12 H, $CH_2CO \times 6$), 2.59–3.01 (brs, 40 H, Asp-βCH_2), 3.52–3.60 (m, 12 H, N-$CH_2 \times 6$), 3.66–3.87 (m,

6 H, Gly-$CH_2 \times 3$), 4.65–4.99 (m, 20 H, Asp-αCH), 5.21 (brs, 48 H, ph-CH_2), 7.15 (brs, 120 H, ph-H). MALDI-TOF MS (m/z): calcd. for 5180.9 ($[M + H]^+$); obsd. 5182.2.

Compound **6c**

Yellow wax (0.26 g, 45%) from **3** (0.03 g, 0.01 mmol). ^{1}H NMR (400 MHz, $CDCl_3$):1.41 (s, 9 H, Boc-H), 2.43–2.56 (m, 12 H, $CH_2CO \times 6$), 2.64–2.92 (brs, 48 H, Asp-βCH_2), 3.56–3.67 (m, 12 H, N-$CH_2 \times 6$), 3.75–3.92 (m, 6 H, Gly-$CH_2 \times 3$), 4.71–5.04 (m, 24 H, Asp-αCH), 5.20 (brs, 56 H, ph-CH_2), 7.14 (brs, 140 H, ph-H). MALDI-TOF MS (m/z): calcd. for 6002.2 ($[M + H]^+$); obsd. 6003.7.

General Procedure for the Synthesis of the Dendron 7

The same procedure as described for preparation of the dendron **5** was used.

General Procedure for the Synthesis of the Dendrons IIIa–c

The naproxen linker acid **2** (1.0 equiv), the dendron **7** (2.0 equiv), o-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate (HBTU) (2.2 equiv), and Hobt (2.2 equiv) were dissolved in dry DMF under N_2. N,N-Diisopropylethylamine (DIPEA) (2.2 equiv) was then added, and the reaction was stirred for 24 h at room temperature. The solvent was evaporated, and the slurry was partitioned between ethyl acetate and water. The aqueous layer was further extracted with ethyl acetate, and the combined organic layers were dried and evaporated to give a yellow foam. The crude product was purified on a silica-gel chromatography column using CH_2Cl_2 and CH_3OH (60:1–20:1, v/v) as an eluent.

General Procedure for the Synthesis of the Dendrons IVa–c

A mixture of dendron **III** (1.0 equiv) and 10% Pd/C (0.1 equiv, 10 wt%) in CH_3OH was stirred at room temperature under an H_2 atmosphere. After 24 h, the mixture was passed through a membrane filter to remove the catalyst and then evaporated under reduced pressure. After concentration, the residue was triturated with pure ethyl ether to afford **IV** as a white powder.

Data

Compound **IVa**

White powder (0.051 g, 36% total yield in two steps) from **2** (0.01 g, 0.03 mmol). ^{1}H NMR (400 MHz, D_2O): 1.46 (d, 3 H, $J = 6.0$ Hz, NapCH$_3$), 2.53–2.74 (brs, 28 H, $CH_2CO \times 14$), 2.77–2.94 (brs, 64 H, Asp-βCH_2), 3.39–3.62 (brs, 28 H, N-$CH_2 \times 14$), 3.81–3.83 (brs, 12 H, Gly-$CH_2 \times 6$), 3.89–3.96 (s, 4 H, NapOCH$_3$ + CH), 4.63–4.96 (m, 32 H, Asp-αCH), 7.32–7.85 (m, 6 H, Nap-phH). MALDI-TOF MS (m/z): calcd. for 5255.5 ($[M + H]^+$); obsd. 5258.3. Anal. calcd. for $C_{196}H_{255}N_{45}O_{126}$: C, 44.78; H, 4.89; N,11.99. Found: C, 44.41; H, 4.81, N, 12.17.

Compound **IVb**

White powder (0.035 g, 21% total yield in two steps) from **2** (0.01 g, 0.03 mmol). ^{1}H NMR (400 MHz, D_2O): 1.45 (d, 3 H, $J = 6.0$ Hz, NapCH$_3$), 2.57–2.73 (brs, 28 H, $CH_2CO \times 14$), 2.80–2.96 (brs, 80 H, Asp-βCH_2), 3.41–3.66 (brs, 28 H, N-$CH_2 \times 14$), 3.80–3.83 (brs, 12 H, Gly-$CH_2 \times 6$), 3.89–3.95 (s, 4 H, NapOCH$_3$ + CH), 4.66–4.95 (m, 40 H, Asp-αCH), 7.32–7.87 (m, 6 H, Nap-phH). MALDI-TOF MS (m/z): calcd. for 6197.7 ($[M + Na]^+$); obsd. 6198.3. Anal. calcd. for $C_{228}H_{295}N_{53}O_{150}$: C, 44.33; H, 4.81; N, 12.02. Found: C, 44.14; H, 4.66; N, 12.19.

Compound **IVc**

White powder (0.055 g, 29% total yield in two steps) from **2** (0.01 g, 0.03 mmol).^{1}H NMR (400 MHz, D_2O): 1.45 (d, 3 H, $J = 6.0$ Hz, NapCH$_3$), 2.44–2.69 (brs, 28 H, $CH_2CO \times 14$), 2.79–3.02 (brs, 96 H, Asp-βCH_2), 3.48–3.57 (brs, 28 H, N-$CH_2 \times 14$), 3.81–3.84 (brs, 12 H, Gly-$CH_2 \times 6$), 3.89–3.92 (s, 4 H, NapOCH$_3$ + CH), 4.54–4.99 (m, 48 H, Asp-αCH), 7.32–7.87 (m, 6 H, Nap-phH). MALDI-TOF MS (m/z): calcd. for 7095.9 ($[M + H]^+$); obsd. 7102.1. Anal. calcd. for $C_{260}H_{335}N_{61}O_{174}$: C, 43.99; H, 4.76; N,12.04. Found: C, 43.65; H, 4.56; N, 12.28.

Hydroxyapatite (HAP) Binding Study

Conjugate solutions in PBS (5 mL, concentration 1 mg/mL) were incubated with HAP (25 mg) for 2 h at 37°C under mild shaking. After centrifugation, the concentration of nonbound conjugates was measured

by UV spectrophotometry in 273 nm (a characteristic absorption of naproxen).

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