Bioconjugate Chemistry

Article

Cite This: Bioconjugate Chem. 2018, 29, 3411−3428

pubs.acs.org/bc

Design of Bifunctional Dendritic 5-Aminolevulinic Acid and Hydroxypyridinone Conjugates for Photodynamic Therapy

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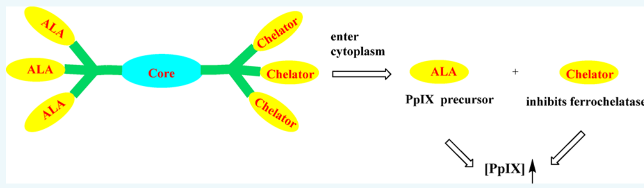
Supporting Information



ABSTRACT: Iron chelators have recently attracted interest in the field of photodynamic therapy (PDT) owing to their role in enhancement of intracellular protoporphyrin IX (PpIX) generation induced by 5-aminolevulinic acid (ALA) via the biosynthetic heme cycle. Although ALA is widely used in PDT, cellular uptake of ALA is limited by its hydrophilicity. In order to improve ALA delivery and enhance the PpIX production, several dendrimers incorporating both ALA and 3-hydroxy-4-pyridinone (HPO) were synthesized. The ability of the dendrimers to enter cells and be metabolized to the PpIX photosensitizer was studied in several human cancer cell lines. The dendrimers were found to be significantly more efficient than ALA alone in PpIX production. The higher intracellular PpIX levels showed a clear correlation with enhanced cellular phototoxicity following light exposure. Dendritic derivatives are therefore capable of efficiently delivering both ALA and HPO, which act synergistically to amplify *in vitro* PpIX levels and enhance PDT efficacy.

■ INTRODUCTION

5-Aminolevulinic acid (ALA) has been shown to be effective at inducing protoporphyrin IX (PpIX) after oral, intravenous (i.v.), and topical applications *in vivo*.[1−4] ALA photodynamic therapy (PDT) currently finds vast use in dermatology for the treatment of actinic keratosis, squamous cell carcinoma, and Bowen's disease,[5,6] as well as cutaneous microbial infections such as acne, onychomycosis, and verrucae.[7,8] Promising results have also emerged from studies in gastroenterology and urology.[9] PpIX from administered ALA may be used not only to directly treat conditions such as Barret's esophagus, inflammatory bowel disease, and bladder cancer by PDT but also as a diagnostic tool for the visualization of pre-cancerous changes in the mucous by fluorescence spectroscopy.[10−13] However, ALA is a hydrophilic molecule, has a limited ability to penetrate biological barriers, and is less efficient at inducing intracellular porphyrin generation compared to esters of ALA.[14−16] Several studies have confirmed that a significant enhancement of cellular uptake can be achieved by esterifying the carboxylic terminal with alkyl or aryl groups, resulting in increased levels of PpIX.[17−21] An attractive alternative is to incorporate ALA into a short peptide which is susceptible to intracellular enzymatic activity.[22,23]

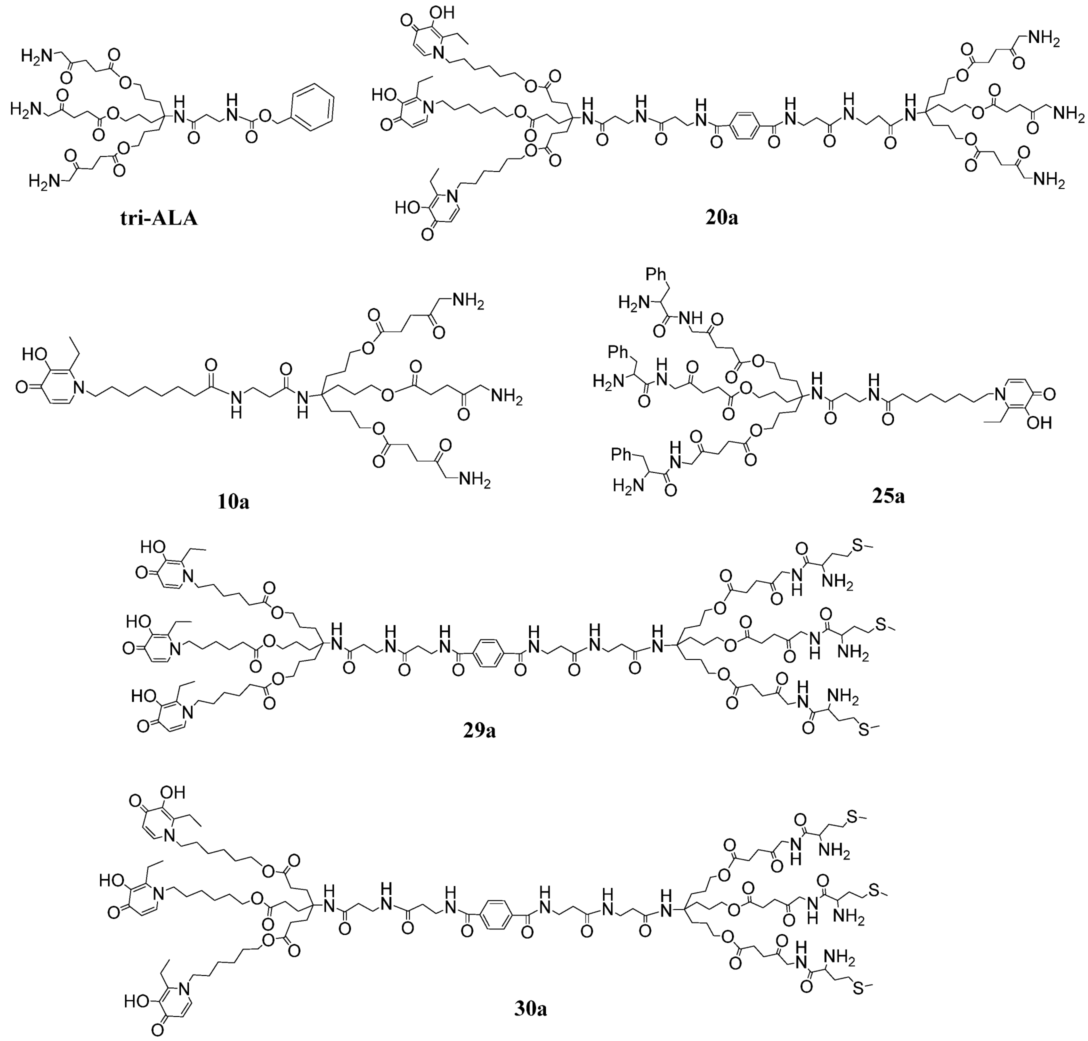
ALA-induced PDT can be modulated in the presence of iron chelators such as EDTA[24,25] and desferrioxamine,[26,27] the chelators inhibiting ferrochelatase by scavenging the intracellular labile iron pool. Several studies have also demonstrated enhancement of PpIX concentration in cells or tissues exposed to a combination of ALA and the membrane-permeable iron chelator 1,2-diethyl-3- hydroxypyridin-4-one (CP94).[28−31] However, optimal tissue accumulation and localization remains a clinical problem. One approach adopted to reduce this problem involves the use of dendrimeric drug carriers which show an appreciable efficacy for ALA delivery and subsequent production of PpIX.[32−36]

Bearing in mind the aforementioned studies and the parameters affecting PpIX levels in tissues, in principle PpIX can be elevated in three different ways: chelation of iron in intracellular compartments, thereby inhibiting ferrochelatase; derivatization of one or both termini of ALA, thereby enabling membrane permeation; and attachment of ALA to macromolecular assemblies, in particular dendrimers. In a previous study, it was demonstrated that ALA−3-hydroxy-4-pyridinone (HPO) conjugates via ester or amido linkages effectively improve the intracellular PpIX production when compared to the use of ALA alone and combination of ALA and HPO.[37−39] Thus, we designed dendritic carriers simultaneously incorporating both ALA and iron-chelating agents (HPOs).

Received: August 11, 2018
Revised: September 19, 2018
Published: September 24, 2018

DOI: 10.1021/acs.bioconjchem.8b00574
Bioconjugate Chem. 2018, 29, 3411−3428

Chart 1



Dendrimers have been shown to rapidly enter cells by triggering endocytotic processes,[40−43] and as such, dendrimers are predicted not to use the same uptake pathway as ALA and simple ALA prodrugs. Dendritic carriers were selected for this study as the synthesis of such molecules can be controlled with respect to the size and loading of both ALA and HPOs. The amino groups of ALA molecules will be protonated under *in vivo* conditions, and the presence of the HPO hydroxyl groups further confers good water solubility of such dendrimers. Dendrimers can be constructed in a stepwise manner, yielding structurally and topologically defined, monodisperse unimolecular entities.[44] The convergent growth strategy adopted for the synthesis of ALA-HPO-containing dendrimers in this study has permitted the dendrimer to be assembled in segments. The dendrons were attached to a di-podent core unit in the final step of dendrimer construction. We have investigated the properties of first-generation dendritic carriers containing three residues of ALA and either one or three residues of HPO. The structure of the dendrimer was designed to enable ALA and the HPO moieties to be attached by controlled synthesis via ester linkages to a multi-podent aromatic core.

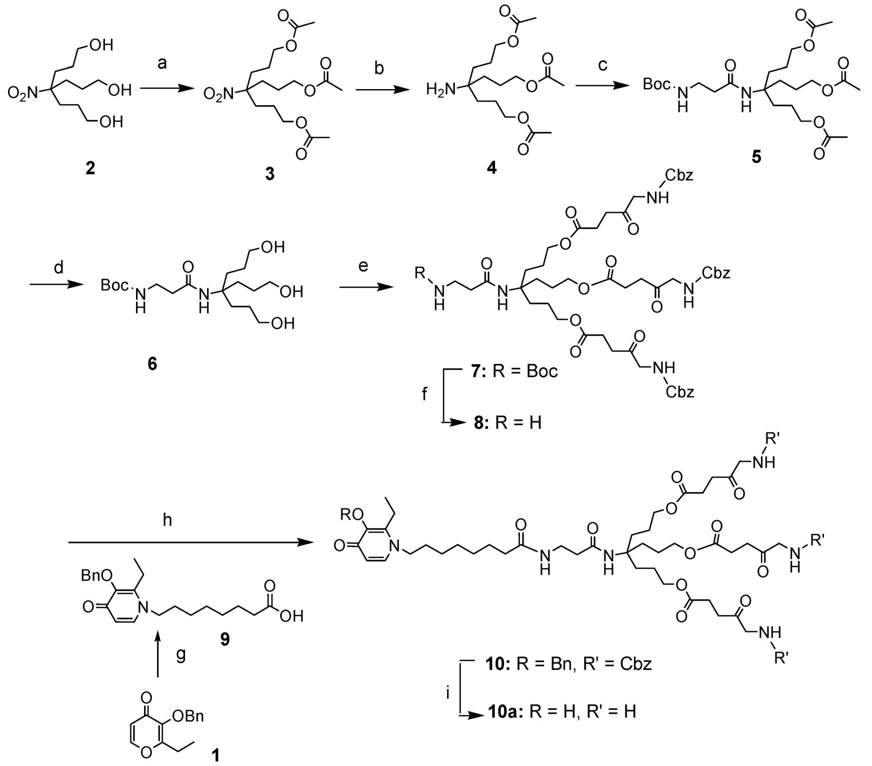
■ RESULTS AND DISCUSSION

Chemistry. Five first-generation dendrimers containing three residues of ALA and with either one residue of HPO (**10a** and **25a**) or three residues of HPO (**20a**, **29a**, and **30a**) were synthesized (Chart 1). The loading of controlled amounts of both ALA and HPO into a well-designed carrier was rendered possible by using standard ester and amide bond-forming reactions and selective protection strategies. The synthetic sequence involves (a) esterification of the three hydroxyl or the three carboxy groups of tripodal molecules; (b) construction of a core molecule with attached β-alanine spacer groups; (c) successive amide bond formation to give dendrons and then dendrimers; and (d) removal of the *N* and *O* protecting groups.

The choice of HPO in this approach was based on Clog*P* values of a range of *N*-(ω-hydroxyalkyl)-HPOs, a compound with appreciable hydrophobicity being necessary to inhibit ferrochelatase.[45] As a result of this analysis, we selected a 3-hydroxypyridinone, with either hexanoic or hexanol substituents at position 1, for the branching building blocks of dendrimers **25a**, **29a**, and **30a**.

The stability of the dendrimers containing the Cbz-ALA dendron was a challenging issue because, subsequent to catalytic hydrogenation of the Cbz protecting group, there was considerable decomposition of the resulting dendrimer, particularly when the hydrogenation step exceeded 30 min. We therefore employed phenylalanine and methionine as protecting groups, which can be cleaved by cytoplasmic

Scheme 1[a]



[a]Reagents and conditions: (a) acetic anhydride, pyridine, rt, 18 h; (b) Raney Ni, MeOH, H_2, 30 psi; (c) DCC, HOBT, DCM/DMF (3:1), rt, argon, 24 h; (d) 2 M NaOH/MeOH (1:1), 10 °C, 30 min; (e) Cbz-ALA, DCC, DMAP, DCM/DMF (3:1), rt, argon, 24 h; (f) 1% TFA in DCM, 30 min; (g) 8-amino-octanoic acid, EtOH/H_2O (1:1), 2 M NaOH, reflux 18 h; (h) DCC, HOBT, Bmim, DCM/DMF (3:1), rt, argon, 24 h; (i) Pd/C, H_2/40 psi, BnCl, MeOH/EtAc (1:4), rt, 30 min.

enzymes. These two amino acids were selected on the basis of their efficacy when conjugated to ALA.[17−19]

*Synthesis of Dendrimer **10a**.* First, the dendron containing three ALA moieties (**8**) was synthesized (Scheme 1). Commercially available nitromethanetrispropanol (**2**) was triacetylated to produce **3**, which was then reduced by hydrogenation to generate amine **4**. The free amino group of **4** was coupled to *N*-Boc-*β*-alanine in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) to afford **5**. The purpose of introducing a small spacer (*β*-alanine) to aminomethanetrispropanol was to reduce steric hindrance in subsequent conjugation reactions. Alkaline hydrolysis of **5** afforded **6**. Cbz-ALA was coupled to the branching unit **6** by formation of an ester bond using DCC/DMAP reagents. The crude product was purified by flash chromatography on silica gel to provide tri-ester **7**, together with mono- and di-esters. Cleavage of the Boc-protected group provided dendron **8**. The structure of dendron **8** was confirmed by ^{1}H and ^{13}C NMR and LC-MS/MS. Dendron **8** was coupled to **9** using the standard amide coupling reagents DCC and HOBt to provide **10** in a reasonable yield (48%). Removal of the protecting groups was accomplished using hydrogen (40 psi) and 10% Pd/C in the presence of benzyl chloride to isolate the corresponding dendrimer **10a** as the tetrahydrochloride. Some decomposition occurred during the hydrogenation step, necessitating further HPLC purification.

*Synthesis of Dendrimer **20a**.* Synthesis of dendrimer **20a** is outlined in Scheme 2. The core for this dendrimer was the dicarboxylic aromatic acid **12**. The synthesis of **12** utilized phthalolyl chloride, providing **11** in good yield by reaction with the ethyl ester of *β*-alanine. Alkaline hydrolysis of **11** afforded core **12**. Coupling of Fmoc-*β*-alanine and aminomethane tris(3-*tert*-butoxycarbonyl propionate) (**13**) in the presence of HOBt and DCC provided **14** in excellent yield (92%). Tri-acid **15** was obtained in quantitative yield by the treatment of **14** with formic acid. 3-(Benzyloxy)-2-ethyl-1-(6-hydroxyhexyl)-pyridin-4(1*H*)-one (**16H**) was coupled to tri-acid **15** to provide tri-ester **17H** in the presence of DCC and DMAP in reasonable yield (42%). Cleavage of Fmoc in **17H** was achieved by treatment with piperidine to yield dendron **18H**. Coupling of **18H** to the dicarboxyl-functionalized core **12** gave **19H** in a moderate yield (56%). **19H** was then coupled with the ALA dendron **8** to provide the protected dendrimer **20**, which on subjection to hydrogenation, yielded dendrimer **20a**. As with **10a**, there was some degradation during the hydrogenation step. After preparative HPLC, the dendrimer was isolated as the hexahydrochloride.

*Synthesis of Dendrimer **25a**.* *N*-Cbz-L-phenylalanine was coupled to ALA methyl ester in the presence of DCC, HOBt, and 1-butyl-3-methylimidazolium bromide (Bmim) to provide **21** in high yield (>95%).[46] **21** was hydrolyzed in alkaline solution to give **22P**. **22P** was coupled to **6** in the presence of DCC and DMAP to provide the tri-branched ALA amino acid moiety **23**, which was then treated with TFA-cleaved Boc, yielding dendron **24**. Dendron **24** was conjugated to HPO **9**, providing the protected dendrimer **25**, which on hydrogenation gave dendrimer **25a** (Scheme 3).

*Synthesis of Dendrimers **29a** and **30a**.* The synthetic route for dendron **19C** is presented in Scheme 4. 6-(3-(Benzyloxy)-2-ethyl-4-oxopyridin-1(4*H*)-yl)hexanoic acid was coupled to

DOI: 10.1021/acs.bioconjchem.8b00574
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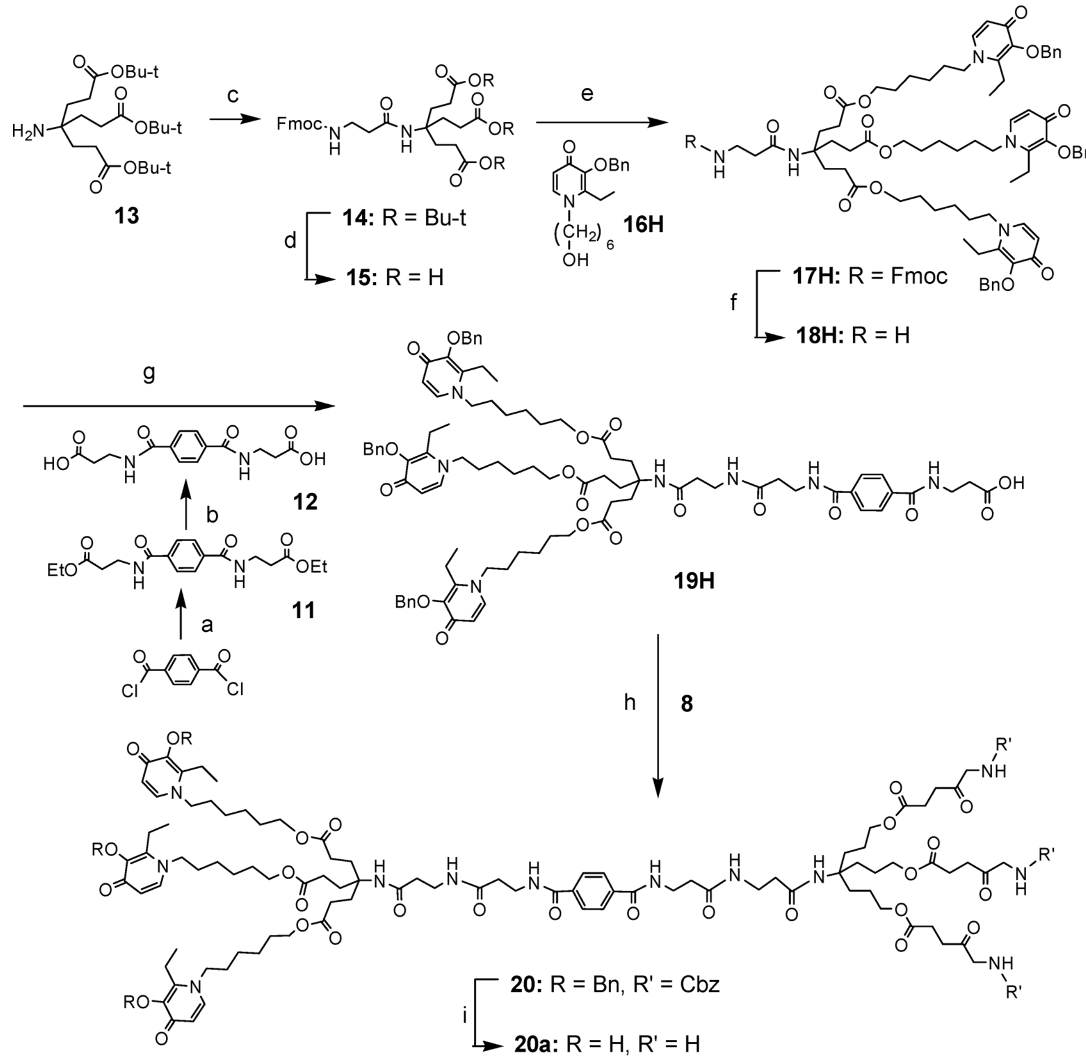
Scheme 2[a]



[a]Reagents and conditions: (a) β-alanine ethyl ester hydrochloride, DCM/DMF (5:1), Et_3N, 0 °C then rt; (b) 2 M NaOH/MeOH (1:1), 10 °C, 30 min, 1 M HCl neutralized to pH 6; (c) Fmoc-β-alanine, DCC, HOBT, DCM/DMF (3:1), rt, argon, 24 h; (d) HCOOH, rt, 24 h; (e) DCC, DMAP, DCM/DMF (3:1), rt, argon, 24 h; (f) piperidine, DMF, rt, 10 min; (g) DCC, HOBT, DMF, Bmim, argon, 0 °C 1 h then rt 24 h; (h) DCC, HOBT, DMF, Bmim, argon, 0 °C 1 h then rt 24 h; (i) Pd/C, H_2/40 psi, BnCl, MeOH/EtAc (1:4), rt, 30 min.

the triol **6** to provide the tri-ester **17C**, which yielded dendron **18C** after removal of Boc. Coupling of **18C** to the dicarboxyl-functionalized core **12** gave **19C** in a yield of 62%. Dendron **28**, which contains three Cbz-Met-functionalized ALA branches and one free amino group, was then synthesized in a similar method to that of **24** (Scheme 3). Coupling of dendron **28** with **19H** and **19C** via amide bond formation in the presence of DCC, HOBt, and Bmim provided the protected dendrimers **29** and **30**, respectively. Hydrogenation of **29** and **30** yielded dendrimers **29a** and **30a** (Scheme 3). Cleavage of *N*-benzyloxycarbonyl and *O*-benzyloxy groups of compounds **29** and **30** was found to be more difficult than with compounds **10**, **20**, and **25**, as both **29** and **30** contain the sulfur group of methionine, which poisons the catalyst.[47] Thus, for these hydrogenation procedures, methanolic ammonia and Pd black were adopted, the dendrimers being isolated as formate salts.[48]

Fluorescence Pharmacokinetics. Several factors control the enhancement of dendrimer-induced PpIX generation: (a) the enhanced rate of drug uptake; (b) the rate of enzymatic conversion of dendrimers to active principles;[13−15] (c) the enhanced retention of dendrimers at the application site; and (d) the accessibility of the HPO moiety to ferrochelatase.[49,50] In this study we compared intracellular PpIX fluorescence levels induced by the dendrimer conjugates compared to ALA as a function of incubation time and concentration in a range of cell lines. MCF-7 cells (human breast adenocarcinoma) and its doxorubicin-resistant subline, MCF-7R, were selected since Feuerstein et al. have shown that the resistant subline exhibits lower ferrochelatase expression.[51] In addition, the human epithelial carcinoma KB cell line of cervical origin was selected, which is relevant to the clinical applications of ALA-PDT.

We initially compared PpIX fluorescence levels induced using a fixed concentration at various incubation times. The intracellular PpIX fluorescence levels following administration to various dendrimers using a concentration of 100 μM increased with time (2−24 h) as shown in Figure 1 for the KB cell line. Higher levels were observed for all the dendrimers compared to ALA, with the most striking enhancement apparent at shorter incubation times (2−4 h), where ALA

DOI: 10.1021/acs.bioconjchem.8b00574
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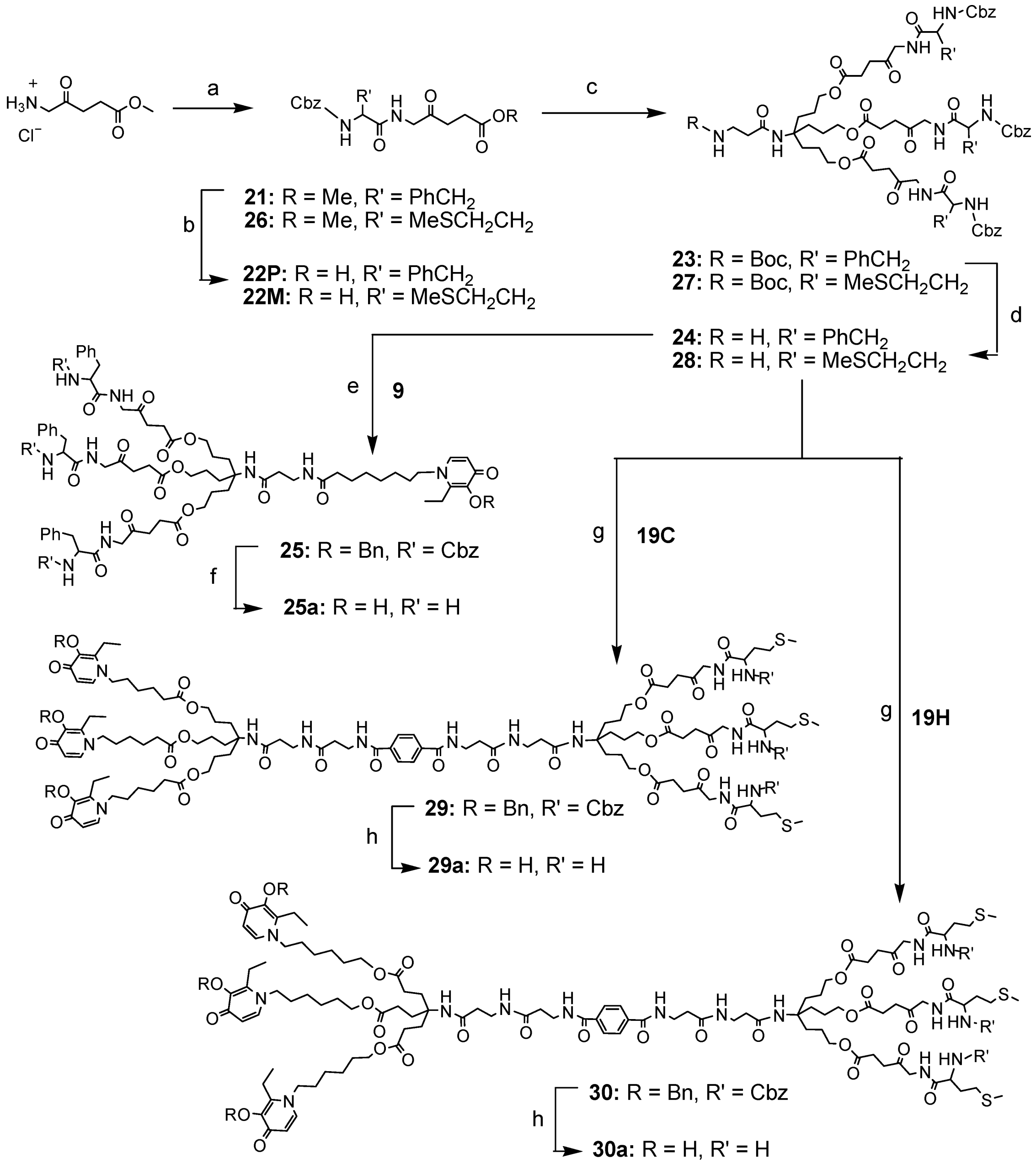
Scheme 3[a]



[a]Reagents and conditions: (a) *N*-Cbz-L-phenylalanine or *N*-Cbz-L-methionine, DCC, HOBT, Bmim, THF, DIPEA, rt, argon, 24 h; (b) 2 M NaOH/MeOH (1:1), 4 °C, 30 min; (c) **6**, DCC, DMAP, DCM/DMF (3:1), rt, argon, 48 h; (d) DCM/HCOOH (10:1), 18 h; (e) **9**, DCC, HOBT, DCM/DMF (3:1), rt, argon, 24 h; (f) Pd/C, H_2/20 psi, EtOAc/MeOH (4:1), 2 min; (g) DCC, HOBt, Bmim, DCM/THF (1:1), rt, argon, 48 h; (h) Pd black, H_2, ammonia in MeOH (7 N) and liquid ammonia, 2 h.

induced very low PpIX levels. The dendrimer which lacked a chelating agent (tri-ALA, Figure 1) was found to be slightly less effective at shorter incubation times than dendrimer **10a** which contains one iron chelating HPO moiety. Dendrimer **20a**, which also contains three ALA residues like **10a** but three HPO chelating moieties, was more effective for PpIX generation than **10a** at all the time points. These data are consistent with a synergistic enhancement in PpIX generation resulting from the presence of the HPO moieties. Inhibition of ferrochelatase activity by the HPO chelator results in the inhibition of PpIX conversion to heme, thereby leading to enhanced intracellular PpIX accumulation, as found in previous studies using co-administration of HPOs and the administration of single conjugates of HPO with ALA where higher PpIX levels were observed in the presence of HPO conjugates compared to ALA alone.[37,39] In a further modification to the dendrimer structure, attachment of a phenylalanine residue at the N-terminus of the ALA (dendrimer **25a**) enhanced the PpIX levels measured at shorter times compared to **20a**. Attachment of methionyl residues (dendrimers **29a** and **30a**) further enhanced PpIX levels and generated approximately 12−15-fold of PpIX fluorescence compared to ALA within 24 h. These results are consistent with a previous study demonstrating enhancement of PpIX generation for single conjugates of ALA with amino acid residues.[23]

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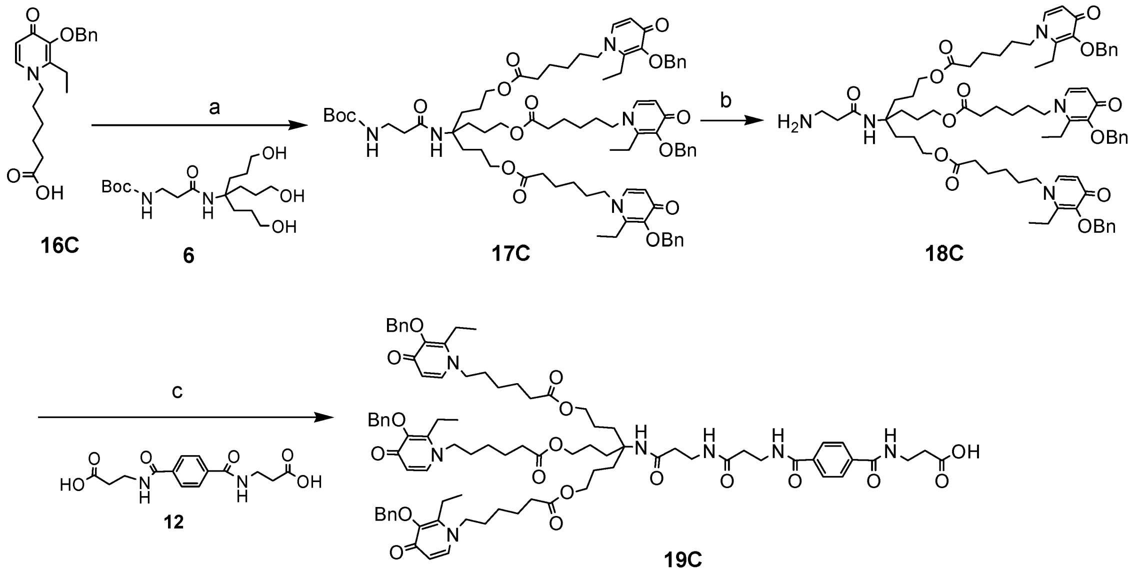
Scheme 4[a]



[a]Reagents and conditions: (a) DCC, DMAP, DCM/DMF (3:1), rt, argon, 48 h; (b) DCM/HCOOH (10:1), rt, 18 h; (c) DCC, HOBT, DMF, Bmim, argon, 0 °C 1 h then rt 24 h.

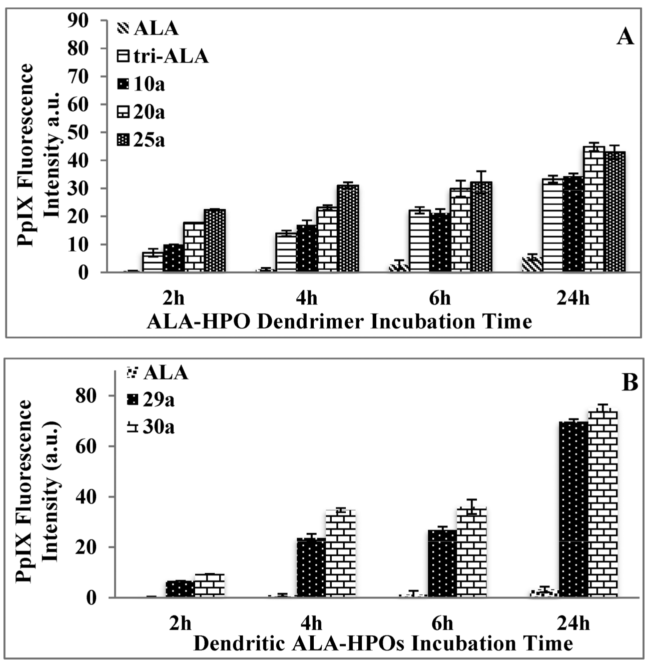


Figure 1. Kinetics of PpIX fluorescence in KB cell line. Time course of fluorescence intensities produced after incubation with 100 μM dendritic ALA-HPO: (A) comparison of **10a**, **20a**, and **25a** with tri-ALA and ALA; (B) comparison of **29a** and **30a** with ALA.

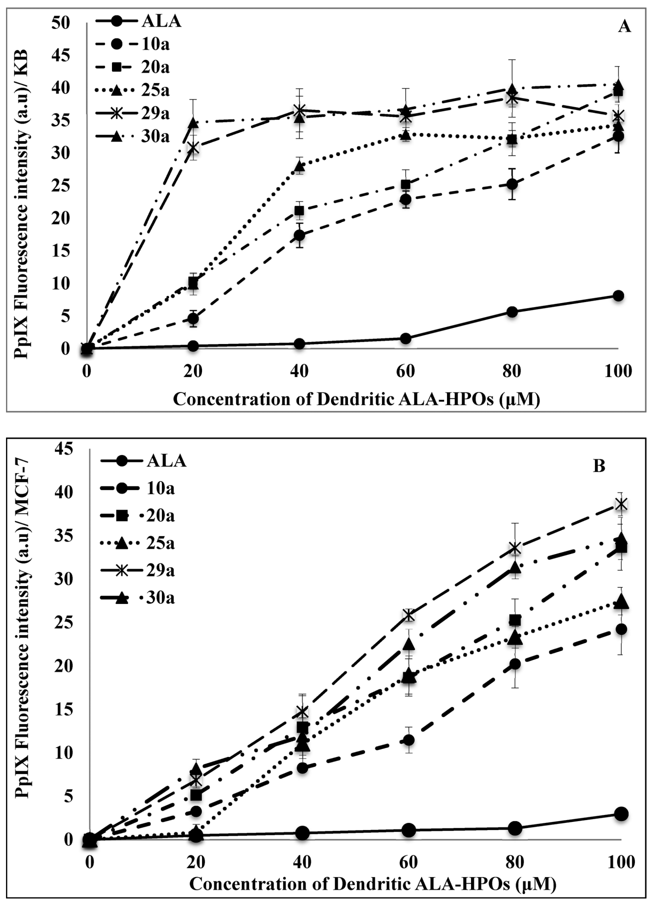


Figure 2. Concentration dependence profile of PpIX generation induced by ALA and the dendritic compounds. Fluorescence measurements were performed after incubation with variable concentration (20−100 μM) of ALA and dendritic ALA-HPO for 4 h: (A) in KB cell line and (B) in MCF-7.

In the next part of the study, a range of concentrations of each compound was investigated for a fixed incubation time point of 4 h, which is a typical exposure time employed clinically for ALA administration. The magnitude of PpIX generation induced by exposure to ALA-containing dendrimers in the KB and MCF-7 cell lines was found to exhibit a dose-dependent response. All dendrimers were found to be more effective than ALA at all concentrations investigated in KB (Figure 2A) and MCF-7 (Figure 2 B). Clearly the protection of the ALA moieties with methionine has an advantage, at lower concentrations the effect being apparently irrespective of whether there is a HPO with either an ω-hydroxyalkyl side chain (**29a**) or an ω-carboxylalkyl side chain (**30a**). We note

DOI: 10.1021/acs.bioconjchem.8b00574
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that previous studies on single conjugates of amino acids with ALA also showed that methione conjugation was effective at improving PpIX efficiency in certain cell lines.[23] The high efficacy of all the dendrimers was evident in a comparative study of three cell lines, KB, MCF-7, and MCF 7R (Figure 3).

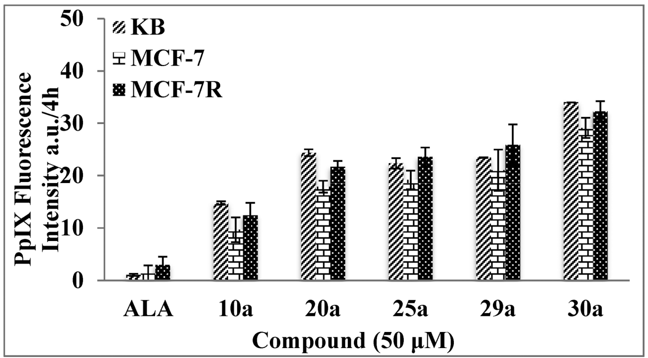

Figure 3. Comparison of PpIX production in three cell lines (KB, MCF-7, and MCF-7R) after incubation for 4 h with 50 μM dendritic ALA-HPO (**10a**, **20a**, **25a**, **29a**, and **30a**) versus ALA.

The dendrimer efficacies (at 50 μM) for each cell line were found to be comparable, which is consistent with Figure 2, where PpIX levels tended toward a common plateau at higher concentrations.

In an attempt to investigate the ability of dendrimers to act as a prolonged source of ALA and chelator, **30a** was incubated with KB cells for various time periods (15–60 min) and the PpIX fluorescence intensity was measured after 4 h (Figure 4A). Exposure for 45 and 60 min led to approximately 50% of the fluorescence being observed when the dendrimers were incubated with cells for the entire period. A similar set of data was observed when the study was extended to 24 h (Figure 4B). This is in complete contrast to incubation with ALA at a 5-fold higher concentration so that the ALA dose to the cells was comparable to the dendrimers containing multiple ALA moieties and shows that the dendrimers are capable of generating ALA and presumably HPO chelators over a prolonged time period. The experiment was repeated with MCF-7 and MCF-7R which gave similar results but with slightly lower fluorescence intensities than KB cell line (Figure S1 in the Supporting Information). These data are consistent with our previous studies of second-generation ALA dendrimers,[35] where we also observed sustained release of ALA over prolonged periods.

Photodynamic Studies. Following illumination of cells incubated with dendrimers containing both ALA and chelators, efficient phototoxicity was observed at the lowest concentrations investigated (2 μM) as shown in Figure 5, where cell viability is plotted versus concentration. In contrast, negligible phototoxicity was observed using ALA. The calculated LC_{50} values are given in Table 1, with the exception of ALA, where we can only set a lower limit since estimation of an LD_{50} value would have required a much higher concentration range. The LD_{50} values for all the dendritic compounds in both cell lines were calculated to be between 2 and 10 μM. From Figure 5 and Table 1, the relative phototoxicities of **10a** and **20a**, which contains 3 times more HPO moieties than **10a**, show that **20a** is more phototoxic in each cell line which is consistent with the greater propensity for PpIX generation by **20a** (Figure 2). The same correlation applies to the other compounds studied and the trend in efficiency for PpIX generation from Figure 2 versus the phototoxicity data shows a clear correlation between higher PpIX generation efficacy and higher phototoxicity, with compounds **29a** and **30a** being the most phototoxic. Moreover higher phototoxicities and correspondingly lower LD_{50} values were observed for the KB cell line than MCF-7 cell line, which is consistent with higher PpIX generation efficiency in the KB cell line, as is evident in Figure 2 for the lower concentrations, which is the relevant range since the phototoxicity studies were conducted with a relatively low concentration of 10 μM. The "dark" toxicity of the dendritic ALA-HPO was also assessed (i.e., their cytotoxicity in the absence of irradiation) and there was negligible toxicity present even at effective ALA levels of 100 μM after 24 h (Figure S2 in the Supporting Information).

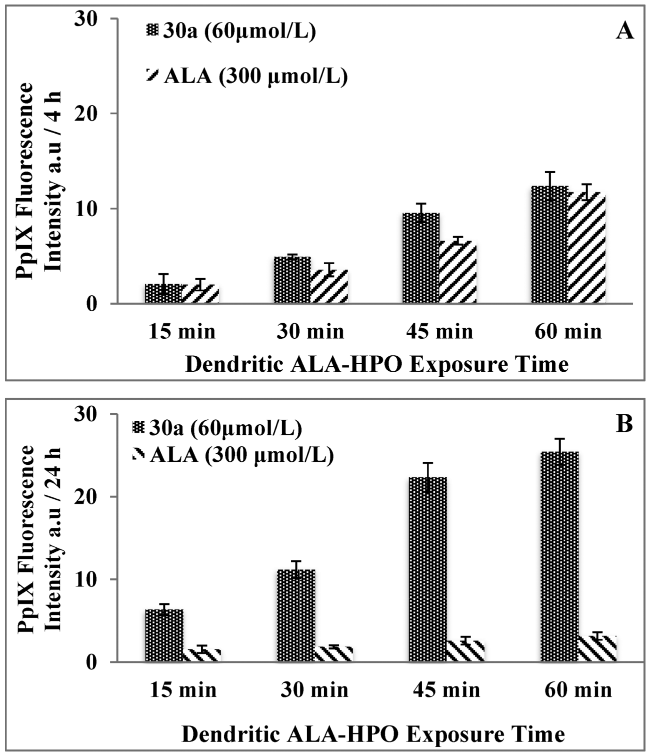

Figure 4. PpIX production in KB cells treated with 300 μM ALA or 60 μM ALA-HPO dendrimer **30a**. Cells were exposed to the compounds for times up to 60 min and then washed to remove the excess compounds. Fluorescence reading were recorded at two later time points to compare porphyrin levels: (A) fluorescence readings taken after 4 h from the time point at which cells were first exposed to the compound; (B) fluorescence readings taken after 24 h.

Mechanistic Investigation of Dendritic ALA-HPO. We also considered the mechanism of cellular uptake. Whereas small drugs are generally taken up via passive diffusion across the cell membrane, larger drug carriers such as dendrimers are taken up via active transport endocytic mechanisms.[52] In our previous study of second generation ALA dendrimers,[35] we found that uptake occurred via an endocytic mechanism (macropinocytosis). In order to provide an insight into utilized endocytic pathways, the cellular uptake mechanism was investigated by measuring PpIX fluorescence induced during incubation of dendritic ALA-HPO following pre-treatment with two endocytosis inhibitors: 5-(*N*-ethyl-*N*-isopropyl)-amirolide (EIPA)[53] and colchicine (Colch).[54] Figure 6 shows that insignificant effects were observed for all investigated compounds with concentration of 50 μM

DOI: 10.1021/acs.bioconjchem.8b00574
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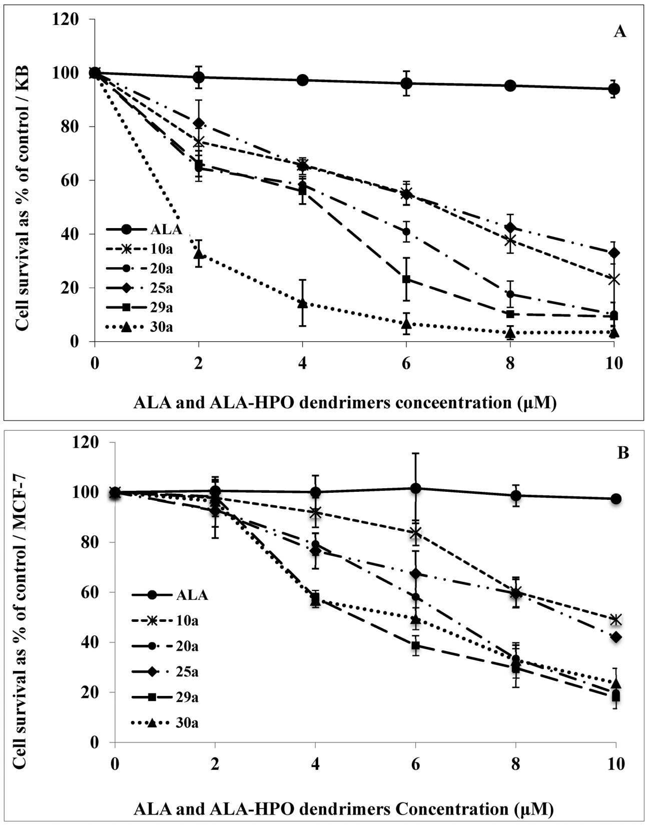

Figure 5. Phototoxicity dependence on concentration after incubation with ALA and dendritic ALA-HPO at a range of concentrations assessed by MTT assay: (A) dependence in KB cells and (B) dependence in MCF-7 cells. Cells were incubated with the compounds for 4 h and illuminated (2.5 J/cm^2).

Table 1. LD_{50} of ALA and ALA-HPO Dendrimers' Phototoxicity in KB and MCF-7 Cell Lines[a]

	LD_{50} (μM)	
	in KB	in MCF-7
ALA	>100	>100
10a	6.9 ± 0.9	10.2 ± 2.1
20a	3.8 ± 0.8	5.6 ± 1.2
25a	6.1 ± 2.3	8.8 ± 3.2
29a	2.8 ± 1.7	5.1 ± 1.9
30a	2.1 ± 1.1	4.6 ± 2.2

[a]Treatment involved 4 h incubation with the compounds and then irradiation/light exposure (2.5 J/cm^2) and further 24 h incubation before MTT tests. LD_{50} values were calculated by using the exponential trendline and then finding the corresponding concentration of each compound that caused 50% of cell death/survival.

employing EIPA or colchicine. PpIX generated by ALA was too weak to assess but it is not likely to be affected with EIPA or colchicine on pre-treatment. These results on first-generation ALA dendrimers contrast with our previous study,[35] where pre-treatment with one of these inhibitors (EIPA) significantly reduced PpIX generation.

However, in support of the present study, we have also investigated a dendrimer containing six ALA residues, albeit in a macrophage cell line instead of cancer cells,[55] where we found that EIPA elicited little effect on porphyrin generation. In that study we used another assay for endocytic uptake by examining the effect of incubation at a lower temperature (18 °C), which inhibits transport between endosomes and lysosomes, and observed a reduction in porphyrin generation by a factor of 2. The first-generation dendrimers studies in this work have a similar size; therefore, active endocytic uptake may be important, but further work including the temperature dependence is needed since both passive and active mechanisms may be relevant.

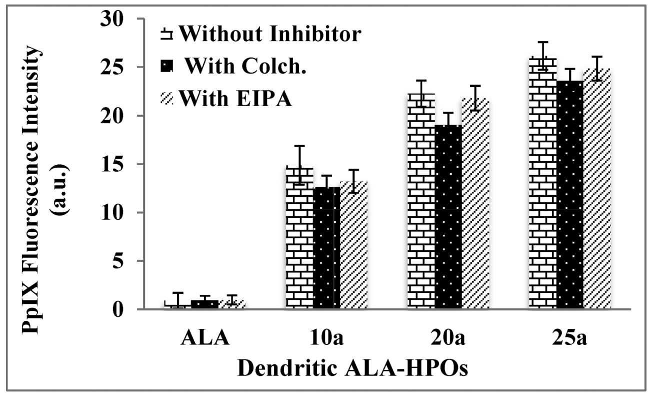

Figure 6. Effect of inhibitors on cellular uptake mechanism of ALA and dendritic ALA-HPO. PpIX fluorescence produced by 50 μM ALA or dendritic ALA-HPO **10a**, **20a**, and **25a** incubated for 4 h in MCF-7R cells. Cells were pre-treated with or without colchicine (100 μM) or EIPA (100 μM) inhibitors for 1 h before prodrugs incubation.

In another mechanistic study on dendrimer uptake, the uptake mechanisms were reported to be dependent on the dendrimer generation (i.e., size).[56] The in vitro cytotoxic and intracellular oxidative stress responses to exposure to poly(propyleneimine) dendritic compounds of increasing generation (G0−G4) identified the threshold between the active endocytic uptake of the larger poly(propyleneimine) generations G3−G4, and passive uptake of the smaller G0 and G1 dendrimers. The G2 dendrimers exhibited intermediate behavior with a contribution of both active and passive uptake.

CONCLUSIONS

Although ALA is a useful agent for PDT studies, it is important to improve the efficiency of its conversion into protoporphyrin IX in order to optimize the phototherapeutic response. The synergistic combination of iron chelating agents with ALA offers a means to improve ALA-PDT efficiency by increasing cellular PpIX accumulation. A series of ALA-HPO dendrimers with an aromatic core with suitable spacers have been synthesized utilizing convergent methodology to enable co-administration of ALA and HPO to cells. By this methodology, the number of ALA molecules, the size, and the molecular weight of the dendrimers were precisely controlled. The concept of using dendrimers as carriers for the delivery of ALA and HPO or other synergistic agents to tumor cells is a novel approach to ALA-PDT. The ALA-HPO dendrimers demonstrated higher efficacies for PpIX generation than ALA and phototoxicities in two human cancer cell lines compared to ALA. In a further modification, conjugation of methionine at the N-terminus of the ALA moieties in the dendrimers amplified PpIX generation. In future the use of other cleavable substituents at the N-terminus could be investigated.[57] In conclusion, ALA-HPO dendrimers provide a promising means for enhancing the efficacy of ALA-PDT.

DOI: 10.1021/acs.bioconjchem.8b00574
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■ EXPERIMENTAL SECTION

General. ^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on Bruker Avance 400 or Bruker Avance 500 spectrometer. Chemical shifts are quoted in ppm measured downfield relative to TMS. Ultraviolet−visible spectra were recorded on a Unicam UV2 spectrometer (Perkin-Elmer, Beaconsfield, UK) in dichloromethane (DCM) solution. An electrospray ionization (ESI) mass spectrometer (Quattro Premier XE, Micromass Technologies) was coupled to a high-performance liquid chromatograph (HPLC, Waters/AcQuity Ultraperformance LC, Waters, Manchester, UK). Analytical thin-layer chromatography was carried out using aluminum-backed, silica-coated plates (VWR, Germany) which were visualized using ultraviolet light (254 nm). Column chromatography was carried out using Alfa Aesar silica gel (220−440 mesh flash grade). R_f values are quoted using the same solvent system as used for column chromatography unless otherwise stated. HPLC was performed on a C18 silica, 250 × 2.1 mm column attached to an instrument from Agilent (Life Sciences & Chemical Analysis Group, UK). All the chemicals were purchased from Aldrich, Fisher, and Alfa Aesar and used without further purification unless otherwise stated; where stated, solvents were purified according to standard laboratory procedures. HPLC purification of the final compounds was performed on a semi-preparative system (Agilent Analytical Systems 1100, Life Sciences & Chemical Analysis Group, UK) that consisted of a G1367A QuatPump and collector 1330B compartment; the column was Hypersil ODS C18 HPLC (Thermo Scientific, UK). The method is described under the synthetic sections below for each final compound.

The purity of the final compounds was analyzed with the Agilent Analytical Systems 1100 instrument (Life Sciences & Chemical Analysis Group, UK). The analytical system consisted of a G1311A QuatPump fitted with an internal vacuum degasser and a WPS-300SL autosampler equipped an H3BDSC10-H column compartment. The separations were performed on a Gemini 5 μm C18 H3BDSC10-H column, 100 mm × 2.1 mm (Phenomenex, UK), equipped with a Security Guard C18 4 mm × 2.1 mm guard column (Phenomenex, UK), at 35 ± 0.1 °C. The mobile phase consisted of 0.05% formic acid in 98% water (solvent A) and 0.05% formic acid in 98% methanol (solvent B). A gradient elution for the mobile phases A and B was carried out in 10 min. An isocratic run was carried out with 0% B for 4 min, gradient from 0 to 100% up to 8 min, and then reconditioning the column to 0% solvent B. The flow rate was 0.2 mL/min, and the elution was monitored at wavelengths 268 and 285 nm. The purity of the products was between 90 and 95% for the final compounds **10a**, **20a**, **25a**, **29a**, and **30a**.

Synthetic Procedures. *Tris(3-acetoxypropyl)nitromethane (**3**).* Tris(3-hydroxypropyl)nitromethane (**2**, 10 g, 42 mmol) was dissolved in pyridine (50 mL). Acetic anhydride was added (25 mL) to the mixture and stirred at room temperature for 18 h. The reaction mixture was quenched with water and stirred for 30 min. The solvents were evaporated, and the residue was dissolved in DCM and washed with sodium carbonate and then water. The solvents were evaporated under vacuum to yield product as an oil (14.2 g, 95% yield). No further purification was required. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.53 (m, 6H, $3CH_2$), 1.97 (m, 6H, $3CH_2$), 2.02 (s, 9H, $3COCH_3$), 4.03 (m, 6H, $3CH_2$). ^{13}C NMR δ: 20.9, 24.0, 32.4, 63.6, 93.3, 171.0. ESI-MS: m/z 362 ($[M+H]^+$), 384 ($[M+Na]^+$).

*Tris(3-acetoxypropyl)aminomethane (**4**).* Compound **3** (10 g, 27.7 mmol) was dissolved in ethanol. Raney nickel was added, and the mixture was stirred at room temperature for 24 h. The catalyst was filtered off, and the solvent was evaporated under vacuum to give an oily product (8.4 g, 94% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.36 (m, 6H, $3CH_2$), 1.57 (m, 6H, $3CH_2$), 2.00 (s, 9H, $3COCH_3$), 4.04 (m, 6H, $3CH_2$). ^{13}C NMR δ: 21.8, 22.9, 36.0, 52.9, 64.6, 171.2. ESI-MS: m/z 332 ($[M+H]^+$), 354 ($[M+Na]^+$).

*4-(3-Acetoxypropyl)-4-(3-((tert-butoxycarbonyl)amino)propanamido)heptane-1,7-diyl Diacetate (**5**).* To a solution of compound **4** (10 g, 30.2 mmol) in THF (100 mL) were added Boc-β-alanine (6.8 g, 36 mmol), DCC (6.8 g, 33 mmol), HOBt (4.5, 33 mmol), and Bmim (6.6 g, 30 mmol). The mixture was stirred under argon at room temperature for 24 h. The precipitate was filtered off, and solvent was evaporated under vacuum to yield an oily residue. The residue was dissolved in DCM and washed with dilute hydrochloric acid, saturated sodium hydrogen carbonate, and water successively. The solvent was dried over anhydrous sodium sulfate, evaporated under vacuum, and then subjected to column chromatography on silica gel using ethyl acetate/methanol (9:1) (12.4 g, 82% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.29 (m, 6H, $3CH_2$), 1.36 (s, 9H, $3CH_3$), 1.60 (m, 6H, $3CH_2$), 2.01 (s, 9H, $3COCH_3$), 2.19 (m, 2H, CH_2), 3.07 (m, 2H, CH_2), 4.03 (m, 6H, $3CH_2$). ^{13}C NMR δ: 21.5, 22.9, 23.4, 27.0, 28.8, 35.9, 53.4, 63.0, 155.9, 171.2, 172.8. ESI-MS: m/z 503 ($[M+H]^+$), 525 ($[M+Na]^+$).

*tert-Butyl (3-((1,7-Dihydroxy-4-(3-hydroxypropyl)heptan-4-yl)amino)-3-oxopropyl)carbamate (**6**).* Compound **5** (5 g, 9.96 mmol) was dissolved in methanol (50 mL) in an ice bath at 0−4 °C. Sodium hydroxide solution (2 M, 100 mL) was added, and the mixture was stirred for 15 min. The alkaline hydrolysis was confirmed with TLC in ethyl acetate and methanol (9:1). The solution was neutralized with Amberlite IR120 to pH 7, and the solvents were evaporated to give a yellow paste (3.46 g, 92% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.29 (m, 6H, $3CH_2$), 1.36 (s, 9H, $3CH_3$), 1.55 (m, 6H, $3CH_2$), 2.21 (m, 2H, CH_2), 3.07 (m, 2H, CH_2), 3.33 (m, 6H, $3CH_2$). ^{13}C NMR δ: 26.0, 28.8, 39.5, 36.0, 37.3, 57.8, 61.9, 78.1, 156.0, 170.2. ESI-MS: m/z 377 ($[M+H]^+$), 399 ($[M+Na]^+$).

*4-(3-((5-(((Benzyloxy)carbonyl)amino)-4-oxopentanoyl)oxy)propyl)-4-(3-((tert-butoxycarbonyl)amino)propanamido)heptane-1,7-diyl Bis[5-(((benzyloxy)carbonyl)amino)-4-oxopentanoate] (**7**).* A mixture of mono-acid (Cbz-ALA) (5 equiv) and DCC (3.3 equiv) in DCM and DMF (3:1) was stirred for 50 min at room temperature under argon. DMAP (0.3 equiv) dissolved in DCM was then added, followed by dropwise addition of Boc-β-alanine tris(propanol) (**6**, 1 equiv) over 1 h. The mixture was allowed to stir for 24 h at room temperature under argon atmosphere. The precipitate was filtered off, the filtrate was concentrated, and the residue was dissolved in DCM and washed once with dilute hydrochloric acid, saturated sodium hydrogen carbonate, and water. The solvent was dried over anhydrous sodium sulfate, evaporated under vacuum, and then subjected to column chromatography on silica gel using ethyl acetate/petroleum ether (3:1). The product was eluted with ethyl acetate/methanol (9:1) after removing 1,3-dicyclohexylurea (DCU) and other byproducts with the first eluent system. The tri-

DOI: 10.1021/acs.bioconjchem.8b00574
Bioconjugate Chem. 2018, 29, 3411−3428

substituted fractions were combined and evaporated to give product **7** as yellow paste. Yield: 36.5%, HPLC purity 97%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.29 (m, 6H, $3CH_2$), 1.43 (s, 9H, $3CH_3$), 1.53 (m, 6H, $3CH_2$), 2.41 (m, 2H, CH_2), 2.56 (m, 6H, CH_2), 2.62 (2m, 6H, $6CH_2$), 3.25 (m, 2H, CH_2), 4.02 (2m, 6H, 6 CH_2), 4.05 (s, 6H, $3NCH_2$), 5.04 (s, 6H, CH_2Ph), 5.82 (br, 3H, 3NH), 5.94 (br, 2H, 2NH), 7.28 (m, 15H, Ph). ^{13}C NMR δ: 23.1, 26.8, 28.8, 34.5, 36.4, 37.1, 49.5, 57.8, 61.9, 66.6, 78.5, 127.1, 127.5, 128.3, 128.6, 136.4, 144.0, 156.0, 171.2, and 204.4. ESI-MS: m/z 1118 ([M+H]$^+$).

*4-(3-Aminopropanamido)-4-(3-((5-(((benzyloxy)-carbonyl)amino)-4-oxopentanoyl)oxy)propyl)heptane-1,7-diyl Bis[5-(((benzyloxy)carbonyl)amino)-4-oxopentanoate] (**8**).* Compound 7 (1 g, 0.895 mmol) was dissolved in DCM (50 mL), and formic acid (5 mL) was added. The mixture was stirred at room temperature for 18 h. The solvents were then evaporated under vacuum to yield yellow paste with HPLC purity of 97%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.28 (m, 6H, $3CH_2$), 1.53 (m, 6H, $3CH_2$), 2.41 (m, 2H, CH_2), 2.56 (m, 6H, CH_2), 2.62 (2m, 6H, $6CH_2$), 3.25 (m, 2H, CH_2), 4.02 (2m, 6H, 6 CH_2), 4.05 (s, 6H, $3NCH_2$), 5.04 (s, 6H, CH_2Ph), 5.82 (br, 3H, 3NH), 5.94 (bs, 2H, 2NH), 7.28 (m, 15H, Ph). ^{13}C NMR δ: 22.5, 26.1, 34.2, 35.6, 36.3, 38.7, 49.3, 56.8, 61.9, 66.5, 127.1, 127.5, 128.3, 128.6, 136.4, 144.0, 171.2, and 204.4. ESI-MS: m/z 1018 ([M+H]$^+$), 1040 ([M+Na]$^+$).

*3-(Benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)octan-amido)propanamide (**9**).* Compound **1** (5g, 22 mmol) was dissolved in ethanol (30 mL)/H_2O (30 mL), followed by the addition of 8-amino-octanoic acid (8.5 g, 54 mmol) and 2 M NaOH solution (2 mL). The mixture was refluxed for 18 h. After cooling to room temperature, the solution was adjusted to pH 1 with concentrated hydrochloric acid and then concentrated to half volume, and then 50 mL of H_2O was added. The solution was washed with diethyl ether (2 × 50 mL), adjusted to pH 6 with 10 M NaOH, and extracted with DCM (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4. After removal of the solvent, the product was obtained by crystallization from ethyl acetate as yellow crystals (6.1 g, 75% yield). ^{1} H NMR ($CDCl_3$, 400 MHz) δ: 1.03 (t, J = 7.5 Hz, 3H, CH_3), 1.32 (m, 6H, CH_2), 1.56 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 2.57 (q, J = 7.5 Hz, 2H, CH_2), 3.64 (t, J = 6.6 Hz, 2H, CH_2), 3.74 (t, J = 7.6 Hz, 2H, CH_2), 5.28 (s, 2H, CH_2), 6.43 (d, J = 7.5 Hz, 1H, C5-H in pyridinone), 7.17 (d, J = 7.5 Hz, 1H, C6-H in pyridinone), 7.29−7.35 (m, 3H, Ph), 7.43 (m, 2H, Ph). ESI-MS: m/z 372 ([M+H]$^+$).

*4-(3-(8-(3-(Benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)-octanamido)propanamido)aminomethane Tris[3-propyl-5-(((benzyloxy)carbonyl)amino)-4-oxopentanoate] (**10**).* Compound **9** (0.50 g, 1.35 mmol) was dissolved in DCM and DMF (50 mL, 3:1) under argon. DCC (0.28 g, 1.35 mmol), HOBt (0.18 g, 1.35 mmol), and Bmim (0.29 g, 1.35 mmol) were added, and the mixture was stirred for 50 min. Compound **8** (1.25 g, 1.3 mmol) was dissolved in DMF (5 mL) and added dropwise to the above mixture over 1 h. The mixture was stirred for 24 h at room temperature, and the precipitate was filtered off. The residue was dissolved in DCM and washed once with dilute hydrochloric acid, saturated sodium hydrogen carbonate, and water. The solvent was evaporated under vacuum and then subjected to column chromatography on silica gel using ethyl acetate/petroleum ether (3:1 ratio). The product was eluted with ethyl acetate/methanol (9:1) after removal of DCU and other byproducts with the first eluent system (0.86 g, 48% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.01 (m, 3H, CH_3), 1.23 (m, 8H, $4CH_2$), 1.45 (m, 2H, CH_2), 1.60 (m, 6H, $3CH_2$), 1.82 (m, 6H, $3CH_2$), 1.86 (m, 2H, CH_2), 2.27 (m, 2H, CH_2), 2.32 (m, 2H, CH_2), 2.53 (m, 2H, CH_2), 2.55 (q, J = 7.4 Hz, 6H, $3CH_2$), 3.35 (m, 2H, CH_2), 3.72 (m, 6H, $3CH_2$), 4.00 (m, 12H, $3CH_2$ and buried $3NCH_2$), 5.04 (s, 8H, $4CH_2Ph$), 5.82 (br, 3H, 3NH), 5.94 (br, 2H, 2NH), 6.56 (d, J = 7.2, 1H, C5-H in pyridinone), 7.28 (m, 21H; 20H from 4Ph and 1H from C6-H in pyridinone). ^{13}C NMR δ: 13.2, 19.6, 22.5, 26.1, 27.8, 28.8, 31.3, 34.4, 49.1, 50.5, 56.8, 61.9, 64.7, 67.0, 71.4, 110.9, 116.9, 118.3, 123.2, 128.0, 128.3, 128.6, 136.4, 138.9, 144.0, 145.6, 156.6, 172.2, 172.4, 173.1, and 204.4. ESI-MS: m/z 1371 ([M+H$^+$]).

*4-(3-(8-(3-(Benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)-octanamido)propanamido)aminomethane Tris[3-propyl-5-(((benzyloxy)carbonyl)amino)-4-oxopentanoate] (**10a**).* Compound **10** was dissolved in methanol and ethyl acetate (50 mL, 1:4). A catalytic amount of palladium on carbon (Pd/C) and benzyl chloride were added. The mixture was stirred for 10 min under 40 psi of hydrogen gas at room temperature. Pd/C was filtered off, and solvents were evaporated to yield dendrimer **10a** as a yellow paste. The product was further purified on an Agilent HPLC semi-preparative column (10.0 mm × 250 mm, C18, 5 μm). Acetonitrile and water (99:1 with 0.05% formic acid) and methanol (0.05% formic acid) were used as mobile phase with gradient in a ratio from 0 to 80% within 12 min. The product was eluted after 4.3 min. The solvents were evaporated under vacuum to yield an oily paste. Yield: 47%. Analytical HPLC-DAD: t_R = 2.1 min; purity 95%). ^{1}H NMR (DMSO, 400 MHz) δ: 0.93 (t, J = 7.4 Hz, 3H, CH_3), 1.17 (m, 8H, $4CH_2$), 1.29 (m, 2H, CH_2), 1.46 (m, 6H, $3CH_2$), 1.69 (m, 6H, $3CH_2$), 1.79 (m, 2H, CH_2), 2.04 (m, 2H, CH_2), 2.25 (m, 2H, CH_2), 2.52 (m, 2H, CH_2), 2.58 (q, J = 7.4 Hz, 6H, $3CH_2$), 3.32 (m, 2H, CH_2), 3.72 (m, 6H, $3CH_2$), 3.97 (m, 12H, $3CH_2$ and buried $3NCH_2$), 6.06 (br, 3H, 3NH), 6.17 (br, 2H, 2NH), 6.21 (d, J = 7.2, 1H, C-5H in pyridinone), 7.64 (br, 9H, $3NH_3^+$), 7.86 (d, J = 7.2, 1H, C-6H in pyridinone), 16.73 (s, 1H, OH). ^{13}C NMR δ: 11.6, 17.5, 20.1, 24.7, 26.4, 27.3, 29.4, 33.1, 44.6, 48.3, 53.1, 59.1, 61.4, 108.3, 114.2, 120.2, 134.6, 143.1, 171.6, 172.3, 172.7, and 203.1. ESI-MS: m/z 294 ([M+3H]$^{3+}$), 879 ([M+H$^+$]), 901 ([M+Na]$^+$); HRMS: calcd for $C_{43}H_{71}N_6O_{13}$, 879.5079 ([M+H]$^+$); found, 879.5093.

*Diethyl 3,3′-(Terephthaloyl-bis(azanediyl))dipropanoate (**11**).* A solution of terephthaloyl chloride (5 g, 24.7 mmol) in DCM (125 mL) and DMF (25 mL) was added to a stirred solution of β-alanine ethyl ester hydrochloride (10 g, 99 mmol) in saturated aqueous sodium hydrogen carbonate solution (125 mL) at 0 °C. Additional amounts of sodium hydrogen carbonate were added to keep the reaction mixture alkaline. Stirring was continued at the same temperature for 3 h and at room temperature for 12 h. The reaction mixture was then extracted with DCM (100 mL). The organic layer was washed with water (50 mL), dried ($MgSO_4$), and evaporated in vacuo to afford **11** as a white powder (6.38 g, 71% yield). Mp: 160−161 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.25 (t, J = 7.2, 6 H, $2CH_3$), 2.66 (m, 4H, $2CH_2$), 3.72 (m, 4H, $2CH_2$), 4.16 (q, J = 6.2, 4H, $2CH_2$), 7.96 (4 H, s, Ar), 9.05 (2 H, t, J = 5.6 Hz, 2 × NH). ^{13}C NMR (100 MHz $CDCl_3$) δ: 14.1, 41.6, 51.6, 61.4, 129.54, 134.63, 166.51, and 172.33. ESI-MS: m/z 365 ([M+H]$^+$).

*3,3′-(Terephthaloyl-bis(azanediyl))dipropanoic Acid (**12**).* To a stirred solution of the diester **11** (1.00 g, 2.75 mmol) in methanol (25 mL) at 0 °C was added 2 M aqueous sodium

DOI: 10.1021/acs.bioconjchem.8b00574
Bioconjugate Chem. 2018, 29, 3411−3428

hydroxide (9.50 mL). The solution was left to stir for 3 h, and then precipitated material was dissolved by the addition of water (20 mL). The mixture was neutralized with Amberlite IR-120 (H^+ form) ion-exchange resin, filtered, evaporated, and dried thoroughly to afford **12** as a white powder (0.82 g, 97% yield). Mp: 257−259 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.68 (m, 4H, CH_2), 3.93 (m, 4H, 2CH_2), 7.96 (s, 4H, Ar), 8.99 (t, J = 7.8 Hz, 2H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 38.6, 41.35, 129.66, 134.57, 166.39, 172.54. ESI-MS: m/z 309 ([M+H]$^+$).

*Di-tert-butyl 4-(3-((((9H-Fluoren-9-yl)methoxy)carbonyl)-amino)propanamido)-4-(3-(tert-butoxy)-3-oxopropyl)-heptanedioate (**14**).* A mixture of Fmoc-β-alanine (2.21g, 7.1 mmol), amine **13** (3.35g, 8.5 mmol), HOBt (1.3 g, 8.5 mmol), and DCC (1.71g, 8.5 mmol) in dry DMF (30 mL) was stirred at room temperature overnight. After filtration and removal of the solvent, the residue was purified with column chromatography using ethyl acetate/cyclohexane (1:1) as an eluent to obtain product as a white solid (4.64 g, 92% yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 1.43 (s, 27H, CH_3), 1.98 (m, 6H, CH_2), 2.21 (m, 6H, CH_2), 2.35 (m, 2H, CH_2), 3.48 (m, 2H, CH_2), 4.21 (m, 1H, CH), 4.35 (m, 2H, CH_2), 5.70 (br, 1H, NH), 6.05 (s, 1H, NH), 7.31 (m, 2H, Ar), 7.39 (m, 2H, Ar), 7.60 (d, J = 7.5 Hz, 2H, Ar), 7.75 (d, J = 7.5 Hz, 2H, Ar). ESI-MS: m/z 709 ([M+H]$^+$).

*4-(3-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-propanamido)-4-(2-carboxyethyl)heptanedioic Acid (**15**).* A mixture of **14** in 96% formic acid was stirred at room temperature for 24 h. After complete removal of the solvent, product **15** was obtained quantitatively. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.83 (m, 6H, CH_2), 2.11 (m, 6H, CH_2), 2.26 (m, 2H, CH_2), 3.17 (m, 2H, CH_2), 4.21 (m, 1H, CH), 4.25 (m, 2H, CH_2), 7.22 (m, 1H, NH), 7.33 (m, 2H, Ar), 7.41 (m, 2H, Ar), 7.68 (d, J = 7.0 Hz, 2H, Ar), 7.88 (d, J = 7.0 Hz, 2H, Ar), 12.08 (br, 3H, COOH). ESI-MS (negative mode): m/z 539 ([M−H]$^-$).

*3-(Benzyloxy)-2-ethyl-1-(6-hydroxyhexyl)pyridin-4(1H)-one (**16H**).* The synthetic procedure was similar to that described for compound **9**. Yield: 86%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.03 (t, J = 7.5 Hz, 3H, CH_3), 1.30−1.44 (m, 4H, CH_2), 1.56 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 2.56 (q, J = 7.5 Hz, 2H, CH_2), 3.64 (t, J = 6.4 Hz, 2H, CH_2), 3.75 (t, J = 7.6 Hz, 2H, CH_2), 5.26 (s, 2H, CH_2), 6.41 (d, J = 7.5 Hz, 1H, C5-H in pyridinone), 7.18 (d, J = 7.5 Hz, 1H, C6-H in pyridinone), 7.27−7.35 (m, 3H, Ph), 7.42 (m, 2H, Ph). ESI-MS: m/z 330 ([M+H]$^+$), 352 ([M+Na]$^+$).

*6-(3-(Benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexanoic Acid (**16C**).* The synthetic procedure was similar to that described for compound **9**. Yield: 88%. Mp: 125.5−126 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.03 (t, J = 7.2 Hz, 3H, CH_3), 1.36 (m, 2H, CH_2), 1.62−1.72 (m, 4H, 2CH_2), 2.34 (t, J = 6.8 Hz, 2H, CH_2), 2.58 (q, J = 7.2 Hz, 2H, CH_2) 3.82 (t, J = 7.6 Hz, 2H, CH_2), 5.23 (s, 2H, CH_2), 6.67 (d, J = 7.6 Hz, 1H, C5-H in pyridinone), 7.27−7.42 (m, 6H; 5H from Ph and 1H from C6-H in pyridinone). ^{13}C NMR ($CDCl_3$) δ: 13.17, 19.59, 24.08, 25.68, 30.88, 34.04, 53.31, 73.03, 116.78, 127.93, 128.24, 128.62, 137.48, 138.74, 145.53, 146.89, 173.16, 176.29. ESI-MS: m/z 344 ([M+H]$^+$), 366 ([M+Na]$^+$).

*Bis(6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)-4-(3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)propan-amido)-4-(3-((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)oxy)-3-oxopropyl)heptanedioate (**17H**).* Fmoc-β-alanine tris(propionic acid) (**15**, 1 equiv) was dissolved in DCM and DMF (1:1) under argon. DCC (3.6 equiv) was added, and the mixture was stirred for 40 min at room temperature. DMAP (0.3 equiv) dissolved in DCM was added, followed by the addition of mono-alcohol compound **16H** (3.6 equiv). The mixture was allowed to stir for 24 h at room temperature under argon atmosphere. The precipitate was filtered off, and the filtrate was concentrated under vacuum and then subjected to column chromatography on silica gel using ethyl acetate/petroleum ether (3:1). The product was eluted with DCM/MeOH (9.5:0.5) after removal of DCU and other byproducts with the first eluent system. The fractions of tri-substituted were combined and evaporated to give product **17H** as a colorless oil (42% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.01 (t, J = 7.4 Hz, 9H, CH_3), 1.28 (m, 12H, CH_2), 1.55 (m, 6H, CH_2), 1.64 (m, 6H, CH_2), 2.02 (m, 6H, CH_2), 2.26 (m, 6H, CH_2), 2.46 (m, 2H, CH_2), 2.57 (q, J = 7.4 Hz, 6H, CH_2), 3.43 (m, 2H, CH_2), 3.73 (t, J = 7.1 Hz, 6H, CH_2), 3.99 (t, J = 6.3 Hz, 6H, CH_2), 4.18 (m, 1H, CH), 4.31 (d, J = 7.0 Hz, 2H, CH_2), 5.26 (s, 6H, CH_2), 5.90 (br, 1H, NH), 6.40 (d, J = 7.5 Hz, 3H, C5-H in pyridinone), 6.79 (br, 1H, NH), 7.16 (d, J = 7.5 Hz, 3H, C6-H in pyridinone), 7.28−7.43 (m, 19H, Ar), 7.59 (d, J = 7.4 Hz, 2H, Ar), 7.74 (d, J = 7.4 Hz, 2H, Ar). ESI-MS: m/z 1474 ([M+H]$^+$), 738 ([M+2H]$^{2+}$), 492 ([M+3H]$^{3+}$).

*Bis(6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)-4-(3-aminopropanamido)-4-(3-((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)oxy)-3-oxopropyl)heptanedioate (**18H**).* To a solution of **17H** (1.8 g) in dry DMF (10 mL) was added piperidine (0.25 mL). The solution was stirred at room temperature for 20 min and then concentrated under reduced pressure. The residue was purified by column chromatography using DCM/MeOH (9:1, 3:1 then 1:1) as eluent to obtain the product **18H** as a colorless oil (1.1 g, yield 72%). ^{1}H NMR (CD_3OD, 400 MHz) δ: 1.09 (t, J = 7.6 Hz, 9H, CH_3), 1.37 (m, 12H, CH_2), 1.63 (m, 6H, CH_2), 1.71 (m, 6H, CH_2), 2.01 (m, 6H, CH_2), 2.29 (m, 6H, CH_2), 2.35 (m, 2H, CH_2), 2.66 (q, J = 7.6 Hz, 6H, CH_2), 2.88 (m, 2H, CH_2), 3.98 (m, 6H, CH_2), 4.06 (m, 6H, CH_2), 5.17 (s, 6H, CH_2), 6.49 (d, J = 7.2 Hz, 3H, C5-H in pyridinone), 7.34 (m, 9H, Ph), 7.41 (m, 6H, Ph), 7.70 (d, J = 7.2 Hz, 3H, C6-H in pyridinone). ESI-MS: m/z 1252 ([M+H]$^+$).

*4-(3-((6-(3-(Benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)-hexanoyl)oxy)propyl)-4-(3-((tert-butoxycarbonyl)amino)-propanamido)heptane-1,7-diyl-bis(6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexanoate) (**17C**).* The synthetic procedure is similar to that described for **7**. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.02 (t, J = 7.6 Hz, 9H, 3CH_3), 1.34 (m, 6H, 3CH_2), 1.42 (s, 9H, 3CH_3), 1.47−1.55 (m, 6H, 3CH_2), 1.61 (t, J = 7.6 Hz, 6H, 3CH_2), 1.65−1.72 (m, 12H, 6CH_2), 2.28 (t, J = 7.2 Hz, 6H, 3CH_2), 2.43 (t, J = 6.4 Hz, 2H, CH_2), 2.58 (q, J = 7.6 Hz, 6H, 3CH_2), 3.33 (q, J = 6.0 Hz, 2H, CH_2), 3.80 (t, J = 7.2 Hz, 6H, 3CH_2), 4.02 (t, J = 6.4 Hz, 6H, 3CH_2), 5.25 (s, 6H, CH_2), 6.42 (d, J = 7.6 Hz, 3H, C5-H in pyridinone), 7.23−7.43 (m, 18H, 3C6-H in pyridinone and 3Ph). ^{13}C NMR ($CDCl_3$) δ: 13.16, 19.40, 22.40, 24.11, 25.58, 29.52, 30.49, 30.85, 33.63, 34.54, 36.13, 52.98, 57.73, 64.40, 72.74, 72.80, 116.91, 127.82, 128.16, 128.44, 137.43, 138.58, 145.54, 146.14, 161.16, 161.59, 170.90, 173.12. ESI-MS: m/z 1352.6 ([M+H]$^+$).

*4-(3-Aminopropanamido)-4-(3-((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexanoyl)oxy)propyl)heptane-1,7-diyl-bis(6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)-hexanoate) (**18C**).* To a solution of **17C** (1.6 g) in DCM (15

DOI: 10.1021/acs.bioconjchem.8b00574
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mL) was added formic acid (5 mL). The mixture was stirred at room temperature overnight and concentrated under reduced pressure. The crude product was obtained in quantitative yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.00 (t, J = 7.5 Hz, 9H, $3CH_3$), 1.22−1.32 (m, 6H, $3CH_2$), 1.49−1.58 (m, 6H, $3CH_2$), 1.60 (t, J = 7.6 Hz, 6H, $3CH_2$), 1.61−1.71 (m, 12H, $6CH_2$), 2.25 (t, J = 7.2 Hz, 6H, $3CH_2$), 2.39 (t, J = 6.2 Hz, 2H, CH_2), 2.56 (q, J = 7.6 Hz, 6H, $3CH_2$), 3.30 (q, J = 6.0 Hz, 2H, CH_2), 3.77 (t, J = 7.2 Hz, 6H, $3CH_2$), 4.03 (t, J = 6.5 Hz, 6H, $3CH_2$), 5.27 (s, 6H, $3CH_2$Ph), 6.40 (d, J = 7.6 Hz, 3H, 3C5-H in pyridinone), 7.20−7.35 (m, 18H, 3C6-H in pyridinone and 3Ph). ESI-MS: m/z 1252 $[M+H]^+$.

*General Procedure for Amide Bond Formation of Monosubstituted Core **12** with First Tri-esters' Amino Terminals of Compounds **19C** and **19H**.* Compound **12** (2 equiv) was dissolved in THF under argon. Compound **18C** or **18H** (1 equiv) was added to the mixture together with DCC (1.1 equiv), HOBt (1.1 equiv), and Bmim (1.1 equiv) all at once, and the mixture was stirred at 0 °C for 1 h and then at room temperature for 24 h. The precipitate was filtered off, and the solvent was evaporated under vacuum. The residue was dissolved in DCM and washed with water (100 mL) five times. The solvent was evaporated, and the residue was purified on silica gel using ethyl acetate/methanol (4:1) to obtain **19C** and **19H**, respectively.

*3-(4-((3-((3-((1,7-Bis((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexanoyl)oxy)-4-(3-((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexanoyl)oxy)propyl)heptan-4-yl)amino)-3-oxopropyl)amino)-3-oxopropyl)carbamoyl)benzamido)propanoic Acid (**19C**).* Yield: 0.42 g, 65%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.96 (t, J = 6.8 Hz, 9H, $3CH_3$), 1.25 (m, 12H, $6CH_2$), 1.54−1.60 (m, 12H, $6CH_2$), 1.97 (m, 6H, $3CH_2$), 2.21 (m, 2H, CH_2), 2.35 (m, 6H, $3CH_2$), 2.50 (m, 2H, CH_2), 2.53 (m, q, J = 7.2 Hz, 6H, $3CH_2$), 3.37 (m, 2H, CH_2), 3.55 (m, 4H, $2CH_2$), 3.68 (m, 4H, $2CH_2$), 3.72 (6H, $3CH_2$), 3.94 (s, 6H, $3CH_2$), 4.83 (s, 6H, $3CH_2$Ph), 6.32 (d, J = 6.8 Hz, 3H, C5-H in pyridinone), 7.22−7.36 (m, 20H, 3Ph, 2H from Ar and 3H from C6-H in pyridinone), 7.78 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$,100 MHz) δ: 13.4, 19.6, 24.3, 25.7, 25.8, 26.1, 28.3, 28.6, 31.3, 33.5, 53.1, 53.3, 57.3, 64.5, 72.9, 117.3, 128.0, 128.4, 128.6, 137.0, 137.8, 138.6, 145.9, 146.1, 166.6, 171.5, 172.2, 173.4, 173.5. ESI-MS: m/z 1542 ($[M+H]^+$), 1564 ($[M+Na]^+$).

*3-(4-((3-((3-((1,7-Bis((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)oxy)-4-(3-((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)oxy)-3-oxopropyl)-1,7-dioxoheptan-4-yl)amino)-3-oxopropyl)amino)-3-oxopropyl)carbamoyl)benzamido)propanoic Acid (**19H**).* ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.98 (t, J = 7.2 Hz, 9H, $3CH_3$), 1.23−1.27 (m, 6H, $3CH_2$), 1.62−1.97(m, 6H, $3CH_2$), 2.00 (m, 6H, $3CH_2$), 2.22 (m, 6H, $3CH_2$), 2.22 (m, 2H, CH_2), 2.36−2.58 (m, 6H, $3CH_2$, m, 4H, $2CH_2$ and q, J = 6.7 Hz, 6H, $3CH_2$), 3.39 (m, 2H, CH_2), 3.56 (m, 4H, $2CH_2$), 3.64 (m, 4H, $2CH_2$), 3.79 (m, 6H, $3CH_2$), 3.95 (m, 6H, $3CH_2$), 4.10 (m, 2H, CH_2), 5.17 (s, 6H, $3CH_2$Ph), 6.53 (d, J = 6.2 Hz, 3H, C5-H in pyridinone), 7.25−7.28 (m, 20H; 15H from 3Ph, 2H from Ar, 3H from C6-H in pyridinone), 7.77 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 13.4, 19.6, 25.4, 26.0, 26.4, 28.4, 28.6, 29.7, 31.3, 53.2, 58.4, 64.7, 72.5, 117.6, 128.0, 128.3, 128.7, 137.8, 137.4, 138.6, 145.9, 147.3, 166.6, 171.8, 172.3, 173.5, 173.8. ESI-MS: m/z 1542 ($[M+H]^+$), 1564 ($[M+Na]^+$).

*General Procedure for Amide Bond Formation of **19C** or **19H** with the Second Triesters Containing Free Amino Group (**8** or **28**).* Compound **19C** or **19H** (monocarboxy free, 1 equiv) was dissolved in THF under argon. Compound **8** or **28** (1.2 equiv) was added to the mixture together with DCC (1.1 equiv), HOBt (1.1 equiv), and Bmim (1.1 equiv) all at once, and the mixture was stirred at 0 °C for 1 h and then at room temperature for 24 h. The precipitate was filtered off, and the solvent was evaporated under vacuum. The residue was dissolved in DCM and washed with water (100 mL) five times. The solvent was evaporated, and the residue was purified on silica gel using ethyl acetate and methanol (4:1). Using this method, compounds **20**, **29**, and **30** were prepared.

*Bis(6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)-4-(3-((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)-oxy)-3-oxopropyl)-4-(3-(3-(4-((14,14-bis(3-((5-(((benzyloxy)-carbonyl)amino)-4-oxopentanoyl)oxy)propyl)-3,6,9,16,20-pentaoxo-1-phenyl-2,10-dioxa-4,15,19-triazadocosan-22-yl)carbamoyl)benzamido)propanamido)propanamido)-heptanedioate (**20**).* Yield: 32%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.96 (t, J = 7.6 Hz, 9H, $3CH_3$), 1.00 (m, 12H, $6CH_2$), 1.24 (m, 6H, $3CH_2$), 1.35−1.54 (m, 12H, $6CH_2$), 1.97 (m, 6H, $3CH_2$), 2.22 (m, 6H, $3CH_2$), 2.25 (m, 4H, $2CH_2$), 2.56 (m, 12H, $6CH_2$), 2.64 (m, 4H, $2CH_2$), 3.1(q, J = 7.2 Hz, 6H, $3CH_2$), 3.59 (m, 6H, $3CH_2$), 3.64 (m, 4H, $2CH_2$), 3.81 (m, 6H, $3CH_2$), 3.95 (m, 6H, $3CH_2$), 3.97 (s, 6H, $3CH_2$ and 4H, $2CH_2$), 4.04 (m, 6H, $3CH_2$), 5.10 (s, 6H, $3CH_2$Ph), 5.28 (s, 6H, $3CH_2$Ph), 6.11 (3H, br 3NH), 6.32 (d, J = 7.2 Hz, 3H, C5-H in pyridinone), 7.22−7.33 (m, 30H, 6Ph; d, J = 7.2 Hz, 3H, C6-H in pyridinone; d, J = 8.0 Hz, 3H, NH), 7.78 (m, 7H; 4H from Ar, 3H from 3NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.3, 22.7, 27.9, 31.2, 34.4, 35.0, 37.0, 37.5, 47.3, 50.6, 58.4, 60.5, 64.7, 66.8, 67.1, 120.1, 127.2, 128.3, 128.6, 136.4, 141.3, 144.1, 146.4, 156.9, 171.2, 172.4, 204. MALDI-TOF: 2542 ($[M+H]^+$), 2564 ($[M+Na]^+$). ESI-MS: m/z 1271 ($[M+2H]^{2+}$).

*Bis(6-(2-ethyl-3-hydroxy-4-oxopyridin-1(4H)-yl)hexyl)-4-(3-(3-(4-((3-((3-((1,7-bis((5-amino-4-oxopentanoyl)oxy)-4-(3-((5-amino-4-oxopentanoyl)oxy)propyl)heptan-4-yl)amino)-3-oxopropyl)amino)-3-oxopropyl)carbamoyl)benzamido)-propanamido)propanamido)-4-(3-((6-(2-ethyl-3-hydroxy-4-oxopyridin-1(4H)-yl)hexyl)oxy)-3-oxopropyl)heptanedioate (**20a**).* After hydrogenation as described for **10a**, the crude product was further purified on an Agilent HPLC semi-preparative column (250 mm × 10.0 mm, RP C18, 5 μm). Acetonitrile and water (99:1 with 0.05% formic acid) and methanol (0.05% formic acid) were used as mobile phase with gradient in a ratio from 0 to 80% within 15 min. The product was eluted after 5.6 min. The solvents were evaporated under vacuum to yield an oily paste with 34% yield. Analytical HPLC-DAD: t_R = 2.5 min; purity 93.7%. ^{1}H NMR (CD_3OD, 400 MHz) δ: 1.12 (t, J = 7.6 Hz, 9H, $3CH_3$), 1.22 (m, 12H, $6CH_2$), 1.29 (m, 6H, $3CH_2$), 1.49−1.72 (m, 12H, $6CH_2$), 1.98 (m, 6H, $3CH_2$), 2.12 (m, 6H, $3CH_2$), 2.33 (m, 4H, $2CH_2$), 2.59 (m, 12H, $6CH_2$), 2.68 (m, 4H, $2CH_2$), 2.3.18 (q, J = 7.2 Hz, 6H, $3CH_2$), 3.83 (m, 6H, $3CH_2$), 3.96 (m, 4H, $2CH_2$), 4.02−4.11 (m, 6H, $3CH_2$, m, 6H, $3CH_2$, s, 6H, $3CH_2$, and 4H, $2CH_2$), 4.21 (m, 6H, $3CH_2$, m 6H, $3CH_2$, and (m, 6H, $3CH_2$), (m, 12H, $6CH_2$), 7.12 (d, J = 7.6 Hz, 3H, C5-H in pyridinone), 7.62 (d, J = 7.6 Hz, 3H, C6-H in pyridinone), 7.84 (m, 4H, Ar). ^{13}C NMR (100 MHz, CD_3OD) δ: 11.4, 22.7, 26.8, 30.7, 33.4, 34.0, 36.0, 36.5, 45.4, 52.6, 57.2, 61.4, 65.2, 66.4, 68.2, 117.3, 126.6, 128.6, 130.2, 137.4, 142.4, 146.4, 156.9, 164.4, 173.4, 203.0. ESI-MS: m/z 624 ($[M+3H]^{3+}$), 1870 ($[M+H]^+$), 1892 ($[M+Na]^+$), 1908 ($[M+K]^+$). HRMS:

DOI: 10.1021/acs.bioconjchem.8b00574
Bioconjugate Chem. 2018, 29, 3411−3428

calcd for $C_{94}H_{141}N_{12}O_{27}$, 1870.0029 ([M+H]$^+$); found, 1870.0051.

*General Procedure for the Synthesis of Methyl ALA-Amino Esters (Compounds **21** and **26**).* *N*-Cbz amino acid (1 equiv), ALA-methyl ester (1.2 equiv), DCC (1 equiv), HOBt (1 equiv), and Bmim (1 equiv) were suspended in THF (100 mL) under argon. The mixture was stirred for 2 h at room temperature and then cooled to 0 °C. DIPEA (1.2 equiv) was added dropwise in THF, and the mixture was stirred overnight at room temperature. Acetone (100 mL) was added, the solid precipitate was filtered off, and the solvent was evaporated under vacuum. The residue was dissolved in DCM and washed with dilute hydrochloric acid three times and once with saturated sodium bicarbonate, and then water. The solvent was evaporated under vacuum to yield a pure white powder.

*Methyl 5-(2-(((Benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxopentanoate (**21**).* Yield: 96%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.58 (t, *J* = 7.0 Hz, 2H, CH_2), 2.74 (t, *J* = 7.0 Hz, 2H, CH_2), 3.14 and 3.47 (m, 2H, CH_2), 3.84 (s, 3H, CH_3), 3.98 (s, 2H, CH_2), 4.36 (m, 1H, CH), 5.08 (s, 2H, CH_2), 6.71 (br, 1H, NH), 7.38−7.47 (m, 10H, Ph), 7.87 (br, 1H, NH). ^{13}C NMR (100 MHz $CDCl_3$) δ: 27.2, 34.5, 37.1, 50.3, 55.1, 58.5, 66.8, 125.4, 127.1, 127.3, 128.3, 128.7, 128.8, 128.9, 136.1, 136.6, 155.7, 172.5, 173.4, 204.5. ESI-MS: *m*/*z* 427 ([M+H]$^+$).

*Methyl 5-(2-(((Benzyloxy)carbonyl)amino)-4-(methylthio)butanamido)-4-oxopentanoate (**26**).* Yield: 93%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.06 (m, 2H, CH_2), 2.14 (s, 3H, CH_3S), 2.60 (m, 2H, CH_2), 2.74 (m, 2H, CH_2), 2.84 (m, 2H, CH_2), 3.68 (s, 3H, CH_3), 4.36 (m, 2H, CH_2), 4.45(m, 1H, CH), 5.09 (s, 2H, CH_2Ph), 6.71 (br, 1H, NH), 7.38−7.47(m, 5H, Ph), 8.03 (s, 1H, NH). ^{13}C NMR δ: 14.1, 14.2, 27.1, 29.3, 34.5, 50.3, 51.9, 57.9, 66.8, 127.1, 127.6, 128.9, 136.1, 155.9, 172.5, 173.1, 204.7. ESI-MS: *m*/*z* 411 ([M+H]$^+$).

*General Procedure for the Hydrolysis of Methyl ALA-Amino Acid Ester (**21** and **26**) To Generate Compounds **22M** and **22P**.* Compound **21** or **26** (1 equiv) was dissolved in methanol and 2 M sodium hydroxide (1:1) at 4 °C. The mixture was stirred at this temperature for 30 min. The solution was then neutralized with concentrated hydrochloric acid to pH 7. The product **22M** or **22P** was precipitated upon neutralization, and further precipitation occurred upon addition of water. The product was filtered and dried on vacuum oven overnight to give a white powder with 93−95% yield.

*5-(2-(((Benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxopentanoic Acid (**22P**).* Yield: 95%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.78 (m, 4H, $2CH_2$), 3.37 and 3.49 (m, 2H, CH_2), 4.07 (s, 2H, CH_2), 4.53 (m, 1H, CH), 5.07 (s, 2H, CH_2), 6.72 (br, 1H, NH), 7.39−7.48 (m, 10H, Ph), 8.02 (br, 1H, NH), 12.3 (br, 1H CO_2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 29.2, 34.6, 37.3, 50.2, 58.5, 66.8, 125.4, 127.1, 127.3, 128.3, 128.7, 128.8, 128.9, 136.1, 136.6, 155.9, 172.2, 173.4, 204.5. ESI-MS: *m*/*z* 413 ([M+H]$^+$).

*5-(2-(((Benzyloxy)carbonyl)amino)-4-(methylthio)butanamido)-4-oxopentanoic Acid (**22M**).* Yield: 93%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.01 (m, 2H, CH_2), 2.04 (s, 3H, CH_3S), 2.58 (m, 4H, $2CH_2$), 2.73 (m, 2H, CH_2), 4.09 (m, 2H, CH_2), 4.35 (m, 1H, CH), 5.06 (s, 2H, CH_2), 6.68 (br, 1H, NH), 7.35−7.56 (m, 5H, Ph), 7.62 (s, 1H, NH). ^{13}C NMR: 15.3, 29.2, 29.5, 34.3, 50.3, 50.4, 57.2, 66.9, 127.1, 127.6, 128.9, 136.2, 155.9, 172.5, 173.1, 204.5. ESI-MS: *m*/*z* 397 ([M+H]$^+$).

*4-(5-Benzyl-3,6,9,12-tetraoxo-1-phenyl-2,13-dioxa-4,7-diazahexadecan-16-yl)-4-(3-((tert-butoxycarbonyl)amino)propanamido)heptane-1,7-diyl-bis(5-(2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxopentanoate) (**23**).* The synthetic procedure is similar to that described for compound 7. Yield: 46.2%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.96 (s, 9H, $3CH_3$), 1.32 (m, 6H, $3CH_2$), 1.54 (m, 6H, $3CH_2$), 2.45 (m, 2H, CH_2), 2.78 (m, 12H, $6CH_2$), 3.37 (m, 6H, $3CH_2$), 3.49 (m, 2H, CH_2), 3.98 (S, 6H, $3CH_2$), 4.08 (m, 6H, $3CH_2$), 4.45 (m, 3H, 3CH), 5.09 (s, 6H, $3CH_2$), 6.72 (br, 5H, 5NH), 7.39−7.48 (m, 30H, Ph), 8.02 (br, 3H, 3NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.5, 21.3, 27.4, 28.6, 34.3, 34.6, 34.8, 37.3, 50.2, 50.4, 58.5, 65.3, 65.6, 66.8, 79.3, 125.4, 127.1, 127.3, 128.3, 128.7, 128.8, 128.9, 136.1, 136.6, 155.4, 155.9, 172.2, 173.4, 203.7. ESI-MS: *m*/*z* 1559 ([M+H]$^+$), 1581 ([M+Na]$^+$).

*4-(3-Aminopropanamido)-4-(5-benzyl-3,6,9,12-tetraoxo-1-phenyl-2,13-dioxa-4,7-diazahexadecan-16-yl)heptane-1,7-diyl-bis(5-(2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxopentanoate) (**24**).* The procedure is similar to that described for **18C**. Yield: 95%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.43 (m, 6H, $3CH_2$), 1.69 (m, 6H, $3CH_2$), 2.61 (m, 2H, CH_2), 2.72 (m, 12H, $4CH_2$), 2.93 (m, 2H, CH_2), 3.37 (m, 3H, 3CH), 3.49 (m, 3H, 3CH), 4.27 (s, 6H, 3CH2), 4.53 (m, 3H, 3CH), 5.09(s, 6H, $3CH_2Ph$), 6.72 (br, 5H, 5NH), 7.39−7.48 (m, 30H, CH_2Ph and Ph), 8.02 (br, 3H, 3NH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 21.4, 27.5, 33.5, 35,6, 36.4, 37.3, 50.2, 58.5, 66.8, 125.4, 127.1, 127.3, 128.3, 128.7, 128.8, 128.9, 136.1, 136.6, 155.9, 171.2, 172.5, 173.4, 203.5. ESI-MS: *m*/*z* 1459 ([M+H]$^+$).

*4-(5-Benzyl-3,6,9,12-tetraoxo-1-phenyl-2,13-dioxa-4,7-diazahexadecan-16-yl)-4-(3-(8-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)octanamido)propanamido)heptane-1,7-diyl-bis(5-(2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxopentanoate) (**25**).* Compound **9** (0.5 g, 1.4 mmol), compound **24** (1.61 g, 1.1 mmol), DCC (0.22 g, 1.1 mmol), HOBt (0.15 g, 1.1 mmol), and Bmim (0.24 g, 1.1 mmol) were all suspended in THF (50 mL) under argon at 0 °C. DIPEA (0.20 mL, 1.1 mmol) was added dropwise in THF, and the mixture was stirred overnight at room temperature. Acetone (100 mL) was added, the solid precipitate was filtered off, and the solvent was evaporated under vacuum. The residue was dissolved in DCM and washed with dilute hydrochloric acid three times and once with saturated sodium bicarbonate, and then water. The solvent was evaporated under vacuum to yield oily product, which was purified on silica gel using ethyl acetate and methanol as mobile phase. The correct fractions were combined and solvents evaporated to give an oily product in 47% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.05 (t, *J* = 7.2 Hz, 3H, CH_3), 1.36 (m, 6H, $3CH_2$), 1.54−1.62 (m, 4H, $2CH_2$), 1.78 (m, 12H, $6CH_2$), 2.12, (m, 2H, CH_2), 2.57−2.78 (m, 12H, $6CH_2$ and m, 2H, CH_2), 2.84, (q, *J* = 7.4, 2H, CH_2), 3.21−3.39 (m, 6H, $3CH_2$), 3.51−3.69 (m, 4H, $2CH_2$), 3.92, (m, 6H, $3CH_2$), 4.01 (s, 6H, $3CH_2$), 4.16, (m, 3H, 3CH), 5.02, (m, 6H, $3CH_2$), 5.06, (s, 2H, CH_2), 6.32, (d, *J* = 7.4, 1H, C5-H in pyridinone), 6.68 (br, 3H, 3NH), 7.26−7.89 (m, 36H, 35 from 7Ph and 1 from C6-H in pyridinone), 7.86 (br, 5H, 5NH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 13.2, 17.3, 21.8, 25.3, 27.6, 27.7, 31.2, 32.7, 33.5, 35.8, 36.3, 36.9, 37.2, 46.4, 50.3, 52.4, 52.8, 53.4, 62.5, 64.3, 66.9, 71.5, 117.2, 119.7, 125.2, 126.9, 127.4, 127.8, 128.2, 128.5, 128.6, 128.9, 136.2, 155.6, 170.0, 171.1, 172.3, 172.4, 172.6, 204.1. ESI-MS: *m*/*z* 1812 ([M+H]$^+$).

DOI: 10.1021/acs.bioconjchem.8b00574
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*4-(3-((5-(2-Amino-3-phenylpropanamido)-4-oxopentanoyl)oxy)propyl)-4-(3-(8-(2-ethyl-3-hydroxy-4-oxopyridin-1(4H)-yl)octanamido)propanamido)heptane-1,7-diyl-bis(5-(2-amino-3-phenylpropanamido)-4-oxopentanoate) (**25a**).* Yield: 28.4%; purity 91.6%. Analytical HPLC-DAD: t_R = 3.2 min. ^{1}H NMR (CD_3OD, 400 MHz) δ: 1.07, (t, J = 7.2 Hz, 3H, CH_3), 1.37 (m, 6H, 3CH_2), 1.53−1.68 (m, 4H, 2CH_2), 1.81 (m, 12H, 6CH_2), 2.13, (m, 2H, CH_2), 2.55−2.82 (m, 12H, 6CH_2 and m, 2H, CH_2), 2.87, (q, J = 7.2, 2H, CH_2), 3.22−3.41 (m, 6H, 3CH_2), 3.52−3.71 (m, 4H, 2CH_2), 4.02, (m, 6H, 3CH_2), 4.11 (s, 6H, 3CH_2), 4.18, (m, 3H, 3CH), 6.43 (br, 3H, 3NH), 6.95, (d, J = 7.6, 1H, C5-H in pyridinone), 7.75 (br, 9H, 3NH_3^+), 7.89 (d, J = 7.6, 1H, C6-H in pyridinone). ^{13}C NMR (100 MHz, CD_3OD) δ: 11.3, 15.2, 21.8, 24.2, 28.7, 28.6, 30.1, 31.5, 33.5, 35.8, 35.6, 37.2, 46.4, 50.3, 52.6, 53.4, 62.4, 65.6, 68.6, 71.7, 115.2, 116.9, 122.8, 126.9, 127.5, 128.6, 128.9, 145.3, 155.6, 165.0, 172.1, 173.5, 173.7, 173.9, 204.7. ESI-MS: m/z 1320 ([M+H]$^+$), 1342 ([M+Na]$^+$). HRMS: calcd for $C_{70}H_{98}N_9O_{16}$, 1320.7132 ([M+H]$^+$); found, 1320.7150.

*4-(3-((tert-Butoxycarbonyl)amino)propanamido)-4-(5-(2-(methylthio)ethyl)-3,6,9,12-tetraoxo-1-phenyl-2,13-dioxa-4,7-diazahexadecan-16-yl)heptane-1,7-diyl-bis(5-(2-(((benzyloxy)carbonyl)amino)-4-(methylthio)butanamido)-4-oxopentanoate) (**27**).* The synthetic procedure is similar to that described for compound **7**. The purity of the products was 97% (32.5% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.09 (s, 9H, 3CH_3), 1.44 (m, 6H, 3CH_2), 1.58 (m, 6H, 3CH_2), 1.87 (m, 6H, 3CH_2), 1.98 (s, 9H, 3SCH_s), 2.25 (m, 2H, CH_2), 2.47 (m, 12H, 6CH_2), 2.62 (m, 6H, 3CH_2), 3.23 (m, 2H, CH_2), 3.96 (m, 6H, 3CH_2), 4.02 (s, 6H, 3CH_2), 4.38 (m, 3H, 3CH), 5.09 (s, 6H, 3CH_2), 6.19 (br, 3H, 3NH), 7.28 (m, 15H, 3Ph), 7.48 (br, 3H, 3NH). ^{13}C NMR δ: 15.4, 22.6, 27.8, 28.5, 30.1, 32.0, 34.5, 37.3, 49.0, 53.6, 45.1, 58.2, 64.5, 67.1, 79.4, 128.1, 128.6, 136.3, 156.4, 156.5, 163.1, 171.3, 172.1, 172.5, 204.0. ESI-MS: m/z 1511 ([M+H]$^+$).

*4-(3-Aminopropanamido)-4-(5-(2-(methylthio)ethyl)-3,6,9,12-tetraoxo-1-phenyl-2,13-dioxa-4,7-diazahexadecan-16-yl)heptane-1,7-diyl-bis(5-(2-(((benzyloxy)carbonyl)-amino)-4-(methylthio)butanamido)-4-oxopentanoate) (**28**).* Compound **27** (1 g, 0.66 mmol) was dissolved in DCM (50 mL), and TFA (1 mL) was added. The mixture was stirred for 1 h at room temperature. The solvents were evaporated under vacuum after confirming completion of the reaction as indicated by TLC, to give the product as a yellow paste. Yield: 95%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.37 (m, 6H, 3CH_2), 1.59 (m, 6H, 3CH_2), 1.96 (m, 6H, 3CH_2), 2.01 (s, 9H, 3SCH_s), 2.26 (m, 2H, CH_2), 2.57 (m, 12H, 6CH_2), 2.62 (m, 2H, CH_2), 2.76 (m, 6H, 3CH_2), 3.96 (m, 6H, 3CH_2), 4.02 (m, 6H, 3CH_2), 4.38 (m, 3H, 3CH), 5.09 (s, 6H, 3CH_2), 6.09 (br, 3H, 3NH), 6.41 (d, 1H, NH), 7.28 (m, 5H, Ph). ^{13}C NMR δ: 15.4, 22.6, 27.6, 30.3, 30.3, 32.0, 34.5, 37.3, 49.2, 53.6, 45.3, 58.2, 64.9, 67.1, 128.1, 128.6, 136.3, 156.5, 163.1, 171.3, 172.2, 172.5, 204.3. ESI-MS: m/z 1411 ([M+H]$^+$), 1433 ([M+Na]$^+$).

*4-(3-((6-(3-(Benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)-hexanoyl)oxy)propyl)-4-(3-(3-(4-((5-(2-(methylthio)ethyl)-17,17-bis(5-(2-(methylthio)ethyl)-3,6,9,12-tetraoxo-1-phenyl-2,13-dioxa-4,7-diazahexadecan-16-yl)-3,6,9,12,19,23-hexaoxo-1-phenyl-2,13-dioxa-4,7,18,22-tetraaza-pentacosan-25-yl)carbamoyl)benzamido)propanamido)-propanamido)heptane-1,7-diyl-bis(6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexanoate) (**29**).* ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.02 (t, J = 6.2 Hz, 9H, 3CH_3), 1.26 (m, 6H, 3CH_2), 1.36 (m, 6H, 3CH_2), 1.37 (m, 6H, 3CH_2), 1.52−1.62 (m, 12H, 6CH_2), 1.96 (m, 6H, 3CH_2), 1.97 (s, 9H, 3CH_2), 2.00 (m, 6H, 3CH_2), 2.21 (m, 8H, 4CH_2 and m, 6H, 3CH_2), 2.51 (m, 12H, 6CH_2), 2.64 (m, 6H, 3CH_2 and m, 6H, 3CH_2), 3.26 (m, 8H, 4CH_2), 3.38−3.41 (m, 12H, 6CH_2), 3.51−3.63 (m, 6H, 3CH_2), 3.95 (m, 6H, 3CH_2), 4.06 (s, 6H, 2CH_2), 4.41 (m, 3H, 3CH), 5.03 (s, 6H, 3CH_2), 5.27 (s, 6H, 3CH_2), 6.32 (d, J = 7.4 Hz, 3H, C-5H in pyridinone), 6.49 (br, 3H, NH), 7.28 (m, 30H, 6Ph), 7.33 (d, J = 7.4 Hz, 3H, C-6H in pyridinone), 7.78 (m, 4H, Ar), 8.09 (br, 5H, 5NH). ^{13}C NMR δ: 8.6, 15.3, 22.6, 30.1, 32.0, 32.5, 34.4, 36.7, 45.9, 49.0, 54.1, 58.5, 64.8, 67.2, 114.5, 118.7, 128.0, 128.3, 128.6, 136.2, 156.5, 161.5, 161.8, 170.2, 172.4, 172.7, 204.5. MALDI-TOF-MS: m/z 2935 ([M+H]$^+$), 2957 ([M+Na]$^+$).

*Bis(6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)-4-(3-((6-(3-(benzyloxy)-2-ethyl-4-oxopyridin-1(4H)-yl)hexyl)-oxy)-3-oxopropyl)-4-(3-(3-(4-((5-(2-(methylthio)ethyl)-17,17-bis(5-(2-(methylthio)ethyl)-3,6,9,12-tetraoxo-1-phenyl-2,13-dioxa-4,7-diazahexadecan-16-yl)-3,6,9,12,19,23-hexaoxo-1-phenyl-2,13-dioxa-4,7,18,22-tetraazapentacosan-25-yl)-carbamoyl)benzamido)propanamido)propanamido)-heptanedioate (**30**).* Yield: 34.5%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.99 (t, J = 7.4 Hz, 9H, 3CH_3), 1.24 (m, 6H, 3CH_2) 1.28 (m, 6H, 3CH_2), 1.52 (m, 6H, 3CH_2), 1.61 (m, 12H, 6CH_2), 1.66−1.78 (m, 12H, 6CH_2), 1.98 (m, 6H, 3CH_2), 2.06 (s, 9H, 3CH_2), 2.12 (m, 6H, 3CH_2), 2.20−2.28 (m, 8H, 4CH_2), 2.54−2.67 (m, 12H, 6CH_2), 2.73 (m, 6H, 3CH_2), 2.77 (m, 6H, 3CH_2), 3.40 (m, 6H, CH_2), 3.62−3.74 (m, 8H, 3CH_2), 3.76 (m, 6H, 3CH_2), 3.93 (m, 6H, 3CH_2), 3.95 (m, 6H, 3CH_2), 4.21 (m, 3H, CH_2), 4.96 (s, 6H, 3CH_2Ph), 5.17 (s, 6H, 3CH_2Ph), 6.40 (br, 3H, NH), 6.45 (br, 3H, 3NH), 7.05 (d, J = 7.2 Hz, 3H, C5-H in pyridinone), 7.25 (m, 30H, 6Ph), 7.33 (d, J = 7.2 Hz, 3H, C6-H in pyridinone), 7.79 (m, 4H, Ar), 7.91 (br, 2H, 2NH). ^{13}C NMR δ: 8.7, 15.5, 22.7, 30.2, 32.1, 32.5, 34.4, 36.8, 45.9, 49.1, 54.5, 58.7, 64.8, 67.5, 114.5, 118.7, 128.0, 128.3, 128.6, 136.2, 156.5, 161.5, 161.8, 170.2, 172.4, 172.7, 204.5. MALDI-TOF-MS: m/z 2935 ([M+H]$^+$), 2957 ([M+Na]$^+$), 2973 ([M+K]$^+$). ESI-MS: m/z 1468 ([M+2H]$^{2+}$), 979 ([M+3H]$^{3+}$).

General Procedure for Removal of Protecting Groups by Catalytic Hydrogenation in Liquid Ammonia. All glassware was dried prior to use. Compound **29** or **30** was dissolved in 7 N ammonia in methanol in a two-necked round-bottomed flask in dry ice−acetone. The cold bath was removed and the flask fitted with a magnetic stirrer. A dry ice reflux condenser was placed on the center neck. Fresh palladium black (0.2−0.5 g) was added in methanol-wet form under nitrogen gas. A stream of hydrogen, dried over concentrated sulfuric gas, was continuously passed through the magnetically stirred solution at the boiling point of ammonia (−33 °C). Reaction progress was followed by thin-layer chromatography. After 20 min, the ammonia was evaporated to dryness under nitrogen, and 0.5 mL of formic acid in 10 mL of toluene was added. The solution was filtered from the catalyst. Evaporation of the solvents under vacuum afforded the product as a oil, which was subjected to HPLC purification. The product was further purified on an Agilent HPLC semi-preparative column (250 mm × 10.0 mm i.d., RP C18, 5 μm). Acetonitrile and water 99:1 (with 0.05% formic acid) and methanol (0.05% formic acid) were used as mobile phases A and B with gradient in a ratio from 0−80% within 12 min. The product was eluted after 6.1 and 6.5 min for **29a** and **30a**, respectively. The solvents

DOI: 10.1021/acs.bioconjchem.8b00574
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were evaporated under vacuum to afford **29a** and **30a** with 28% and 25% yield.

*4-(3-(3-(4-((5-Amino-17,17-bis(3-((5-(2-amino-4-(methylthio)butanamido)-4-oxopentanoyl)oxy)propyl)-6,9,12,19,23-pentaoxo-13-oxa-2-thia-7,18,22-triazapentacosan-25-yl)carbamoyl)benzamido)propanamido)propanamido)-4-(3-((6-(2-ethyl-3-hydroxy-4-oxopyridin-1(4H)-yl)hexanoyl)oxy)propyl)heptane-1,7-diyl-bis(6-(2-ethyl-3-hydroxy-4-oxopyridin-1(4H)-yl)hexanoate) (**29a**).* Analytical HPLC-DAD: t_R = 3.7 min, purity 90%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.13 (t, *J* = 6.4 Hz, 9H, $3CH_3$), 1.27 (m, 6H, $3CH_2$), 1.38 (m, 6H, $3CH_2$), 1.57−1.68 (m, 12H, $6CH_2$), 2.01 (m, 6H, $3CH_2$), 2.07 (s, 9H, $3CH_2$), 2.11 (m, 6H, $3CH_2$), 2.25 (m, 14H, $7CH_2$), 2.58 (m, 12H, $6CH_2$), 2.69 (m, 12H, $6CH_2$), 3.28 (m, 8H, $4CH_2$), 3.36−3.47 (m, 12H, $6CH_2$), 3.58−3.69 (m, 6H, $3CH_2$), 4.01 (m, 6H, $3CH_2$), 4.10 (s, 6H, $3CH_2$), 4.47 (m, 3H, 3CH), 6.31 (br, 3H, NH), 7.05 (d, *J* = 7.6 Hz, 3H, C-5H in pyridinone), 7.62 (d, *J* = 7.6 Hz, 3H, C-6H in pyridinone), 7.78 (m, 4H, Ar), 8.09 (br, 5H, 5NH). ^{13}C NMR δ: 13.2, 16.3, 17.4, 19.4, 22.5 25.6, 27.4, 27.8, 30.1, 32.0, 32.5, 34.4, 36.7, 45.9, 49.0, 54.1, 58.5, 64.8, 67.2, 114.5, 118.7, 128.0, 128.3, 128.6, 136.2, 156.5, 161.5, 161.8, 170.2, 172.4, 172.7, 204.5. MALDI-TOF-MS: 2263 ($[M+H]^+$), 2285 ($[M+Na]^+$). ESI-MS: *m*/*z* 755 ($[M+3H]^{3+}$). HRMS: calcd for 1/2($C_{109}H_{169}N_{15}O_{30}S_3$+2H), 1132.0661; found, 1132.0672 ($[M+2H]^{2+}$).

*Bis(6-(2-ethyl-3-hydroxy-4-oxopyridin-1(4H)-yl)hexyl)-4-(3-(3-(4-((5-amino-17,17-bis(3-((5-(2-amino-4-(methylthio)-butanamido)-4-oxopentanoyl)oxy)propyl)-6,9,12,19,23-pentaoxo-13-oxa-2-thia-7,18,22-triazapentacosan-25-yl)-carbamoyl)benzamido)propanamido)propanamido)-4-(3-((6-(2-ethyl-3-hydroxy-4-oxopyridin-1(4H)-yl)hexyl)oxy)-3-oxopropyl)heptanedioate (**30a**).* Analytical HPLC-DAD: t_R = 3.6 min, purity 90.1%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.04 (t, *J* = 7.4 Hz, 9H, $3CH_3$), 1.27 (m, 6H, $3CH_2$) 1.29 (m, 6H, $3CH_2$), 1.55 (m, 6H, $3CH_2$), 1.66 (m, 12H, $6CH_2$), 1.71−1.82 (m, 6H, $3CH_2$ and m, 6H, $3CH_2$), 2.02 (m, 6H, $3CH_2$), 2.13 (s, 9H, $3CH_3$), 2.15 (m, 6H, $3CH_2$), 2.27−2.34 (m, 8H, $4CH_2$), 2.56−2.69 (m, 12H, $6CH_2$), 2.77 (m, 6H, $3CH_2$), 2.82 (m, 6H, $3CH_2$), 3.45 (m, 6H, $3CH_2$), 3.63−3.76 (m, 8H, $4CH_2$), 3.79 (m, 6H, $3CH_2$), 3.98 (m, 6H, $3CH_2$), 4.02 (m, 6H, $3CH_2$), 4.25 (m, 3H, CH), 6.40 (br, 2H, NH), 7.13 (d, *J* = 7.6 Hz, 3H, C5-H in pyridinone), 7.41 (br, 2H, 2NH). 7.79 (m, 4H, Ar), 7.82 (d, *J* = 7.6 Hz, 3H, C6-H in pyridinone). ^{13}C NMR δ: 13.2, 16.3, 17.4, 19.4, 22.5 25.6, 27.4, 27.8, 30.1, 32.0, 32.8, 34.6, 36.7, 45.9, 49.2, 54.7, 57.9, 65.9, 67.8, 113.9, 117.8, 128.1, 128.3, 128.7, 136.4, 156.4, 161.3, 161.8, 170.5, 172.7, 172.9, 204.3. MALDI-TOF MS: *m*/*z* 2263 ($[M+H]^+$), 2285 ($[M+Na]^+$), 2301 ($[M+K]^+$). ES-MS: *m*/*z* 1132 ($[M+2H]^{2+}$), 755 ($[M+3H]^{3+}$); HRMS: calcd for 1/2-($C_{109}H_{169}N_{15}O_{30}S_3$+2H), 1132.0661; found, 1132.0678 ($[M+2H]^{2+}$).

Biological Evaluation. *Cell Culture.* The human breast adenocarcinoma cell lines MCF-7 (obtained from the European Collection of Authenticated Cell Cultures, ECACC, Porton Down, UK) and MCF-7R (donated by Professor John Masters, University College London) were employed for this study. Both cell lines were cultured in DMEM-F12 containing L-glutamine (20 μM) and phenol red, under aseptic conditions in a class II laminar flow cabinet. The media was supplemented with 10% fetal calf serum, which was standardized to give an iron concentration between 450 and 600 μg/100 g in order to reach the physiological iron levels, and gentamycin (500 units/mL; Life Technologies). The human carcinoma KB cell line (obtained from ECACC) was cultured in Eagle's minimum essential medium (EMEM) with 10% fetal calf serum (standardized to give an iron concentration between 450 and 600 μg/100 g), L-glutamine 2% (200 μM), and streptomycin solution 2% (200 U mL^{-1}, 200 μg mL^{-1}). The cells were grown as monolayers in sterile, vented-capped, angle-necked cell culture flasks (Corning) and maintained at 37 °C in a humidified 5% CO_2 incubator (IR Autoflow Water-Jacketed Incubator; Jencons Nuaire) until confluent.

Fluorescence Kinetics. Cells were seeded into gamma-sterilized 96-well plates (Orange Scientific, Triple Red Laboratory Technologies) at a density of approximately 5 × 10^4 cells per well for 48 h. After removal of the culture medium, the wells were washed with PBS. The cells were incubated with freshly prepared solutions of ALA and dendritic ALA-HPO conjugates in the presence of serum-free medium. Each plate contained control wells (cells without added conjugate for determination of the background reading) and reference wells (cells incubated with the equivalent ALA concentrations). For conjugate incubation, serum-free medium was used since serum is known to cause release of PpIX from cells, thus resulting in loss of the fluorescence signal.[19]

The fluorescence signal from each well was measured with a Perkin-Elmer LS 50B fluorescence spectrometer coupled to an automated plate reader (Perkin-Elmer, Beaconsfield, UK) using 410 nm excitation and 635 nm emission wavelengths with slit widths set to 10 nm and an internal 530 nm long-pass filter used on the emission side; spectral scans were recorded between 600 and 750 nm to check for the presence of any porphyrins other than PpIX. The mean fluorescence was calculated after subtraction of the control values.

Photodynamic Treatment. Cells were prepared in 96-well plates at a density of approximately 5 × 10^4 cells per well. Following incubation for 48 h, the cells were washed with PBS. Each well plate contained three control wells without dendrimer, and 0.1 mL of dendrimer at several concentrations (2−10 μM) in triplicate designated wells. The plates were incubated for 4 h and then irradiated with a fluence of 2.5 J/cm^2 using a LumiSource lamp (PCI Biotech) which emits a uniform field of low-power blue light over an area of 14 cm × 32 cm. Peak output is around 420 nm, which overlaps well with the porphyrin Soret band. Following irradiation, the medium was replaced with 0.1 mL of RPMI containing 10% FCS, and cells were incubated for a further 24 h. Cell cytotoxicity was determined using the MTT assay: cells were incubated with medium containing 3-(4,5-dimethylthiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (1 mg/mL dissolved in full RPMI-1640 medium) for 2 h. The insoluble end product (formazan derivatives) was dissolved in 0.1 mL of DMSO after the medium was removed. UV absorption was quantified at 570 nm using a 96-well plate reader (MR 700 Dynatech). The mean cell survival was calculated for each prodrug at every concentration tested and expressed as a percentage of control cell survival values. For determination of "dark" toxicity, well plates were prepared in the same manner as above without irradiation. The cells were kept in dark at 37 °C for 24 h prior to the MTT assay.

Cellular Uptake Mechanism. Experiments were conducted using low concentrations of ALA and dendritic ALA-HPO exposed to KB, MCF-7, and MCF-7R cells for short periods of time to assess their cellular penetration ability and their metabolism over time. Cells were seeded in 96-well plates (5 ×

DOI: 10.1021/acs.bioconjchem.8b00574
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10^5) as described above for 48 h. Later cells were washed with PBS and incubated with 300 μM concentration of ALA or 60 μM dendritic ALA-HPO concentrations. Cells were seeded in 96-well plates for temporary exposure to the investigated compounds for periods of 15, 30, 45, and 60 min. The compounds were removed after the temporary exposure, and the cells were washed three times with PBS and incubated for a further 4 and 24 h.

The possibility of the involvement of endocytosis in the cellular uptake mechanism of dendritic ALA-HPOs was investigated by employing direct reading of fluorescence intensity measurements for examination of the effect of macropinocytosis or endocytosis inhibitors (colchicine 100 μM and 5-(*N*-ethyl-*N*-isopropyl)amirolide (EIPA, 100 μM)) on ALA and ALA-HPO uptake. Cells (5×10^4) were pre-incubated with fresh medium without FCS or PR containing the inhibitors for 1 h at 37 °C. The inhibitors were removed, and cells were washed three times with cold PBS. Cells then were incubated with 100 μM ALA or dendritic ALA for a further 4 h. PpIX fluorescence measurements were obtained for the cells after 4 h incubation with ALA or ALA-HPO in the presence and absence of the inhibitors.

Statistical Analysis. The results are displayed graphically or tabulated with error bars representing the standard deviations of quadruplicate measurements. Differences are considered to be significant using the unpaired *t* test with $P < 0.05$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00574.

PpIX production in MCF-7 and MCF-7R cells were treated with ALA or ALA-HPO dendrimer **30a**; dark toxicity after incubation with ALA and dendritic ALA-HPO in KB, MCF-7, and MCF-7R for 24 h (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank EPSRC (EP/G005087; EP/G004986; EP/G004382), Science Technology Department of Zhejiang Province (No. 2013C24006), and Natural Science Foundation of Zhejiang Province (LY12B02014) for financial support.

■ ABBREVIATIONS USED

ALA, 5-aminolevulinic acid; Cbz-ALA, *N*-benzyloxycarbonyl aminolevulinic acid; DCC, *N,N*-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole hydrate; DMAP, 4-dimethylaminopyridine; DCM, dichloromethane; TFA, trifluoroacetic acid; DMF, dimethylformamide; Bmim, 1-butyl-3-methylimidazolium bromide; DMSO, dimethyl sulfoxide; HPO, hydroxypyridinone; Pd-C, palladium on activated carbon; HPLC, high-performance liquid chromatography; DIPEA, diisopropylethylamine; PpIX, protoporphyrin IX; PDT, photodynamic therapy; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amirolide

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DOI: 10.1021/acs.bioconjchem.8b00574
Bioconjugate Chem. 2018, 29, 3411−3428