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RESEARCH ARTICLE

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Synthesis of carboxy-polyethylene glycol-amine (CA (PEG)$_n$) and [1-^{14}C]-CA (PEG)$_n$ via oxa-Michael addition of amino-polyethylene glycols to propiolates vs to acrylates

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Abstract

Synthesis of carboxy-polyethylene glycol-amine (CA (PEG)$_n$) via oxa-Michael addition of amino-polyethylene glycols to either acrylates or propiolates was investigated. Compared with the oxa-Michael addition to acrylates, the corresponding addition to propiolates was found to proceed under mild reaction conditions and afford the adducts in high yields from a broad scope of substrates. A two-step efficient and convenient synthesis of benzyl [1-^{14}C]-propiolate from $^{14}CO_2$ was therefore developed and utilized as a common synthon to afford practical and high yielding access to [1-^{14}C]-CA (PEG)$_n$.

KEYWORDS

[1-^{14}C]-propiolate, bifunctional crosslinker, carbon-14, carboxy-polyethylene glycol-amine, oxa-Michael addition, radiolabeling

1 | INTRODUCTION

Carboxy-polyethylene glycol-amine (CA (PEG)$_n$, **1**) compounds are pegylated amino acids with a short and well-defined PEG spacer between the carboxy and amino termini (Figure 1). CA (PEG)$_n$ has been used as bifunctional crosslinkers in constructing bioconjugates for targeted drug delivery,[1a, b] targeted radionuclide therapy and imaging,[2] targeted protein degradation,[3] and bivalent modulator design.[4a, b] Radiolabeled bioconjugates provide an understanding of their pharmacokinetics and biodistribution during therapeutic development; however, unlike small molecules, introducing radiolabels into biologics remains a formidable challenge. In the case of conjugates with noncleavable linkers, an alternative approach can be applied whereby placing the radiolabel into the linker portion thus reduces the synthetic complexity and in turn affords a common fragment that can be applied to a broader range of bioconjugates. Therefore, the development of a carbon-14 label synthesis of CA (PEG)$_n$ would provide a common carbon-14 labeling approach to the bioconjugates bearing the CA (PEG)$_n$ as noncleavable linker. Herein, we report a practical, efficient, and general synthesis of CA (PEG)$_n$ via the robust oxa-Michael addition of amino-polyethylene glycols to benzyl propiolate. Then, a two-step convenient and efficient synthesis of benzyl [1-^{14}C]-propiolate from readily available $^{14}CO_2$ was developed and utilized as a common synthon to provide a practical access to [1-^{14}C]-CA (PEG)$_n$.

2 | RESULTS AND DISCUSSION

CA (PEG)$_n$ has been synthesized via oxa-Michael addition of polyethylene glycols to acrylates to install the terminal propionate functional group (Scheme 1). Addition of polyethylene glycol **2** to acrylate **4** provides adduct **5**. [5, 6a–g] The *O*-terminus of **5** is then converted to an amino group giving intermediate **6**, a protected CA

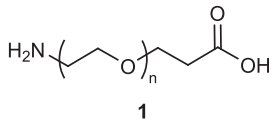


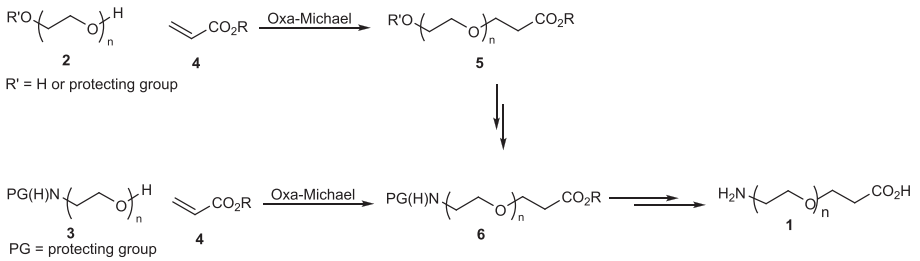
FIGURE 1 CA $(PEG)_n$

$(PEG)_n$. Alternatively, addition of amino-polyethylene glycol **3** to acrylate **4** gives directly the intermediate **6**. [7a–e] Deprotection and hydrolysis of the intermediate **6** complete the synthesis of CA $(PEG)_n$ (**1**). The synthesis starting from **2** has been more extensively explored in the literature; however, the synthesis starting from **3** involves fewer reaction steps after the oxa-Michael addition. With both commercially available **2** and **3**, the synthesis starting from **3** is shorter and thus **3** was selected as the starting material for the label synthesis of CA $(PEG)_n$.

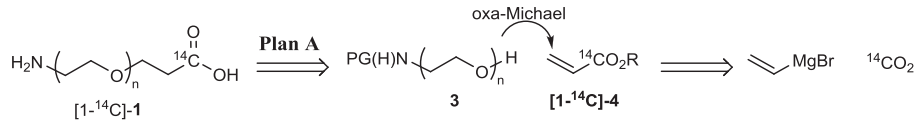
Exploiting the known preparative methods for CA $(PEG)_n$, the initial carbon-14 label synthesis plan **A** introduces the ^{14}C isotope into the carboxylate carbon to make [1-^{14}C]-CA $(PEG)_n$ (Scheme 2). It could be prepared via the oxa-Michael addition of amino-polyethylene glycol **3** to acrylate [1-^{14}C]-**4**. In turn, [1-^{14}C]-acrylate **4** can be prepared via the addition of vinyl magnesium bromide to $^{14}CO_2$.[8]

To be efficient and practical, synthesis plan **A** demands that the acrylate [1-^{14}C]-**4** be the limiting reagent in the oxa-Michael addition and the yield of the adduct be good. It came to our attention that the reported oxa-Michael addition of amino-polyethylene glycol **3** to acrylate **4** has been achieved using an excess (greater than or equal to 1.5 Eq) of acrylate.[7a–e] Furthermore, the yield of the adduct in the limited number of reported cases was generally moderate,[7a–d] yet low yields and failed reactions have also been reported.[9e] In response, the use of phase transfer catalysts has addressed such issues, resulting in excellent yield; however, large excesses (up to 5 Eq) of acrylate were normally used.[7e] Therefore, we investigated the effect of the oxa-Michael addition of amino-polyethylene glycol **3** to acrylate **4**, with **4** as the limiting reagent, on the yield of the adduct.

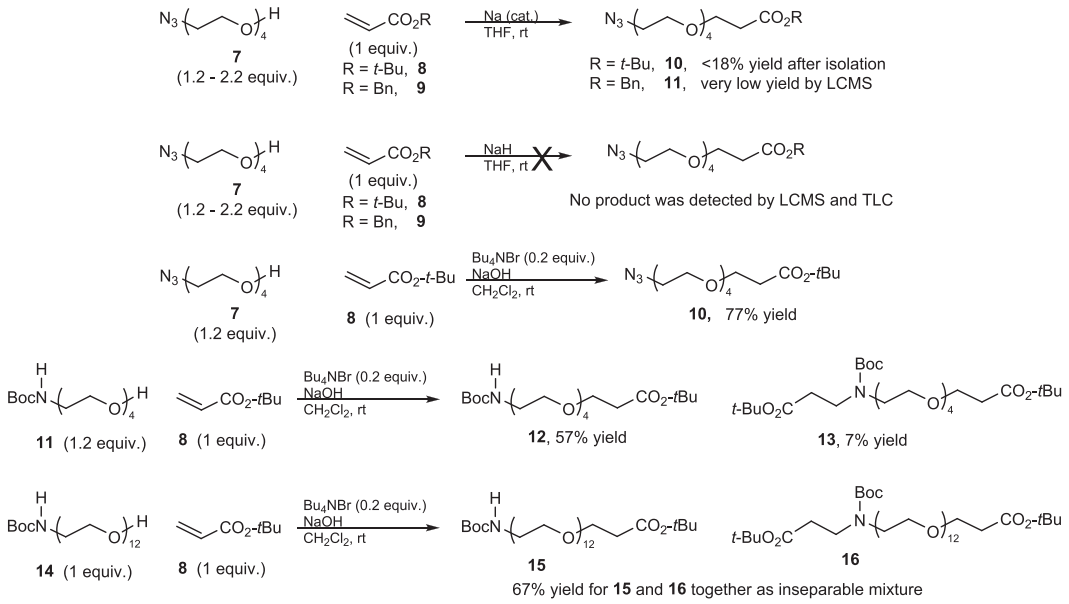
As shown in Scheme 3, treatment of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-ol (**7**, 1.2 Eq) with a catalytic amount of sodium in THF, followed by 1 Eq of either *t*-butyl acrylate **8** or benzyl acrylate **9**, gave the adduct **10** or **11** but only in very low yield because of an incomplete reaction and formation of several uncharacterized byproducts. When sodium hydride was used in place of sodium for the oxa-Michael addition, no desired adduct was detected by liquid chromatography–mass spectrometry (LCMS) or thin-layer chromatography (TLC) analysis of the reaction aliquot. Low yielding or failed oxa-Michael additions to acrylate have been reported for similar amino-polyethylene glycols bearing an amino terminus, such as BocNH—, Bn_2N—, or phthalimide.[9e] In order to improve the yield of the oxa-Michael addition of **7** to **8** or **9**, phase transfer catalysts were explored.[9e] Remarkably, treatment of **7** (1.2 Eq) with **8** (1 Eq) in the presence of tetrabutyl ammonium bromide and sodium hydroxide provided the adduct **11** in 77% yield. Encouraged by this result, amino-polyethylene glycol **11** with a deprotonatable BocNH— terminus was subjected to the same phase transfer reaction conditions. Other than the desired adduct **12**, which was isolated in a moderate 57%



SCHEME 1 Preparative methods for CA $(PEG)_n$ in literature



SCHEME 2 Labeling synthesis plan **A** for [1-^{14}C]-CA $(PEG)_n$

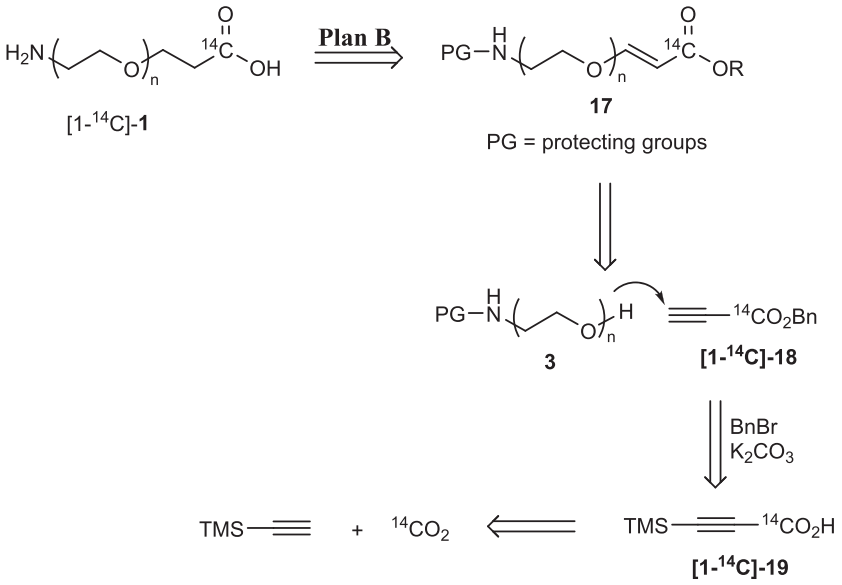


SCHEME 3 Oxa-Michael addition of amino-polyethylene glycols to acrylates under various conditions

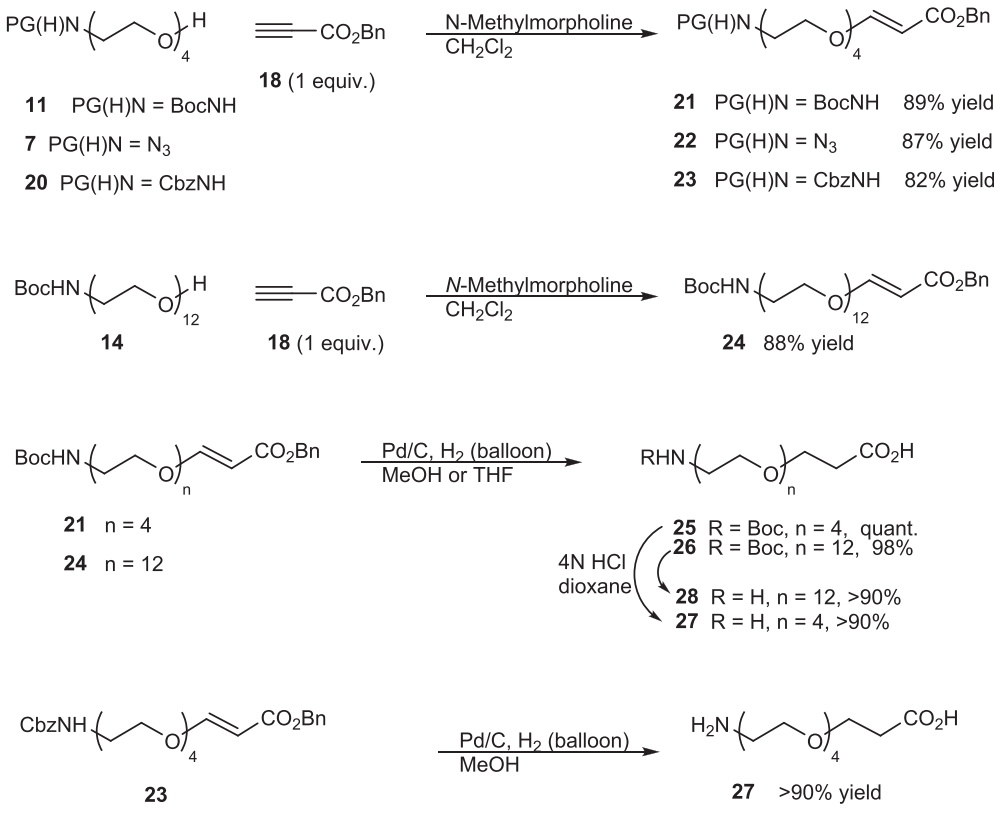
yield, the bis-Michael addition product **13** was also received in 7% yield, which made the chromatographic purification of **12** difficult. In a similar manner, addition of **14** to **8**, adduct **15** was formed in moderate yield but isolated as an inseparable mixture with the bis-Michael addition byproduct **16**. The formation of the bis-Michael addition byproduct not only posed a purification challenge but also caused another issue. For example, 7% yield of the byproduct **13** translates to a 14% loss of the precious [1-^{14}C]-acrylate and also results in an increase of radioactive waste. Even though the oxa-Michael addition of **7** to **8** under phase transfer conditions gave adduct **10** in a satisfactory yield, the moderate yields and challenging product purification of the similar reactions with substrates **11** and **14** render the oxa-Michael addition to acrylate nonoptimal to be used in labeling synthesis of [1-^{14}C]-CA $(PEG)_n$. Therefore, a high-yielding oxa-Michael addition with a broad substrate scope was sought.

It was recognized that a propiolate such as [1-^{14}C]-**18** could replace acrylate serving as an alternative oxa-Michael acceptor for the synthesis of [1-^{14}C]-CA $(PEG)_n$ as shown in synthesis plan **B** (Scheme 4). Specifically, 1-[^{14}C]-CA $(PEG)_n$ could be prepared via hydrogenation of a pegylated alkoxylacrylate **17**, which in turn could be synthesized by oxa-Michael addition of amino-polyethylene glycol **3** to benzyl [1-^{14}C]-propiolate (**18**). Propiolate [1-^{14}C]-**18** could be synthesized from trimethylsilyl acetylene and $^{14}CO_2$ via intermediate **19**.

While the oxa-Michael addition of an alcohol to propiolate has been reported,[9-11] to the best of our knowledge, it has not been applied to the synthesis of CA $(PEG)_n$-type molecules. To our delight, treatment of compound **11** (1.0 to 1.5 Eq) with 1 Eq of benzyl propiolate (**18**) in the presence of *N*-methylmorpholine in CH_2Cl_2 at room temperature gave adduct **21** smoothly in 89% isolated yield (Scheme 5).Dibenzyl 3,3'-oxy-diacrylate, formed due to the presence of trace amount of water, was also isolated in 4% yield from the reaction and it was easily removed chromatographically.[12] It should be noted that the problematic bis-Michael addition byproduct, observed in the addition to acrylate, was not detected in the addition to propiolate with substrates **11** bearing a deprotonatable BocNH— terminus. Similarly, adducts **22** and **23** were isolated in good yields from the reaction of benzyl propiolate with azido-polyethylene glycol **7** and Cbz-amino-polyethylene glycol **20**, respectively. Noteworthy is that Boc-protected amino PEGylated alcohol **14**, with a much longer PEG of 12 ethylene glycol units, effectively added to benzyl propiolate producing the adduct **24** in 88% yield. The oxa-Michael addition of amino-polyethylene glycols to propiolate therefore appears to be a mild, robust, and efficient reaction whose generality extends across a range of amino-polyethylene



SCHEME 4 Labeling synthesis plan **B** for 1-[^{14}C]-CA $(PEG)_n$



SCHEME 5 Oxa-Michael addition of amino-polyethylene glycols to propiolate and synthesis of CA $(PEG)_n$

glycols bearing different nitrogen protecting groups and of varied PEG chain length.

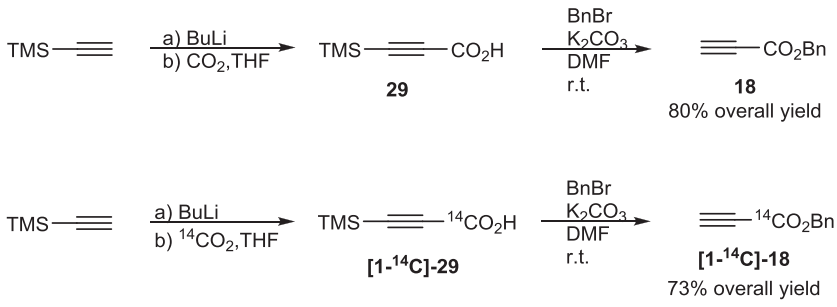
Adducts **21**, **23**, and **24** were then converted to the target products CA $(PEG)_n$ in good to excellent yields as follows. Treatment of compound **21** with Pd/C catalyst under H_2 atmosphere (1 atm) provided Boc-protected CA $(PEG)_4$ **25** quantitatively (Scheme 5). After removal of Boc, CA $(PEG)_4$ **27** was isolated in greater than 90% yield as the hydrochloride salt. In the same way, compound **24** was converted smoothly to **28** in good yield. Treatment of

compound **23** with Pd/C catalyst under H_2 atmosphere saturated the C?C, cleaved the benzyl ester, and removed the Cbz protecting group to afford the product **27** in one step as the neutral zwitterion in good yield.

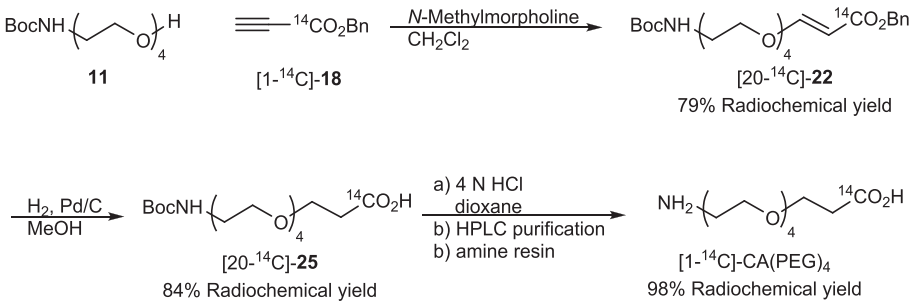
We then turned our attention to developing a convenient and cost-effective synthesis of benzyl [1-^{14}C]-propiolate ([1-^{14}C]-**18**). Benzyl propiolate has been synthesized from propiolic acid via coupling with benzyl alcohol or via alkylation with benzyl halide.[13,14] Because of the increased risk of personnel and environmental contamination when handling semi-volatile and difficult-to-vacuum-transfer [1-^{14}C]-propiolic acid (b.p. 62°C/20 mmHg),[15] we investigated the less volatile and protected form, 3-(trialkylsilyl)propiolic acid, as our labeling intermediate. Therefore, we conceived a two-step synthesis of benzyl propiolate (**18**) using trimethylsilylacetylene and $^{14}CO_2$ as the starting materials (Scheme 6). From the many available silyl protecting groups for alkynes, we selected the smallest trimethylsilyl (TMS) for two reasons: First, the boiling point of the intermediate 3-(trimethysilyl)propiolic acid (**29**) at 108 to 110°C/10 mmHg[16] is high enough to be cautiously but safely processed in flasks without the need for vacuum transfer. Secondly, we envisioned that removal of the trimethysilyl protecting group and the carboxylic acid alkylation of intermediate **29** could be achieved simultaneously in one step under basic conditions, thus directly converting intermediate **29** to the product benzyl propiolate (**18**).

Using a modified literature procedure,[17] trapping of the in situ generated trimethylsilylacetylene anion (1.1 Eq) with 1 Eq of CO_2 produced 3-(trimethylsilyl)propiolic acid (**29**) cleanly by ^{1}H NMR analysis of the crude reaction product (Scheme 6). Without further purification, the trimethylsilylpropiolic acid was immediately alkylated with benzyl bromide in the presence of K_2CO_3 in DMF. Both the carboxylic acid alkylation and the removal of TMS group proceeded smoothly under the conditions giving benzyl propiolate (**18**) in 80% overall yield. Following the two-step procedure and using a $^{14}CO_2$ manifold, [1-^{14}C]-benzyl propiolate (**18**) was obtained in 73% overall radiochemical yield, which was dissolved and stored in CH_2Cl_2. No significant decomposition of [1-^{14}C]-benzyl propiolate (**18**) was detected by high-performance liquid chromatography (HPLC) after storage of it at −20°C for 3 weeks.

Utilizing the developed route to CA $(PEG)_n$ and the prepared benzyl [1-^{14}C]-propiolate ([1-^{14}C]-**18**), the synthesis of [1-^{14}C]-CA $(PEG)_4$ was conducted as shown in Scheme 7. Boc, instead of Cbz or azide, was chosen as the amino protecting group for the starting material **11**, because it provided the opportunity to purify intermediate [20-^{14}C]-**25** just prior to the final deprotection, which facilitated the purification of the final compound [1-^{14}C]-



SCHEME 6 Two-step synthesis of benzyl propiolate **18** or [1-^{14}C]-**18** from CO_2 or $^{14}CO_2$



SCHEME 7 Synthesis of 1-[^{14}C]-CA $(PEG)_4$

CA $(PEG)_4$ ([1-^{14}C]-**27**). The labeled compound [1-^{14}C]-**27** was converted to its zwitterionic form by treatment of the TFA salt received after HPLC purification with an amine resin. The overall radiochemical yield for the three steps illustrated in Scheme 7 was 65%.

In conclusion, a practical, high yielding, and general synthesis of CA $(PEG)_n$ via a robust oxa-Michael addition of amino-polyethylene glycols to benzyl propiolate has been developed. A two-step efficient and convenient preparation of the benzyl [1-^{14}C]-propiolate from the readily available $^{14}CO_2$ was also achieved and utilized as a common synthon to provide a practical access to [1-^{14}C]-CA $(PEG)_n$. In light of the frequent application of CA $(PEG)_n$ as a linker in bioconjugate pharmaceutical research and development, the synthesis of [1-^{14}C]-CA $(PEG)_n$ provides an efficient and expeditious way to radiolabel such bioconjugates for pharmacokinetics studies.

3 | EXPERIMENTAL

All reagents were purchased from commercial sources and used without further purification. The PEGylated alcohols were purchased from BroadPharm, San Diego, CA, USA. ^{1}H NMR spectra were recorded on a Bruker AC-400 (400 MHz) spectrometer using tetramethylsilane as an internal standard. Electrospray mass spectra (MS-ES) were recorded on a Hewlett-Packard 59987A spectrometer. Radiochemical yield was determined by liquid scintillation counting on a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter. Specific activity was determined by LC/MS on the basis of isotopic peak intensity distribution. Analytic HPLC method for compounds [1-^{14}C]-**18**, [20-^{14}C]-**22**, and [20-^{14}C]-**25**: Agilent 1200 series HPLC with a Waters XBridge C18 5 μm 150X4.6 mm column; mobile phase: A: 0.05% TFA in water, B: 0.05% TFA in CH_3CN; gradient 10%B to 95%B in 0 to 20 minutes, isocratic at 95%B in 20 to 25 minutes; flow rate 1 mL/min, injection volume 5 μL. Radioactive detection was accomplished using the above HPLC system and a Perkin Elmer radioactive flow detector Radiomatic 625TR equipped with a 0.5-mL flow cell and Ultima Flo M cocktail at a flow rate of 2 mL/min. Analytic HPLC method for compound [15-^{14}C]-**27**: Agilent 1200 series HPLC with a Waters XBridge C18 5 μm 250X4.6 mm column; mobile phase: A: 0.1% TFA in water, B: 0.1% TFA in CH_3CN; gradient 5%B to 10%B in 0 to 15 minutes, gradient 10% B to 95%B in 15 to 20 minutes, isocratic at 95%B in 20 to 28 minutes, gradient 95%B to 5%B in 28 to 28.1 minutes, isocratic at 5%B in 28.1 to 30 minutes, flow rate 1 mL/min, injection volume 5 μL; column temperature 30°C. Radioactive detection was accomplished using the same above radioactive flow detector with the same settings.

3.1 | *tert*-Butyl 1-azido-3,6,9,12-tetraoxapentadecan-15-oate (10)

To a solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy) ethan-1-ol (**7**, 132 mg, 0.6 mmol) and *tert*-butyl acrylate (**8**, 64 mg, 0.5 mmol) in CH_2Cl_2 (1.5 mL) at room temperature were added Bu_4NBr (32 mg, 0.1 mmol) and NaOH (50 wt% in H_2O, 0.13 mL). The mixture was stirred at room temperature for 3 hours. Water (2 mL) was added, and the mixture was extracted with CH_2Cl_2 (1 mL × 3). The combined extracts were washed with H_2O (2 mL) and dried over $MgSO_4$. Filtration and concentration of the filtrate gave 171.4 mg of the crude product as colorless oil. Flash chromatography on silica gel (gradient 0% to 100% EtOAc in heptane) gave **10** (132.9 mg, 77%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.71 (t, J = 8.0 Hz, 2H), 3.65-3.69 (m, 10H), 3.60-3.65 (m, 4H), 3.39 (t, J = 4.0 Hz, 2H), 2.50 (t, J = 8.0 Hz, 2H), 1.45 (s, 9H); MS (ES, m/z) 370 ($M + Na^+$).

3.2 | *tert*-Butyl 2,2-dimethyl-4-oxo-3,8,11,14,17-pentaoxa-5-azaicosan-20-oate (12) and di-*tert*-butyl 4-(tert-butoxycarbonyl)-7,10,13,16-tetraoxa-4-azanonadecanedioate (13)

To a solution of *tert*-butyl (2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)carbamate (**11**, 176 mg, 0.6 mmol) and *tert*-butyl acrylate (**8**, 64 mg, 0.5 mmol) in CH_2Cl_2 (1.5 mL) at room temperature were added Bu_4NBr (32 mg, 0.1 mmol) and NaOH (50 wt% in H_2O, 0.13 mL). The mixture was stirred at room temperature for 3 hours. Water (2 mL) was added, and the mixture was extracted with CH_2Cl_2 (2 mL × 4). The combined extracts were washed with H_2O (2 mL) and dried over $MgSO_4$. Filtration and concentration of the filtrate gave the crude product as colorless oil. Flash chromatography on silica gel (gradient 0% to 100% EtOAc in heptane) gave **13** (21 mg, 7.5%) as colorless film stuck on flask wall, followed by **12** (121 mg, 57%) as colorless oil. Compound **13**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.71 (t, J = 8.0 Hz, 2H), 3.31-3.67 (m, 18H), 2.51 (t, J = 8.0 Hz, 4H), 1.45 (s, 27H); MS (ES, m/z) 572 ($M + Na^+$). Compound **12**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.06 (brs, 1H), 3.71 (t, J = 8.0 Hz, 2H), 3.60-3.67 (m, 12H), 3.54 (t, J = 4.0 Hz, 2H), 3.32 (q, J = 4.0 Hz, 2H), 2.51 (t, J = 8.0 Hz, 2H), 1.45 (s, 18H); MS (ES, m/z) 422 ($M + Na^+$).

3.3 | *tert*-Butyl 2,2-dimethyl-4-oxo-3,8,11,14,17,20,23,26,29,32,35,38,41-tridecaoxa-5-azatetratetracontan-44-oate (15) and di-*tert*-butyl 4-(tert-butoxycarbonyl)-7,10,13,16,19,22,25,28,31,34,37,40-dodecaoxa-4-azatritetracontanedioate (16)

To a solution of *tert*-butyl (35-hydroxy-3,-6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl)carbamate (**14**, 229 mg, 0.36 mmol) and *tert*-butyl acrylate (46 mg, 0.36 mmol) in CH_2Cl_2 (1.5 mL) at room temperature were added Bu_4NBr (23 mg, 0.07 mmol) and NaOH (50 wt% in H_2O, 0.1 mL). The mixture was stirred at room temperature for 3 hours. Water (2 mL) was added, and the mixture was extracted with CH_2Cl_2 (2 mL × 4). The combined extracts were washed with H_2O (2 mL) and dried over $MgSO_4$. Filtration and concentration of the filtrate gave the crude product as colorless oil. Flash chromatography on silica gel (gradient EtOAc to 10% MeOH in CH_2Cl_2) gave 184 mg of colorless oil, which was a mixture of **15** and **16**. Compound **15**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.07 (brs, 1H), 3.71 (t, J = 8.0 Hz, 2H), 3.59-3.68 (m, 44H), 3.54 (t, J = 4.0 Hz, 2H), 3.32 (q, J = 4.0 Hz, 2H), 2.50 (t, J = 8.0 Hz, 2H), 1.45 (s, 18H); MS (ES, m/z) 796 (M + Na^+). Compound **16**: MS (ES, m/z) 925 (M + Na^+).

3.4 | Benzyl (*E*)-2,2-dimethyl-4-oxo-3,8,11,14,17-pentaoxa-5-azaicos-18-en-20-oate (21)

To a stirred solution of benzyl propiolate (**18**, 400 mg, 2.5 mmol) in CH_2Cl_2 (15 mL) were added *tert*-butyl (2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)carbamate (**11**, 1.099 g, 3.75 mmol) and *N*-methylmorpholine (379 mg, 3.75 mmol). The solution was stirred at room temperature for 18 hours and then concentrated. Flash chromatography on silica gel (gradient 0% to 100% EtOAc in heptane) gave **21** (1.007 g, 89%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.66 (d, J = 12.6 Hz, 1H), 7.29-7.38 (m, 5H), 5.27 (d, J = 12.6 Hz, 1H), 5.16 (s, 2H), 5.01 (brs, 1H, NH), 3.98-4.04 (m, 2H), 3.74-3.79 (m, 2H), 3.58-3.71 (m, 8 H), 3.53 (t, J = 5.1 Hz, 2 H), 3.27-3.35 (m, 2H), 1.44 (s, 9H); MS (ES, m/z) 476 (M + Na^+).

3.5 | Benzyl (*E*)-1-azido-3,6,9,12-tetraoxapentadec-13-en-15-oate (22)

To a mixture of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy) ethan-1-ol (**7**, 66 mg, 0.3 mmol) and *N*-methylmorpholine (30 mg, 0.3 mmol) in CH_2Cl_2 (0.5 mL) was added a solution of benzyl propiolate (**18**, 40 mg, 0.25 mmol) in CH_2Cl_2 (0.5 mL). The solution was stirred at room temperature for 18 hours and then concentrated. Flash chromatography on silica gel (gradient 0% to 100% EtOAc in heptane) gave **22** (82.6 mg, 87%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.66 (d, J = 12.6 Hz, 1H), 7.28-7.38 (m, 5H), 5.27 (d, J = 12.6 Hz, 1H), 5.16 (s, 2H), 3.98-4.02 (m, 2H), 3.74-3.78 (m, 2H), 3.63-3.69 (m, 10 H), 3.38 (t, J = 5.1 Hz, 2 H); MS (ES, m/z) 402 (M + Na^+).

3.6 | Benzyl (*E*)-3-oxo-1-phenyl-2,7,10,13,16-pentaoxa-4-azanonadec-17-en-19-oate (23)

To a stirred solution of benzyl propiolate (**18**, 30 mg, 0.19 mmol) in CH_2Cl_2 (1 mL) at room temperature were added benzyl (2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy) ethyl)carbamate (**20**, 93 mg, 0.29 mmol) and *N*-methylmorpholine (29 mg, 0.29 mmol). The solution was stirred at room temperature for 3.5 hours and then concentrated. Flash chromatography on silica gel (gradient 0% to 100% EtOAc in heptane) gave **23** (75.7 mg, 82%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.63 (d, J = 12.1 Hz, 1H), 7.26-7.38 (m, 10H), 5.31 (brs, 1H, NH), 5.25 (d, J = 12.1 Hz, 1H), 5.15 (s, 2H), 5.09 (s, 2H), 3.91-3.98 (m, 2H), 3.67-3.74 (m, 2H), 3.58-3.66 (m, 8 H), 3.55 (t, J = 5.1 Hz, 2 H), 3.39 (q, J = 5.2 Hz, 2H); MS (ES, m/z) 488 (M + H^+).

3.7 | Benzyl (*E*)-2,2-dimethyl-4-oxo-3,8,11,14,17,20,23,26,29,32,35,38,41-tridecaoxa-5-azatetratetracont-42-en-44-oate (24)

To a stirred solution of benzyl propiolate (**18**, 52 mg, 0.33 mmol) in CH_2Cl_2 (1 mL) at room temperature were added *tert*-butyl (35-hydroxy-3,-6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl)carbamate (**14**, 210 mg, 0.33 mmol) and *N*-methylmorpholine (49 mg, 0.49 mmol). The solution was stirred at room temperature for 5 hours and then concentrated. Flash chromatography (gradient CH_2Cl_2 to 10% MeOH in CH_2Cl_2) gave **24** (229.7 mg, 88%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.66 (d, J = 12.6 Hz, 1H), 7.28-7.39 (m, 5H), 5.27 (d, J = 12.6 Hz, 1H), 5.16 (s, 2H), 5.06 (brs, 1H, NH), 3.97-4.02 (m, 2H), 3.73-3.78 (m, 2H), 3.57-3.73 (m, 40 H), 3.54 (t, J = 5.1 Hz, 2 H), 3.26-3.35 (m, 2H); MS (ES, m/z) 828 (M + Na^+).

3.8 | 2,2-Dimethyl-4-oxo-3,8,11,14,17-pentaoxa-5-azaicosan-20-oic acid (25)

A mixture of compound **21** (985 mg, 2.17 mmol) and Pd/C (10 wt%, 231 mg, 0.22 mmol) in 30 mL of THF was

stirred under H_2 (balloon) for 24 hours. The mixture was filtered through Celite, and the filtrate was concentrated to give a colorless oil. Flash chromatography on silica gel (10% MeOH in CH_2Cl_2) gave **25** (790.5 mg, quantitative) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.78 (t, J = 6.1 Hz, 2H), 3.60-3.74 (m, 12H), 3.50-3.60 (m, 2H), 3.23-3.37 (m, 2H), 2.61 (t, J = 5.6 Hz, 2 H), 1.45 (s, 9H); MS (ES, m/z) 388 (M + Na^+).

3.9 | 2,2-Dimethyl-4-oxo-3,8,11,14,17,20,23,26,29,32,35,38,41-tridecaoxa-5-azatetratetracontan-44-oic acid (26)

A mixture of compound **24** (197 mg, 0.24 mmol) and Pd/C (10 wt%, 52 mg, 0.05 mmol) in 5 mL of THF was stirred under H_2 (balloon) for 18 hours. The mixture was filtered through Celite, and the filtrate was concentrated to give compound **26** (172 mg, 98%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.76 (t, J = 6.2, 2H), 3.59-3.71 (m, 44H), 3.54 (t, J = 4.9 Hz, 2H), 3.25-3.36 (m, 2H), 2.60 (t, J = 6.2 Hz, 2H), 1.45 (s, 9H); MS (ES, m/z) 718 (M + H^+).

3.10 | 1-Amino-3,6,9,12-tetraoxapentadecan-15-oic acid (27) through intermediate 25

A solution of compound **25** (767 mg, 2.10 mmol) in 4N HCl in dioxane (20 mL) was stirred at room temperature for 2 hours. After concentration on rotary evaporator, the slightly yellowish sticky residue was purified by flash chromatography on C18 pre-packed column eluted with 4% ACN in H_2O to give compound **27** (848 mg) as colorless oil. The product was in HCl salt form and might contain water. ^{1}H NMR (CD_3OD, 400 MHz) δ 3.71-3.76 (m, 4H), 3.61-3.71 (m, 12 H), 3.13 (t, J = 5.1, 2H), 2.57 (t, J = 6.0 Hz, 2H); MS (ES, m/z) 266 (M + H^+).

3.11 | 1-Amino-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxanonatriacontan-39-oic acid hydrochloride (28)

A solution of compound **26** (52 mg, 0.07 mmol) in 4N HCl in dioxane (1 mL) was stirred at room temperature for 2 hours. After concentration under high vacuum, it gave compound **28** (47 mg, quantitative) as yellowish oil: ^{1}H NMR (D_2O, 400 MHz) δ 3.48-3.75 (m, 48H), 3.06-3.12 (m, 2 H), 2.54 (t, J = 5.5 Hz, 2H); MS (ES, m/z) 618 (M + H^+).

3.12 | 1-Amino-3,6,9,12-tetraoxapentadecan-15-oic acid (27) through intermediate 23

A mixture of **23** (69 mg, 0.14 mmol) and Pd/C (10 wt%, 30 mg, 0.03 mmol) in 5 mL of MeOH was stirred under H_2 (balloon) for 18 hours. The mixture was filtered through Celite, and the filtrate was concentrated to give compound **27** (36.6 mg, 98%) in the neutral form as a colorless oil. ^{1}H NMR (CD_3OD, 400 MHz) δ 3.74-3.79 (m, 2H), 3.58-3.73 (m, 14H), 3.10-3.15 (m, 2H), 2.41 (t, J = 5.5 Hz, 2H); MS (ES, m/z) 266 (M + H^+).

3.13 | Benzyl propiolate (18)

Trimethylsilylacetylene (166 mg, 1.69 mmol) in THF (4.5 mL) was cooled to −78°C under N_2. BuLi (2.5M in hexanes, 0.65 mL, 1.62 mmol) was added dropwise. After addition, the solution was stirred at −78°C for 40 minutes. CO_2 (33 mL at room temperature, measured with a syringe, 1.47 mmol) in a sealed flask was cooled in a liquid N_2 bath. The above solution of trimethylsilylacetylene anion was added using a syringe. The reaction mixture was then put in a dry-ice/acetone bath and stirred at −78°C for 1 hour. The reaction was quenched by the addition of 0.2 mL of MeOH, followed by a mixture of 1.8 mL of 1N HCl and 3.6 mL of H_2O. Et_2O (4 mL) was added and the stirred mixture was allowed to warm to room temperature. The two layers were separated, and the aqueous layer (pH = 7) was extracted with Et_2O (5 mL × 2). The combined organic layers were washed with H_2O (5 mL) and dried (Na_2SO_4). Filtration and concentration of the filtrate at room temperature provided the crude product of 3-(trimethylsilyl)propiolic acid (**29**) as slightly yellowish oil (204 mg, 98%).

The above 3-(trimethylsilyl)propiolic acid (**29**) crude product was dissolved in DMF. The solution was cooled to 0°C and treated with K_2CO_3 (198 mg, 1.43 mmol). The mixture was stirred at 0°C for 5 minutes. Benzyl bromide (245 mg, 1.43 mmol) was added. After stirring at room temperature for 6 hours, water (5 mL) was added and the mixture was extracted with Et_2O (5 mL × 3). The combined extracts were washed with H_2O (5 mL × 2) and brine (5 mL) sequentially. After drying over Na_2SO_4 and filtration, the filtrate was concentrated to give a brown oil. Chromatography on silica gel (gradient 10% to 30% EtOAc in heptane) gave **18** (190 mg, 81% overall yield for the two steps) as colorless oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34-7.41 (m, 5H), 5.22 (s, 2H), 2.89 (s, 1H). No molecular ion was detected by MS.

3.14 | 3-(Trimethylsilyl)propiolic-1-^{14}C acid ([1-^{14}C]-29)

Trimethylsilylacetylene (74 mg, 0.75 mmol) in THF (2 mL) was cooled to −78°C under N_2. BuLi (2.5M in hexane, 0.28 mL, 0.7 mmol) was added dropwise. After addition, the solution was stirred at −78°C for 40 minutes and then cooled in a liquid nitrogen bath. $^{14}CO_2$ gas (0.5 mmol, SA = 59.4 mCi/mmol, 29.7 mCi) was measured from a $^{14}CO_2$ manifold at 507 mbar pressure and vacuum transferred to the reaction mixture cooled in liquid nitrogen bath. Use caution when handling radioactive and volatile $^{14}CO_2$. After vacuum transfer, the mixture was stirred at −78°C for 1 hour. The reaction was quenched by the addition of 0.1 mL of MeOH, followed by a mixture of 0.7 mL of 1N HCl and 2 mL of H_2O. Two milliliters of Et_2O was added, and the stirred mixture was allowed to warm to room temperature. Any volatile radioactivity was then carried by a stream of N_2 and trapped in a phenyl magnesium bromide trap. The organic layers were separated, and the aqueous layer was extracted with Et_2O (3 mL × 3). The combined organic layers were washed with H_2O and dried over Na_2SO_4. Filtration and concentration of the filtrate at room temperature provided 148 mg of a slightly yellowish oil. The crude product was used immediately in the next step without further purification. MS (ES, m/z) 145 ($M + H^+$), 57 mCi/mmol by MS analysis.

3.15 | [1-^{14}C]-Benzyl propiolate ([1-^{14}C]-18)

To the above crude product in 0.5 mL DMF at 0°C was added K_2CO_3 (69 mg, 0.5 mmol). The mixture was stirred at 0°C for 5 minutes. Then, benzyl bromide (85 mg, 0.5 mmol) was added. After stirring at room temperature for 6 hours, water (5 mL) was added and the mixture was extracted with Et_2O (5 mL × 3). The combined extracts were washed sequentially with H_2O (4 mL) and brine (3 mL). After drying over Na_2SO_4 and filtration, the filtrate was concentrated to give 149 mg of light-yellow liquid. Flash chromatography on silica gel (gradient 10% to 30% EtOAc in heptane) gave compound [1-^{14}C]-**18** (21.7 mCi, 57 mCi/mmol based on the used starting material [1-^{14}C]-**29**, 73% radiochemical yield over two steps). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34-7.41 (m, 5H), 5.22 (s, 2H), 2.89 (s, 1 H). No molecular ion was detected by MS.

3.16 | Benzyl (*E*)-2,2-dimethyl-4-oxo-3,8,11,14,17-pentaoxa-5-azaicos-18-en-20-[^{14}C]-oate ([20-^{14}C]-22)

To a stirred solution of [1-^{14}C]-benzyl propiolate ([1-^{14}C]-**18**, 8.0 mCi, 57 mCi/mmol, 0.14 mmol) in CH_2Cl_2 (0.82 mL) were added *tert*-butyl (2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)carbamate (**11**, 62 mg, 0.21 mmol) and *N*-methylmorpholine (21 mg, 0.21 mmol). The solution was stirred at room temperature for 18 hours. Flash chromatography on silica gel (heptane to EtOAc) gave [20-^{14}C]-**22** (6.3 mCi, 57 mCi/mmol by MS analysis, 79% radiochemical yield, 98% radiochemical purity by HPLC) as colorless oil. MS (ES, m/z) 478 ($M + Na^+$).

3.17 | 2,2-Dimethyl-4-oxo-3,8,11,14,17-pentaoxa-5-azaicosan-20-[^{14}C]-oic acid ([20-^{14}C]-25)

A mixture of compound [20-^{14}C]-**22** (6.3 mCi, 57 mCi/mmol, 0.11 mmol) and Pd/C (10 wt%, 27 mg, 0.03 mmol) in 5 mL of MeOH was stirred under H_2 (balloon) for 18 hours. The mixture was filtered through Celite and the filtrate was concentrated to give a colorless oil. Flash chromatography on silica gel (10% MeOH in CH_2Cl_2) gave [20-^{14}C]-**25** (5.3 mCi, 57 mCi/mmol by MS analysis, 84% radiochemical yield, 99% radiochemical purity by HPLC) as colorless oil. MS (ES, m/z) 390 ($M + Na^+$).

3.18 | [1-^{14}C]-CA $(PEG)_4$

A solution of compound [20-^{14}C]-**25** (5.3 mCi, 57 mCi/mmol, 0.09 mmol) in 4N HCl in dioxane (3 mL) was stirred at room temperature for 2 hours. After concentration on rotary evaporator, the slightly yellowish sticky residue was purified by HPLC equipped with a C18 column using ACN/H_2O containing 0.1% TFA as mobile phase. After HPLC purification, the product fractions were combined and concentrated. The residue was dissolved in H_2O and treated with Amerlyst 21 free amine resin. Filtration and concentration of the filtrate gave [1-^{14}C]-CA $(PEG)_4$ (5.2 mCi, 57 mCi/mmol by MS analysis, 98% radiochemical yield, 99% radiochemical purity by HPLC). MS (ES, m/z) 268 ($M + H^+$).

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