A New Aliphatic Amino Prodrug System for the Delivery of Small Molecules and Proteins Utilizing Novel PEG Derivatives

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A new amino PEG prodrug system, based entirely on aliphatic structures, has been designed using ester derivatives easily synthesized from *N*-modified bis-*N*-2-hydroxyethylglycinamides. Hydrolysis of the various promoiety bonds, in vivo, regenerated amine in a predictable manner. Thus, a novel new methodology for controlled release of amino-containing drugs, peptides, and proteins has been accomplished. This work demonstrates the usefulness of a PEG prodrug strategy that results in solubilization of insoluble amino-containing drugs and provides prodrugs with relatively long circulating half-lives. It can be appreciated that this novel system should also be applicable for nonpolymer-containing prodrugs as well.

Introduction

In 1997, a report appeared by Suggs[1] that described the rapid hydrolysis of C-terminal amides of glycine at 25 °C and pH 7 when the *N*-terminus is *N*-hydroxyethylated. The $t_{1/2}$ of bis-*N*-2-hydroxyethylglycinamide (**1**) in 0.1 M phosphate buffer was determined to be 3 h, which is exceptionally better than the 7 yr reported for the unsubstituted glycinamide.[1] Amide derivatives with a single hydroxyethyl group underwent hydrolysis more slowly than would be predicted by statistical factors alone, suggesting that both hydroxyethyl groups assist in the hydrolysis of the amide and that **1** is a simple serine protease mimic cyclizing to a morpholinolactone (**2**, detected by NMR analysis), which is then rapidly hydrolyzed by water to the acid **4** (bicine or bicin), as shown in Scheme 1.

Of late, there has been a great deal of interest in prodrug strategies, especially those that can solubilize and release amine-containing bioactive molecules and macromolecules.[2−10] From inspection of **1**, it was immediately apparent that a PEG prodrug system for amino-containing drugs could be designed that would be based on this novel substituted bicin amide hydrolysis and would of necessity be entirely based on aliphatic chemistries. In the case of polypeptides, this would be especially desirable. Since very few amino prodrug strategies[2−5] are practical, and few of these are aliphatic-based,[8−10] successful development of a bicin-based prodrug would be of major importance to the field of drug delivery, especially if PEG could be incorporated into this system to add solubility and increased circulating half-life ($t_{1/2}$) to the prodrug. The tactical approach used to increase the release rates of the promoiety was either to introduce an α-heteroatom on the acid portion of the ester or to provide anchimeric assistance when an even more rapid $t_{1/2}$ was desired. Lengthening the $t_{1/2}$ was accomplished by the introduction of steric hindrance and/or by adding α-substituents.[2,3] Not only did this basic strategy work but it led to the synthesis of novel branched PEG prodrug derivatives of both proteins and small organic molecules. As the PEG prodrugs of small molecules underwent self-destruction, a clear demonstration of the effect of PEG molecular weight (MW) on in vivo activity was again observed.[11]

Chemistry. The design of PEG prodrugs of the basic bicin molecule was founded on ester chemistry. Thus, cleavage of the esters produces the sidearm alcohols, which in turn eliminate amine. The first ester synthesis incorporates an α-*N* (Scheme 2) and broadly illustrates the sequence of reactions used to prepare the series of esters designed as bicin prodrugs. First the *tert*-butyl ester of bicin, **7**, was prepared by a displacement reaction using *tert*-butyl bromoacetate. Condensation of **7** with Boc-glycine in the presence of EDC/DMAP consistently produced mixtures of mono- and disubstituted esters that were not easily separated. A likely explanation for this incomplete reaction is that steric hindrance is introduced after the first acylation and retards introduction of the second ester group. Scandium triflate and DMAP have been found to accelerate the acylation of hindered alcohols,[12] and it was also found to be an effective reagent for the diesterification of **7**: using the same reaction conditions in the presence of scandium triflate resulted in high yields of the diester **9**. Subsequently, the BOC-glycine ester, compound **9**, was deblocked using mild conditions (15% TFA for 2 h) to give the amine salt **10** without affecting the *tert*-butyl ester. Condensation of **10** with mPEG-succinimidyl carbonate (**11**, SC−PEG, MW 12 000)[13] gave the branched PEG bicin derivative, **12**, which was hydrolyzed using more concentrated TFA (33%) in DCM for a 12 h period, producing the branched PEG acid **13**. Activation of **13** by EDC/NHS gave the NHS-activated ester **14**, while 2-mercaptothiazoline (2-MT) provided the desired linker **15**. In a similar fashion, the more hindered alanine-derived linker **22** was also prepared (Scheme 3). Next, placement of an α-alkoxy moiety was accomplished using the known heterobifunctional Boc-amino acid **26**,[10] as shown in Scheme 4. Anchimeric assistance was introduced using linker **36**, prepared from the mono-Boc diamine **32**[14] and diglycolic anhydride **33** in the sequence shown in Scheme 5, and resulted in the most rapidly hydrolyzed ester prepared in this study (Tables

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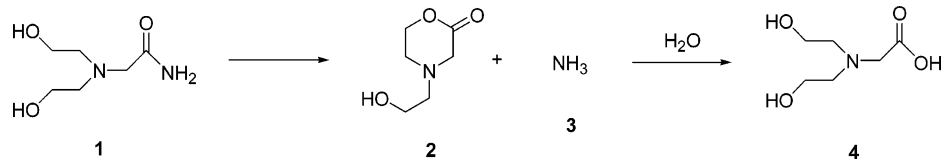
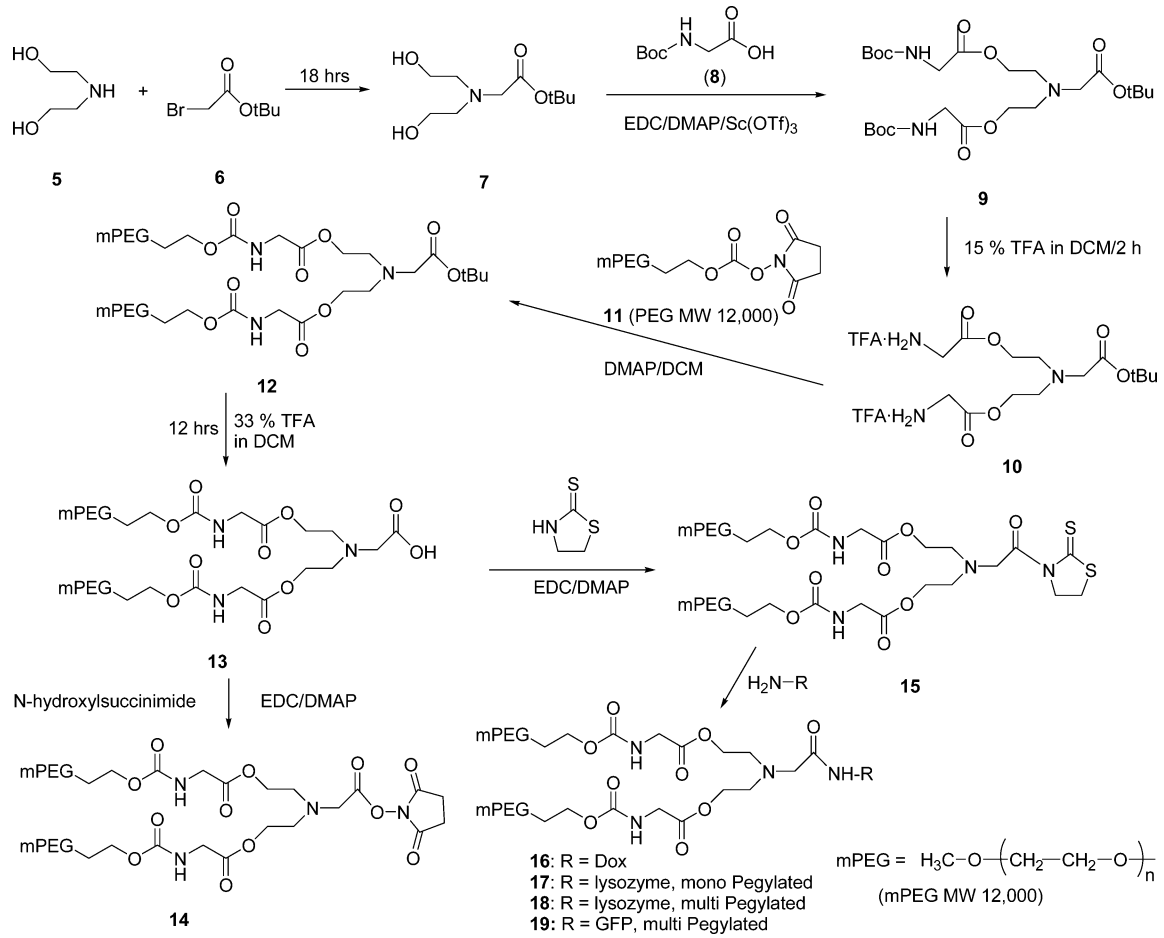
Scheme 1

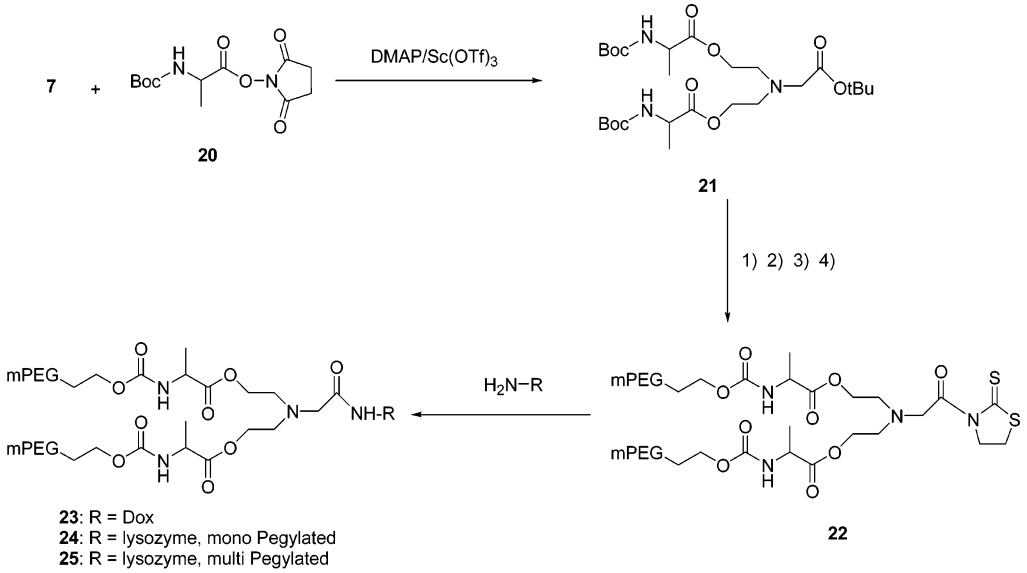


Scheme 2



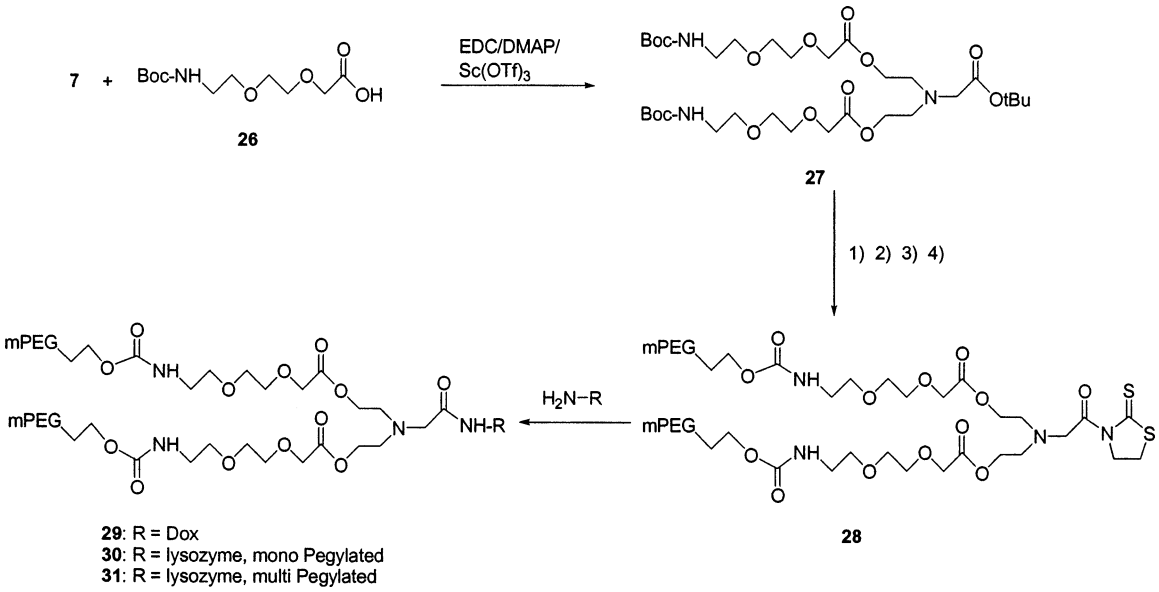
1, 3, and 4). The PEG linkers were easily conjugated to small molecules such as doxorubicin (Dox, Table 1) and the protein model lysozyme. Linker **15** was also used for conjugation with green fluorescent protein (GFP) in

Scheme 3



1) 15 % TFA/DCM/2 h; 2) **11** / DMAP/DCM; 3) 33 % TFA /DCM/3 h; 4) 2-MT/EDC/DMAP

Scheme 4



order to easily obtain pharmacokinetic (PK) data.[15] Linker **14**, an NHS ester, can also be used to accomplish the conjugation.

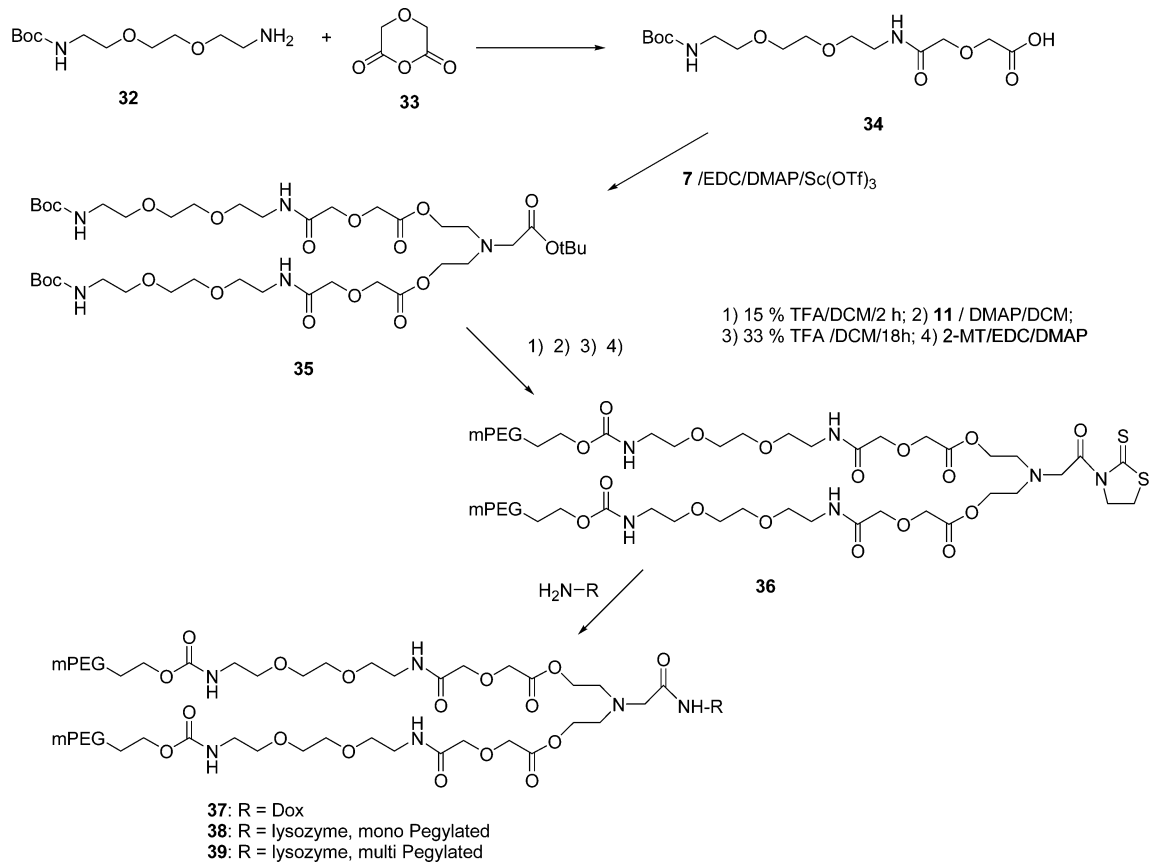
Discussion

The goal of this study was to develop prodrugs of amino-containing compounds based on the finding that *N*-bis-hydroxyethyl derivatives of glycinamide can hy-

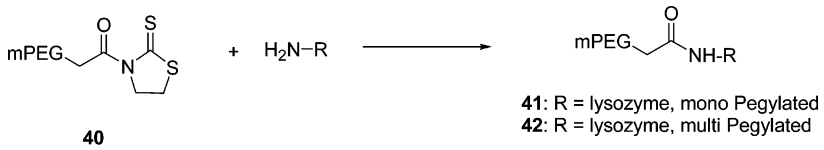
Table 1. Properties of rU-PEG−Dox Conjugates

compound	$t_{1/2}$ (h) (rat plasma)	MW	IC_{50} (nM) (P388)
doxorubicin HCl		580	17
16	10	24 855	428
23	20	24 883	316
29	5.5	25 031	1100
37	3.2	25 233	455

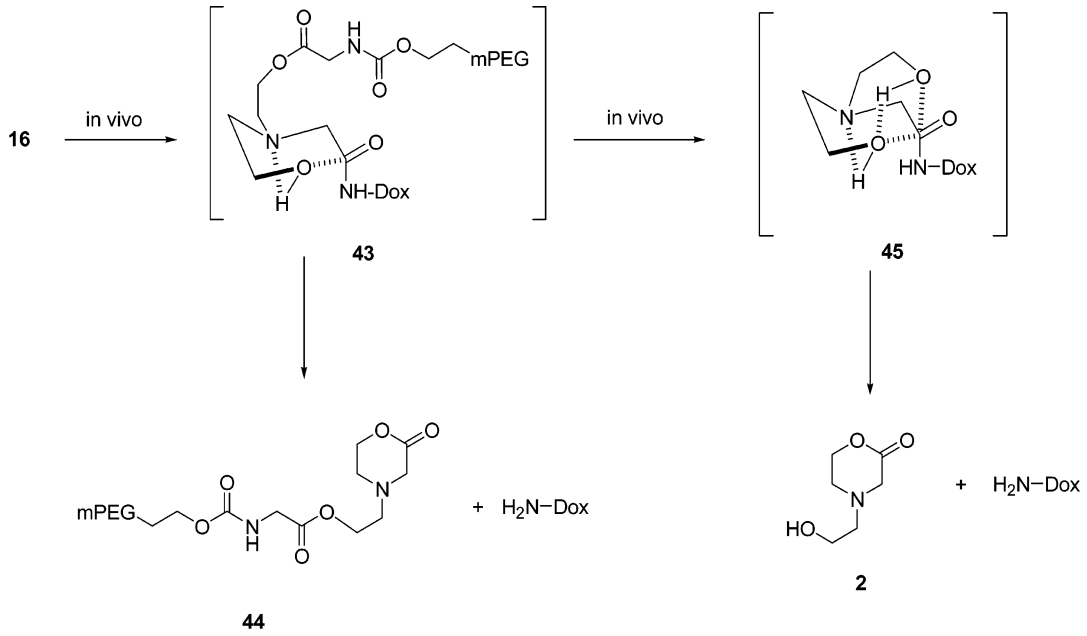
Scheme 5



Scheme 6



Scheme 7



drolyze relatively rapidly. In fact, the structure of the PEG prodrugs designed in this work present novel examples of branched PEG, previously described as "umbrella" or U-PEG.[16] The original design incorporates *permanent* PEG strands (through amide or carbamate linkages) that increase the MW and $t_{1/2}$ of protein conjugates; *note that there can be no release of native drug from this type of system*. However, in the case of PEG-substituted bicins, attachment utilizes ester bonds and thus the conjugated PEG strands are labile. This can be confirmed by examination of Table 1, which reports on the properties of the doxorubicin (Dox) substituted bicin derivatives, **16**, **23**, **29**, and **37**. The rates of plasma hydrolysis varied between about 3 and 20 h, releasing native Dox, with the IC_{50} values shown in Table 1, thus demonstrating that not only were these esters prodrugs but the rates of decomposition could be directed by the chemical modifications outlined in the Chemistry section. However, diester **16** is a double prodrug, and a proposed mechanism for the release of Dox (which is representative for any amine) is shown in Scheme 7. The first step in the breakdown must, of necessity, be the hydrolysis of one ester bond, producing **43**. Although monoester **43** is capable of amide-assisted hydrolysis to free amine (Dox) and compound **44**, this reaction is probably on the order of 30−100 times slower than the doubly assisted breakdown of **45** to Dox and **2**, which arises after a second ester hydrolysis.[1] Although the IC_{50} values indicated that the prodrugs were active in vitro (Table 1), unexpectedly, these Dox derivatives, all based on a 24 000 molecular weight (MW) U-PEG (two 12 000 strands), were found to be inactive in a M109 murine tumor model when compared to native Dox. An explanation for this unusual behavior is that in the first step of prodrug breakdown, ester hydrolysis, one PEG 12 000 MW strand is lost, leaving a prodrug (compound **43**, Scheme 6) that now can be seen to be a linear PEG prodrug with an MW of 12 000. It has been clearly demonstrated that low MW PEG (<20 000) attached to drugs (specifically paclitaxel) undergo rapid renal excretion and show no drug activity.[11] Therefore, it appears that the lack of in vivo activity of **16** is due to the generation of lower MW polymeric species arising from the initial self-destruction of the diester. Circumvention of this obstacle for the use of small organic medicinal agents by employing higher MW PEG is currently under investigation and will be the subject of a future publication.

Application of the U-PEG prodrug [hereafter referred to as rU-PEG to correspond to the term rPEG that has previously been applied to monomethoxy poly(ethylene glycol) (mPEG) terminated prodrugs[17]] technology to proteins was entirely satisfactory with MW 24 000, since the loss of one PEG strand still leaves a high MW protein conjugate that is capable of continued circulation with limited renal elimination. The use of lysozyme and green fluorescent protein (GFP) as protein models has already been described in detail for rPEG linkers with aromatic rings[2,3] and will not be further detailed. Using the series of rU-PEGs, in a manner similar to that described previously for the rPEG systems,[15] it was found that release of native proteins from their conjugates took place in a comparable fashion. In mono rU-PEG-lysozyme, one of seven amino groups (six lysines and one N-terminus) was randomly modified with the PEG linker. In multi rU-PEG−lysozyme, as many as four amino groups were modified. The analysis of the conjugates was accomplished using MALDI-TOF, and

Table 2. Pharmacokinetic Parameters of GFP and rU-PEG−GFP in Rats

	$t_{1/2}$ (h)	AUC (h μg/mL)	CL (mL/h/kg)	MRT (h)	% correln (obs vs est)
GFP	0.15 ± 0.013	18.86 ± 0.26	265.24 ± 7.20	0.22 ± 0.04	93
19	23.46 ± 2.61	878.56 ± 4.69	5.69 ± 0.03	33.85 ± 3.76	96

Table 3. Release Rates of Mono-rU-PEGylated Lysozyme in Rat Plasma and in Buffer[a]

		PBS			pH 8.5,
compd	plasma	4 °C	25 °C	37 °C	37 °C
17	20	>168	158	48	5.2
30	14	>168	101	33	3.2
38	5	>144	36	12	1.3
41	N/R	N/R	N/R	N/R	N/R

[a] The data are expressed as $t_{1/2}$ in hours. The release in plasma was monitored for 3 days, the release in pH 8.5 buffer for 5 days, and the release in PBS for 7 days. PBS contains 138 mM NaCl, 2.7 mM KCl, and 10 mM phosphate, pH 7.4. The release of lysozyme was detected by regeneration of lysozyme activity and confirmed by gel electrophoresis. N/R stands for "no release".

Table 4. Release Rates of Multi-rU-PEGylated Lysozyme in Rat Plasma and in Buffer[a]

		PBS			pH 8.5,
compd	plasma	4 °C	25 °C	37 °C	37 °C
18	36	N/R	>168	114	17
31	29	N/R	>168	84	16
39	6	>144	48	15	2

[a] The data are expressed as $t_{1/2}$ in hours. The release in plasma was monitored for 3 days, the release in pH 8.5 buffer for 5 days, and the release in PBS for 7 days. PBS contains 138 mM NaCl, 2.7 mM KCl, and 10 mM phosphate, pH 7.4. The release of lysozyme was detected by regeneration of lysozyme activity and confirmed by gel electrophoresis. N/R stands for "no release".

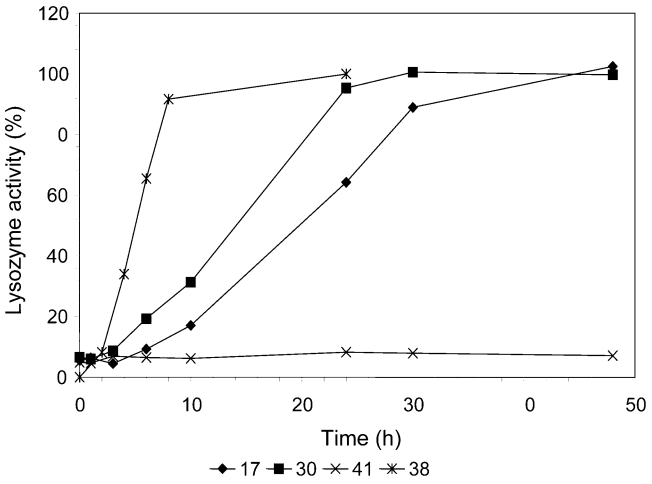


Figure 1. Regeneration of lysozyme activity from mono-PEG−lysozyme conjugates in rat plasma. Mono-PEG−lysozyme conjugates (0.15 mg/mL) were incubated in rat plasma at 37 °C under N_2. At the time indicated, an aliquot was withdrawn and analyzed for lysozyme activity.

lysozyme activity was determined using *Micrococcus lysodeikticus*.[15] The data for lysozyme is presented in Tables 3 and 4, and graphically in Figures 1−4. The activity of permanently PEGylated lysozyme compounds **41** and **42** (Scheme 6), as expected, showed no activity (Figures 1−4). rU-PEG−lysozyme activities are given in Figures 1−4 and are zero at time zero. The results clearly demonstrate once again that monosubstituted PEG conjugates release protein at a more rapid rate than multisubstituted conjugates. An SDS−PAGE analysis, performed according to published procedures,[15] of lysozyme release from the monoconjugates **17**, **30**, and **38** supports this finding. Both **17** and **30** have comparable half-lives. The multisubstituted derivatives **18** and **31** are, as expected, longer lived and hydrolyze at only about half the rate of that of the single strand conjugate. The anchimerically derived linker, **36**, yields the rapidly cleaving lysozyme conjugates **38** and **39** with a $t_{1/2}$ of 5 and 6 h, respectively (Table 3). Generally, rapid removal, in vitro, of both the mono- and multi-promoieties can be accomplished by raising the pH to 8.5 during a processing step (Figure 3) if in vivo decomposition is not desired. All conjugates were found to have great stability in PBS buffer at 4 °C and reasonable stability at 25 °C (Tables 3 and 4), thus allowing long-term storage and prior dissolution of lyophilized PEG−protein conjugates. A comparison of hydrolysis rates of **17** and **30** (Table 3)

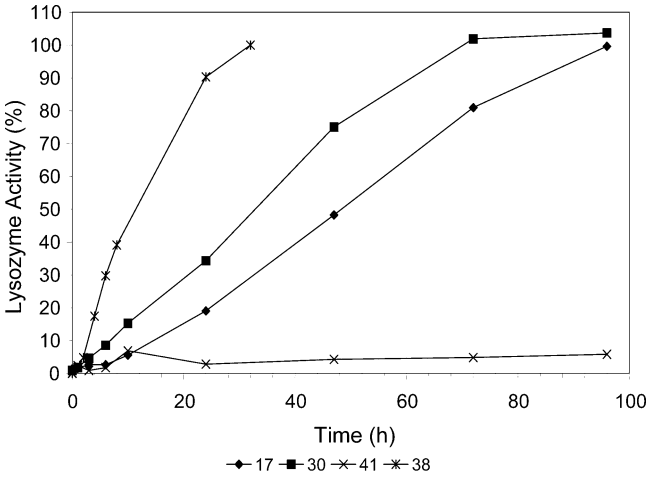


Figure 2. Release of lysozyme from mono-PEG−lysozyme conjugates at pH 7.4. Mono-PEG−lysozyme conjugates (0.15 mg/mL) were incubated in PBS buffer, pH 7.4, at 37 °C. At the time indicated, an aliquot was withdrawn and analyzed for lysozyme activity

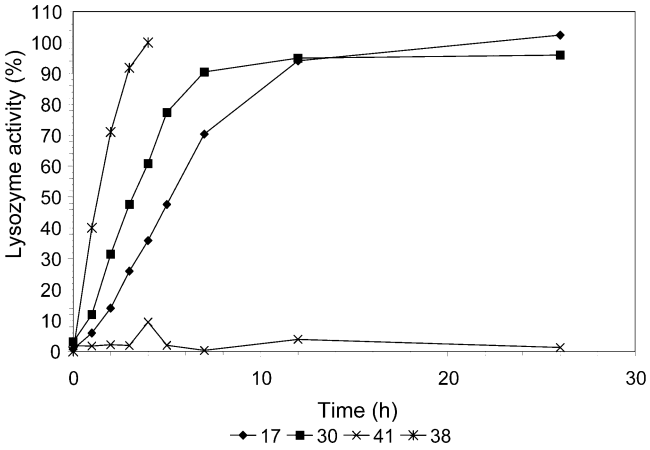


Figure 3. Release of lysozyme from mono-PEG−lysozyme conjugates at pH 8.5. Mono-PEG−lysozyme conjugates (0.15 mg/mL) were incubated in 50 mM Tris buffer, pH 8.5, at 37 °C. At the time indicated, an aliquot was withdrawn and analyzed for lysozyme activity.

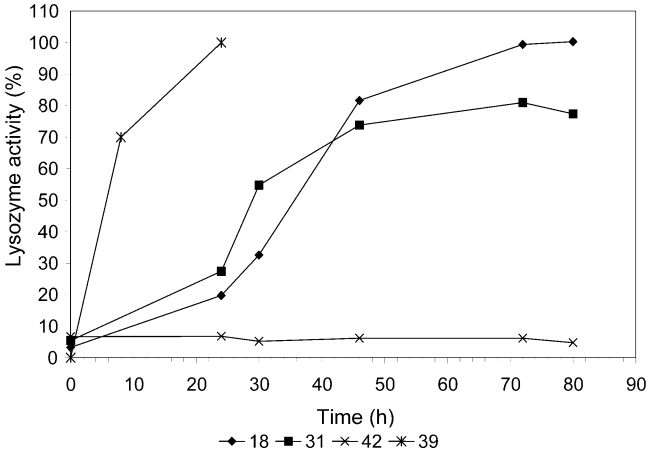


Figure 4. Regeneration of lysozyme activity from multi-PEG−lysozyme conjugates in rat plasma. Multi-PEG−lysozyme conjugates (0.15 mg/mL) were incubated in rat plasma at 37 °C under N_2. At the time indicated, an aliquot was withdrawn and analyzed for lysozyme activity.

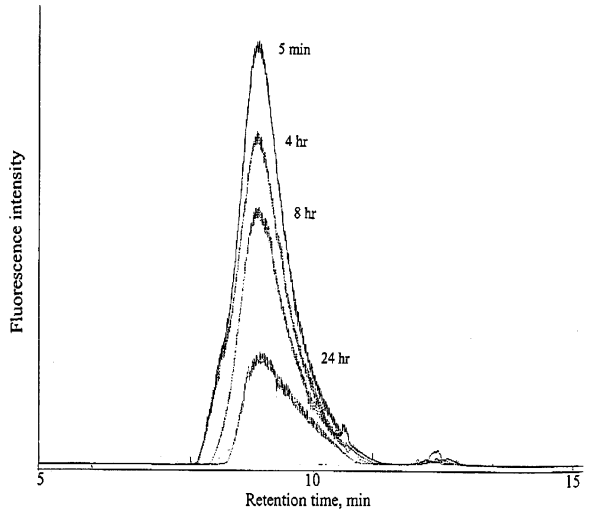


Figure 5. Release profile of GFP from PEG−GFP (**19**) conjugate in rats. Plasma samples collected at various time points were fractionated on SEC. The fluorescence intensity of GFP and PEG−GFP conjugates eluted from the column was measured using an on-line fluorescence detector. The tailing of the peak toward the small molecule side indicates the release process of GFP from **19**.

with **18** and **31** in PBS buffer at 37 °C (Table 4) clearly illustrates that enzymatic activity in plasma is responsible for the more rapid half-lives observed. A PK study employing GFP, easily followed by fluorescent detection, was performed in a similar fashion as previously reported.[15] The rate of release of GFP from the rU-PEG−GFP conjugate **19** is given in Figure 5, where it can be appreciated that the half-life of **19** is considerably greater than the native protein.

Conclusion

The design of long-circulating, aliphatic PEG prodrugs of amino-containing organic compounds and proteins was accomplished by carrying out esterification of the hydroxyl groups of bis-*N*-2-hydroxyethylglycinamide derivatives and takes the form of branched, or U-PEG, structures. By the use of α-substituents on the ester moiety, the half-life of the prodrugs can be adjusted for various applications. In the case of small organic molecules, the stepwise release of PEG from the higher MWrU-PEG leads initially to a monosubstituted linear PEG-ylated species and the MW of the new PEG conjugate becomes the determining factor in the circulating half-life of the PEG prodrug and thus of the overall in vivo biological activity. When the α- or ϵ-amino groups of proteins are modified with the rU-PEG−bicin, the PEG MW of the resulting prodrug (proprotein) is not of primary importance, since the protein conjugate has a high enough total MW to prevent rapid clearance from the circulatory system. These aliphatic derived rU-PEG−protein conjugates are suitable for regenerating native protein in a controlled fashion and for possible site-directed applications as well.

Experimental Section

Chemistry. All reactions were run under an atmosphere of dry nitrogen or argon. Commercial reagents were used without further purification. All PEG compounds were dried under vacuum or by azeotropic distillation from toluene prior to use. NMR spectra were obtained using a Varian Mercury 300 NMR spectrometer and deuterated chloroform as the solvent, unless otherwise specified. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS).

HPLC Methods. The reaction mixtures and the purity of intermediates and final products were monitored by a Beckman Coulter System Gold HPLC instrument employing a ZOBAX 300 SB C-8 reversed phase column (150 × 4.6 mm) or a Phenomenex Jupiter 300A C18 reversed phase column (150 × 4.6 mm) with a multiwavelength UV detector, using a gradient of 30−90% of acetonitrile in 0.5% trifluoroacetic acid (TFA) at a flow rate of 1 mL/min.

Kinetic Studies in Rat Plasma. The PEG−bicin derivative, 15 mg, was dissolved in 1000 μL of a mixture of MeCN and MeOH (1:1, v/v). The solution (100 μL) was transferred to each vial (8 vials total, StepVial System II). Solvent was removed under reduced pressure and 100 μL of rat plasma was added to each vial. The vial was sealed and vortexed for 1 min, after which time the vial was incubated at 37 °C for 0, 0.5, 1, 2, 4, 6, and 20 h, respectively. To each vial was added again 400 μL of MeCN and MeOH (1:1, v/v), and the vial was vortexed for 1 min. The mixture was filtered through a 0.45 μm filter membrane, and 50 μL of the filtrate was injected directly into the HPLC system.

Compound 7. A solution of **5** (24.0 g, 0.228 mol) and **6** (12.0 g, 0.061 mol) in anhydrous methylene chloride (DCM, 400 mL) was stirred at room temperature for 18 h. The reaction mixture was washed with water (4 × 150 mL), and the organic layer dried over anhydrous sodium sulfate, followed by filtration and removal of the solvent in vacuo to yield **7** (6.1 g, 0.0279 mol, 46%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 172.1, 81.4, 59.5, 57.0, 56.3, 27.8; HRMS (ESI) *m/z* calcd for $C_{10}H_{27}NO_4$ 219.28, found 220.22 ($M + H^+$).

Compound 9. A solution of compound **7** (0.50 g, 2.28 mmol), *N*-Boc-glycine (4.26 g, 13.4 mmol), 4,4′-(dimethylamino)pyridine (DMAP, 2.18 g, 17.8 mmol), and scandium triflate (0.65 g, 1.32 mmol) in anhydrous DCM (25 mL) was cooled to −8 °C in an ice−salt bath for 30 min. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 3.0 g, 15.6 mmol) was added and the reaction mixture stirred at −8 °C for 30 min and then at room temperature for 12 h. The reaction mixture was filtered, the filtrate was washed with 0.1 N HCl (3 × 20 mL) and distilled water (20 mL) and dried ($MgSO_4$), and the solvent was evaporated in vacuo. The product was further purified by silica gel column chromatography to give **9** (1.40 g, 1.71 mmol, 75%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 172.4, 170.7, 145.5, 128.7, 128.0, 126.6, 81.1, 70.7, 63.1, 56.2, 52.7, 45.8, 28.0; HRMS (ESI) *m/z* calcd for $C_{24}H_{43}N_3O_{10}$ 533.60, found 534.30 ($M + H^+$).

Compound 10. Compound **9** (0.240 g, 0.292 mmol) was dissolved in 1% solution of trifluoroacetic acid (TFA) in DCM (10 mL) and stirred at room temperature for 30 min. The solvent was partially removed under reduced pressure and ethyl ether (50 mL) was added to precipitate the product. After decanting the ether, the residue was again dissolved in DCM and evaporated in vacuo to give **10** (0.148 g, 0.263 mmol, 90%): ^{13}C NMR (67.8 MHz, $CDCl_3/CD_3OD$) δ 167.9, 166.8, 83.4, 61.8, 55.1, 52.8, 39.8, 27.5; HRMS (ESI) *m/z* calcd for $C_{14}H_{27}N_3O_6$ 333.38, found 334.20 ($M + H^+$).

Compound 12. To a solution of monomethoxypoly (ethylene glycol) succinimidyl carbonate (**11**, SC−PEG, 12 kDa, 6.0 g, 0.49 mmol) and DMAP (0.12 g, 0.98 mmol) in anhydrous DCM (35 mL) was added dropwise the solution of compound **10** (0.17 g, 0.30 mmol) in DCM/dimethylformamide (DMF) (32 mL/3 mL) over a time period of 30 min. The resulting mixture was stirred at room temperature for another 12 h. The solvent was partially removed under reduced pressure, followed by precipitation of the PEG derivative with ethyl ether. Filtration gave the crude PEG product, which was crystallized from DCM/ethyl ether (24 mL/96 mL) and then from 2-propanol (IPA, 120 mL), respectively, to give **12** (5.6 g, 0.23 mmol, 76%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 170.2, 169.7, 156.1, 80.8, 63.1, 58.7, 58.5, 52.5, 42.2, 27.8.

Compound 13. PEG derivative **12** (5.6 g, 0.23 mmol) was dissolved in TFA/DCM (30 mL/60 mL) and stirred at room temperature for 12 h. The solvents were evaporated under reduced pressure. The residue was redissolved in 10 mL of DCM, precipitated with ethyl ether, filtered, and washed with several portions of ether to give **13** (5.1 g, 0.21 mmol, 91%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 171.3, 169.7, 156.2, 62.5, 58.7, 58.59, 52.8, 42.2.

Compound 14. To a solution of **13** (2.90 g, 0.12 mmol), DMAP (15 mg, 0.12 mmol), and *N*-hydroxysuccinimide (0.041 g, 0.36 mmol) in anhydrous DCM (60 mL) cooled to 0 °C were added DMAP (0.044 g, 0.36 mmol) and EDC (0.069 g, 0.36 mmol), and the reaction mixture was stirred overnight as the temperature gradually rose from 0 °C to room temperature. The solvent was subsequently removed in vacuo and the PEG derivative was precipitated with ethyl ether, filtered, and crystallized from IPA (150 mL) to yield **14** (2.55 g, 0.10 mmol, 87%): ^{13}C NMR (300 MHz, $CDCl_3$) δ 169.5, 168.7, 165.9, 156.0, 69.9−71.6 (PEG), 63.9, 63.2, 58.7, 52.8, 52.6, 42.4, 25.3.

Compound 15. A solution of **13** (5.1 g, 0.21 mmol), DMAP (0.076 g, 0.63 mmol), and 2-mecaptothiazoline (2-MT, 0.075 g, 0.63 mmol) in anhydrous DCM (70 mL) was cooled to 0 °C in an ice bath for 30 min, and EDC (0.12 g, 0.63 mmol) was added in one portion. The mixture was allowed to warm to room temperature slowly and stirred for 12 h. The solvent was partially removed under reduced pressure and the PEG linker precipitated with ethyl ether (200 mL). The crude product was obtained by filtration and crystallized from DCM/ethyl ether (20 mL/80 mL) and then from 2-propanol (IPA, 100 mL) respectively to give **15** (4.5 g, 0.18 mmol, 80%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 201.1, 172.7, 169.6, 156.0, 63.2, 58.4, 55.2, 52.4, 42.1, 28.5.

Compound 16. A solution of **15** (2.0 g, 0.082 mmol), doxorubicin hydrochloride (0.095 g, 0.16 mmol), and DMAP (0.040 g, 0.33 mmol) in anhydrous DMF/DCM (20 mL/20 mL) was stirred at room temperature for 12 h. The solvents were partially removed under reduced pressure, and the final product was precipitated with ethyl ether (80 mL). The solid was filtered and recrystallized from DMF/methanol (35 mL/25 mL) to give **16** (1.75 g, 0.070 mmol, 86%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 212.9, 186.1, 170.0, 169.5, 160.7, 156.7, 156.0, 155.4, 135.7, 135.4, 133.8, 133.6, 119.8, 118.7, 111.5, 101.1, 75.6, 69.4−74.0 (PEG), 68.3, 65.5, 64.9, 63.2, 61.9, 60.3, 59.6, 57.4, 55.4, 45.5, 43.3, 36.5, 34.7, 30.7, 17.91.

Compound 21. A solution of compound **7** (2.00 g, 9.12 mmol), DMAP (7.80 g, 63.8 mmol), and scandium triflate (3.14 g, 6.38 mmol) in anhydrous DCM (45 mL) was cooled to −8 °C in an ice−salt bath for 30 min. *N*-α-*tert*-Boc-L-alanine *N*-hydroxysuccinimate ester (**20**, 18.3 g, 63.8 mmol) was added and the temperature gradually rose from −8 °C to room temperature, and the reaction mixture stirred overnight under dry nitrogen. The reaction mixture was diluted with DCM; washed with 0.1 N HCl (3 × 60 mL), H_2O (2 × 50 mL), and saturated brine solution (60 mL); and dried over $MgSO_4$. The solvent was removed in vacuo and the product further purified by silica gel column chromatography to yield **21** (4.80 g, 8.55 mmol, 94%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 173.1, 170.4, 154.9, 81.2, 79.7, 63.7, 56.4, 53.0, 49.3, 28.1, 28.3, 18.7; HRMS (ESI) *m/z* calcd for $C_{26}H_{47}N_3O_{10}$ 561.66, found 562.33 ($M + H^+$).

Compound 22. Compound **22** was made similarly to **15** in 90% yield: ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 201.0, 172.7, 172.3, 155.1, 69.1−72.2 (PEG), 63.8, 63.6, 60.5, 58.7, 55.3, 52.6, 49.3, 28.8, 18.2.

Compound 23. A solution of doxorubicin hydrochloride (0.123 g, 0.21 mmol) in anhydrous DMF (15 mL) was added to a stirred solution of **22** (2.60 g, 0.11 mmol) in anhydrous DCM. DMAP (52 mg, 0.43 mmol) was added, and the resulting solution was stirred at room temperature overnight. The reaction solution was filtered through a bed of Celite, the filtrate was concentrated in vacuo, and the PEG derivative was precipitated with ethyl ether, filtered, and crystallized from 20% DMF/IPA (150 mL) to yield **23** (2.27 g, 0.09 mmol, 86%): ^{13}C NMR (300 MHz, $CDCl_3$) δ 213.2, 186.4, 186.2, 172.5, 169.4, 160.5, 155.7, 155.5, 155.2, 135.3, 135.0, 133.3, 133.2, 120.4, 119.3, 118.1, 111.1, 110.9, 100.4, 69.0−71.5 (PEG), 67.2, 65.1, 64.0, 63.8, 62.6, 58.6, 56.4, 54.6, 49.2, 44.6, 65.4, 33.6, 29.5, 17.8, 16.6.

Compound 27. A solution of **7** (0.10 g, 0.45 mmol), **26** (0.70 g, 2.66 mmol), DMAP (0.90 g, 7.38 mmol), and scandium triflate (0.022 g, 0.045 mmol) in anhydrous DCM (35 mL) was cooled to −8 °C in an ice−salt bath for 30 min, EDC (0.67 g, 3.49 mmol) was added, and the reaction mixture was stirred at −8 °C for another 30 min and then at room temperature for 4.5 h. The reaction mixture washed with 0.1 N HCl (3 × 30 mL), 0.1 N $NaHCO_3$ (3 × 30 mL), and distilled (30 mL) water and dried (Na_2SO_4), and the solvent was evaporated to give compound **27** (0.33 g, 0.45 mmol, 100%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 170.3, 170.1, 155.7, 81.1, 79.0, 70.8, 70.2, 68.4, 63.0, 56.2, 52.9, 40.3, 28.4, 28.2;

HRMS (ESI) *m/z* calcd for $C_{32}H_{59}N_3O_{14}$ 709.81, found 710.54 ($M + H^+$).

Compound 28. Compound **28** was made similarly to **15** in 83% yield: ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 28.7, 40.3, 52.5, 53.6, 55.3, 58.5, 62.8, 63.4, 68.0, 69.1, 69.6−73.0 (PEG), 155.8, 169.6, 172.6 and 200.9; HRMS (ESI) *m/z* calcd for $C_{32}H_{59}N_3O_{14}$ 709.81, found 710.54 ($M + H^+$).

Compound 29. To a solution of **28** (1.0 g, 0.041 mmol) and doxorubicin hydrochloride (0.047 g, 0.081 mmol) in a mixture of DCM/DMF (10 mL/10 mL) was added DMAP (0.020 g, 0.162 mmol). This mixture was stirred under nitrogen for 18 h, followed by partial removal of the solvent under reduced pressure. The PEG derivative was precipitated with ethyl ether, collected by filtration, and crystallized twice from DMF/IPA (4 mL/16 mL) to yield **29** (0.44 g, 0.018 mmol, 44%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 16.8, 29.8, 33.8, 35.6, 38.6, 40.7, 44.6, 54.2, 55.3, 56.5, 58.8, 63.7, 67.2, 67.3, 68.3, 69.4, 69.7−73.2 (PEG), 100.7, 111.3, 118.3, 119.6, 133.4, 133.5, 135.3, 135.5, 155.4, 155.9, 156.1, 160.8, 169.9, 170.1, 186.4, 186.7, and 213.4.

Compound 32. To a solution of di-*tert*-butyl dicarbonate (15 g, 0.086 mol) in 1,4-dioxane (150 mL) cooled to 5 °C in an ice bath was added dropwise a solution of 2,2′-(ethylenedioxy)-bis(ethylamine)[14] (25.85 g, 174.4 mmol) in 1,4-dioxane (100 mL) over a period of 1 h. The reaction mixture was allowed to warm to room temperature and stirred for two more hours. The solvent was removed under reduced pressure and the residue dissolved in methylene chloride (DCM, 150 mL), washed with water (3 × 150 mL), dried ($MgSO_4$), and filtered, and the solvent was evaporated under reduced pressure to yield **32** (13.84 g, mmol, 80%): ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 115.8, 79.0, 73.5, 70.1, 40.3, 28.4. HRMS (ESI) *m/z* calcd for $C_{11}H_{24}N_2O_4$ 248.32, found 249.26 ($M + H^+$).

Compound 34. A solution of **32** (3.0 g, 12.1 mmol), diglycolic anhydride (**33**, 1.26 g, 10.9 mmol), and DMAP (1.4

g, 11.5 mmol) in anhydrous DCM (30 mL) was stirred at room temperature for 18 h. The mixture was washed with 0.1 N HCl (30 mL), and the organic layer was dried (anhydrous sodium sulfate) and filtered, and the solvent removed under reduced pressure to yield **34** (1.5 g, 4.14 mmol, 38%): ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 173.4, 171.3, 169.9, 169.6, 157.8, 156.0, 81.2, 79.3, 71.8−68.8 (m), 41.6, 40.1, 38.9, 38.7, 28.3; HRMS (ESI) *m*/*z* calcd for $C_{15}H_{28}N_2O_8$ 364.45, found 364.18 ($M + H^+$).

Compound 35. Compound **34** (0.5 g, 1.37 mmol), **7** (0.090 g, 0.41 mmol), DMAP (0.46 g, 3.8 mmol), and scandium triflate (0.04 g, 0.023 mmol) were dissolved in anhydrous DCM (10 mL) cooled to 0 °C, and EDC (0.35 g, 1.8 mmol) was added. The mixture was allowed to warm to room temperature overnight. The reaction mixture was washed with water and then with 0.1 N HCl. The organic layer was dried (anhydrous sodium sulfate) and filtered, and the solvent was removed under reduced pressure to give **35** (0.37 g, 0.41 mmol, ∼100%): ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 170.2, 169.4, 168.6, 155.7, 81.2, 79.1, 71.0, 69.9, 69.6, 68.3, 67.6, 63.2, 53.1, 52.9, 40.3, 38.6, 28.4, 28.1; HRMS (ESI) *m*/*z* calcd for $C_{40}H_{71}N_5O_{18}$ 912.02, found 912.73 ($M + H^+$).

Compound 36. Compound **36** was made in a manner similar to **15** in 87% yield: ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 201.1, 172.5, 169.1, 168.3, 155.9, 71.5−67.1 (PEG), 63.5, 63.1, 60.5, 58.6, 55.3, 52.6, 40.4, 38.3, 28.7.

Compound 37. To a solution of **36** (2.0 g, 0.089 mmol) and doxorubicin hydrochloride (0.103 g, 0.179 mmol) in a mixture of DCM/DMF (20 mL/20 mL) was added DMAP (0.043 g, 0.35 mmol). This mixture was stirred under nitrogen for 18 h, followed by partial removal of the solvent under reduced pressure. The PEG derivative was precipitated with ethyl ether, collected by filtration, and crystallized twice from DMF/IPA (8 mL/32 mL) to yield **37** (1.6 g, 0.065 mmol, 73%): ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 313.3, 186.6, 186.2, 169.5, 168.9, 168.6, 160.6, 156.0, 155.9, 155.2, 135.4, 135.1, 133.5, 133.3, 120.5, 119.4, 118.2, 111.1, 110.9, 100.5, 72.0−69.0 (PEG), 68.0, 65.2, 63.7, 62.7, 58.7, 56.4, 54.1, 40.5, 38.4, 35.5, 33.6, 29.7, 16.7.

Biology. (1) Lysozyme. Materials. Chicken egg white lysozyme (EC 3.2.1.17), lysozyme substrate bacteria (*Micrococcus lysodeikticus*), and PBS (10 mM phosphate, pH 7.4, 138 mM NaCl, and 2.7 mM KCl) were purchased from Sigma Inc. (St. Louis, MO). Precast 4−20% Tris-glycine SDS electrophoresis gel and the gel running buffer were obtained from Invitrogen (Carlsbad, CA). Rat plasma in EDTA was received on dry ice the same day.

Preparation of Single-Stranded PEG-Lysozyme Conjugates. Permanent PEG-linker **40** (Scheme 6) or the rU-PEG linker (**14**, **15**, **22**, **28**, and **36**), at a reaction molar ratio of 1:1 (PEG:lysozyme), was added to a lysozyme solution of 5 mg/mL in 0.1 M phosphate buffer, pH 7.4, with fast stirring. After 45 min at 25 °C, the reaction was treated with 0.2 M sodium phosphate (pH 5.1) to a final pH of 6.8. The reaction mixture was dialyzed against 20 mM sodium phosphate, pH 5.1, at 4 °C, using a 6000−8000 MW cutoff membrane. The isolation of single-stranded rU-PEG−lysozyme conjugate was performed on a cation exchange column (Poros, HS) using a solvent system of 20 mM sodium phosphate at pH 5.1 and a NaCl gradient. The peak of the product was collected and concentrated using the ultrafree centrifugal filter device with 10k NMWL membrane (Millipore Corp., Bedford, MA).

Preparation of Multistranded PEG−Lysozyme Conjugates. Either permanent linker **40** or an activated rU-PEG linker, at a reaction molar ratio of 20:1 (PEG:lysozyme), was added to a lysozyme solution of 5 mg/mL in 0.1 M phosphate buffer, pH 7.4, with fast stirring. After 45 min at ambient temperature, the reaction was treated with 0.2 M sodium phosphate (pH 5.1) to final pH of 6.8. The reaction mixture was diluted with H_2O, filtered through 0.2 μM membrane, and separated on a Hiload Superdex 200 column which was equilibrated with 20 mM sodium phosphate, pH 6.0, 140 mM NaCl. The fractions of the peak were pooled and concentrated using the ultrafree centrifugal filter device with 30k NMWL membrane (Millipore Corp., Bedford, MA) to give the multistranded rU-PEG−lysozyme conjugate.

Release in Rat Plasma and in Chemical Buffer. rU-PEG−lysozyme conjugates underwent buffer exchange with PBS, pH 7.4, to monitor release in rat plasma and stability in PBS at different temperatures. The conjugates also underwent buffer exchange with H_2O for the release in Tris buffer, pH 8.5. CentriCon 10K centrifuge tubes (Millipore Corp., Bedford, MA) were used for the single-stranded rU-PEG−lysozyme conjugates while CentriCon 30K was used for the multistranded rU-PEG−lysozyme conjugates. The release of lysozyme from single-stranded or multistranded PEG−lysozyme conjugates was conducted at 0.15 mg/mL, under N_2. At the time indicated, an aliquot was withdrawn, neutralized with 0.2 M phosphate, pH 5.1 to 6.5, and stored at −20 °C until further analysis.

Determination of Release of Lysozyme from PEG−Lysozyme Conjugates. Under the reaction conditions mentioned above, lysozyme activity disappeared after conjugation with only single PEG. The release of the lysozyme was indicated by regeneration of the lysozyme activity under various release conditions and confirmed on SDS electrophoresis gel.

Enzyme Activity Assay. PEG−lysozyme conjugate concentration was determined by UV at 280 nm using an extinction coefficient of 2.39 mL/mg cm in 0.1 M sodium phosphate, pH 7.4. Activity was performed as previously reported. [15]

(2) Green Fluorescent Protein (GFP). Conjugation of PEG to GFP and Purification of GFP−PEG Conjugate. This was carried out in a similar fashion as previously reported,[15] employing rU-PEG linker **15**.

Characterization of PEG−GFP Conjugates. GFP has 21 free amines, 20 from lysine side chains and one from the N-terminus. Purified rU-PEG−GFP conjugate **19** had an apparent molecular weight of higher than 200 000 on 10% SDS electrophoresis gel when the reaction was carried out at a 30:1 molar ratio (PEG:GFP). The PEG-ylation number estimated on the gel was 10−14 PEG molecules per GFP.

Pharmacokinetics and Pharmacokinetic Parameters of GFP and PEG−GFP Conjugate in Rats. The pharmacokinetic parameters, such as half-life ($t_{1/2}$), area under curve (AUC), clearance (CL), and mean residence time (MRT) of GFP and PEG−GFP conjugates were estimated using WinNonlin software (Pharsight Corp., Mountainview, CA), using 5 mg/kg dosing (Table 2). The data show that the half-life of GFP can increase to 156-fold and the clearance can decrease to 47-fold after it is PEG-ylated at a 30:1 molar ratio. A one-compartment model was used to predict the curves of the dose−plasma concentration versus time course following a single intravenous dose administration. Results showed that the correlation between predicted and observed curves was above 93% for native GFP and 96% for **19**.

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