Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 3341–3346

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

CATHEPSIN B-SENSITIVE DIPEPTIDE PRODRUGS. 1. A MODEL STUDY OF STRUCTURAL REQUIREMENTS FOR EFFICIENT RELEASE OF DOXORUBICIN

Gene M. Dubowchik,* and Raymond A. Firestone[1]

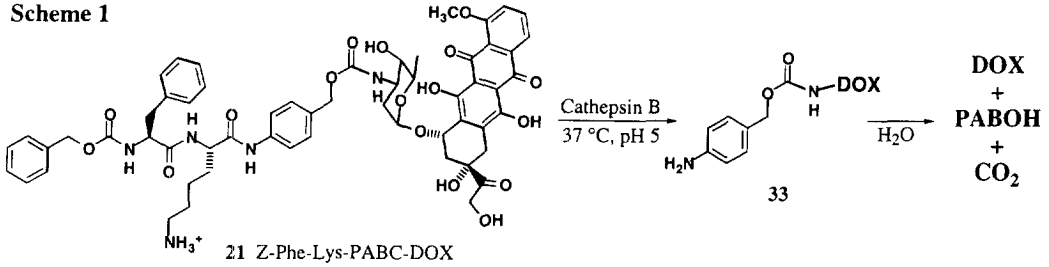
Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, P.O. Box 5100, Wallingford, CT 06492-7660, U.S.A.

Received 22 July 1998; accepted 12 October 1998

Abstract A series of lysosomal protease-sensitive peptides attached to doxorubicin (DOX) was prepared as model substrates for internalizing anticancer immunoconjugates and potential antimetastasis prodrugs. Rates of cathepsin B-mediated release of free drug was measured for each, and human plasma stabilities for representative examples.

The selective release of cytotoxic drugs from macromolecular or small molecule prodrugs at a tumor site relies on systemic stability as well as lability at the target tissue. The immunoconjugate BR96-DOX (BMS-182248), in which doxorubicin (DOX)‡ is attached to an internalizing antibody (BR96), depends upon a hydrazone link for selective hydrolysis inside tumor cell lysosomes (pH ca. 5, $t_{1/2}$ = 4 h) and relative stability in circulation (pH ca. 7, $t_{1/2}$ = 120 h).[2] We sought to use cathepsin B-sensitive peptides as drug-carrier linkers that would have increased systemic stability, could be used to carry a greater variety of drugs (i.e., drugs that contain amino or hydroxyl groups), and would release those drugs quickly at the target tissue following internalization.[3] Cathepsin B is a cysteine protease found in all mammalian cell lysosomes.[4] In addition, we are interested in the potential utility of these linkers as small molecule prodrugs to target metastatic sites that express cathepsin B (or L).[5] Success in the latter application would particularly depend on speed of drug release. Other known lysosomally cleavable peptides, specifically Gly-Phe-Leu-Gly[6] and Ala-Leu-Ala-Leu,[7] were judged to be less suitable for our purposes for two reasons: (1) lysosomal drug release is relatively slow, and (2) the hydrophobicity of the linkers would likely cause precipitation or aggregation of our antibodies, especially with a full complement of eight drugs per antibody. A general substrate for the assay of cathepsins B and L is Z-Phe-Arg-X, where X is a fluorogenic moiety.[8] In using this as a starting point for substrate design, we replaced Arg with Lys. This was done for synthetic ease as well as potential systemic stability in a series of compounds designed to determine structural requirements for efficient drug release and plasma stability. We also investigated two protected Arg substrates and a series of compounds containing citrulline (Cit),[9] which is isosteric and isoelectronic with Arg. Concern that drugs such as DOX would be too bulky to fit in the active site of cathepsin B led us to prepare substrates containing the self-immolative *p*-aminobenzylcarbonyl (PABC) spacer,[10] which spontaneously releases free drug upon deacylation in protic solvents (Scheme 1).

Scheme 1



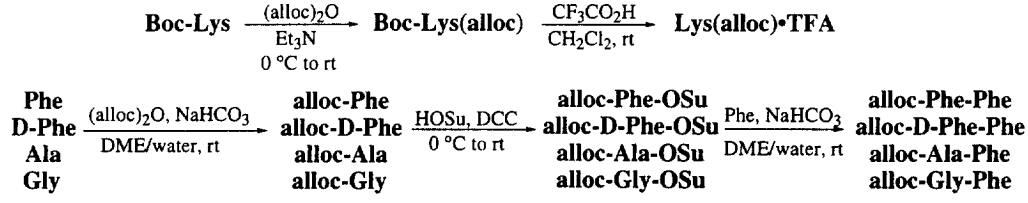
21 Z-Phe-Lys-PABC-DOX

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00609-X

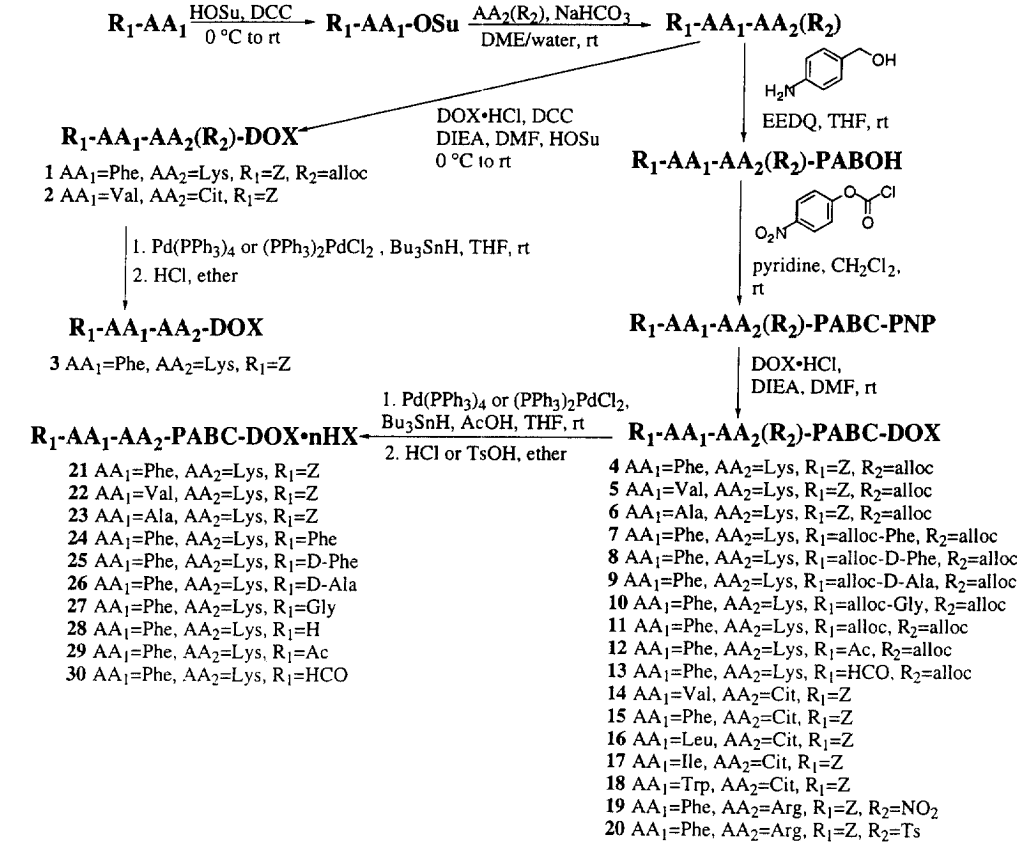
Synthesis

The substrates were prepared according to the general protocol outlined in Schemes 3 and 4, for the most part using modifications of methods well-known in peptide chemistry.[11] For reversible amine protection of Lys and N-terminal amino acids allyloxycarbonyl (alloc) was used (Scheme 2). Deprotection of **4** - **13** and **31** was effected by treatment with $Pd(PPh_3)_4$ or $(PPh_3)_2PdCl_2$ and quenching of the allyl-Pd complex with Bu_3SnH.[12] Triethylsilane was also tried as a hydride source but was found to be insufficiently reactive. Careful addition of either 1 M HCl/ether or *p*-toluenesulfonic acid (TsOH) precipitated the crude ammonium salts. Satisfactory purification often involved simply dissolving the solid as far as possible in methanol and filtering off insoluble by-products, but could also be carried out by chromatography on celite, eluting with progressively higher fractions of CH_3OH/CH_2Cl_2. The latter was always necessary for the bis-amino substrates **24** - **28**.

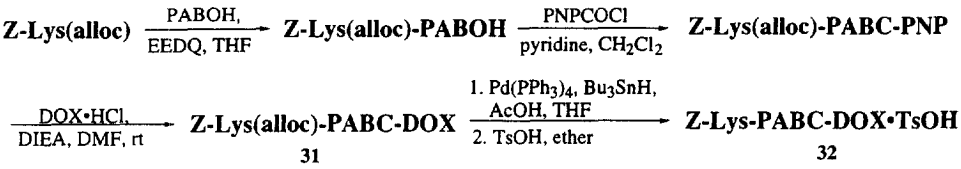
Scheme 2



Scheme 3



Scheme 4



Cathepsin B Assays

Assay conditions (enzyme concentration, pH, etc.) were chosen to approximate the lysosomal medium as a model for intracellular drug release. Bovine spleen cathepsin B (Sigma) was activated at rt with a solution of 30 mM DTT/15 mM EDTA for 15 min, and then diluted with 25 mM acetate/1 mM EDTA buffer (pH 5.0, preincubated at 37 °C),[13] followed immediately by the substrate in methanol giving the following initial concentrations: [cathepsin B] = ca. 40 nM, [substrate] = 0.04 mM. The mixture was incubated at 37 °C and aliquots were periodically removed and injected into the HPLC (15 cm C-18 column, 8:2 methanol/50 mM Et_3N-HCO_2H buffer (pH 2.8) at 1 mL/min, λ = 495 nm). The time between injections varied depending on speed of drug release. The ratios of DOX/substrate peak areas were used to calculate half-lives ($t_{1/2}$) of release (Table 1). At 495 nm the appearance of DOX correlated precisely with the disappearance of substrate. When Z-Phe-Lys-PABC-DOX **21** was run at high concentrations (≥ 5 mM) a small, transient peak eluted between the substrate and DOX that might have been the intermediate PABC-DOX **33** (Scheme 1). Stabilities were also measured without cathepsin B at pH 5. No release of DOX was ever observed within 6 - 7 h.

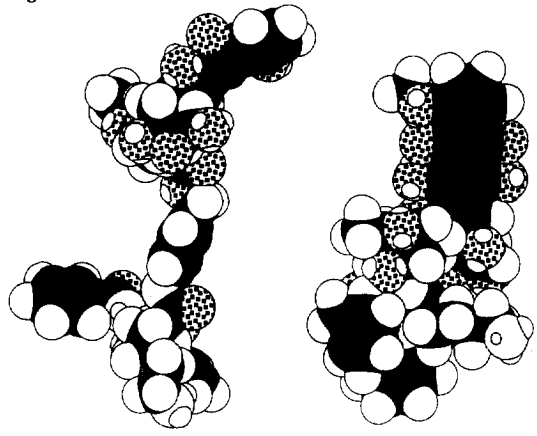
Human Plasma Stability Assays

A solution of the substrate (40 μM) in freshly drawn human plasma was incubated at 37 °C. Aliquots (50 μL) were periodically removed and diluted with cold methanol (100 μL). The samples were centrifuged for 10 min and the supernatants injected into the HPLC. Half-lives were calculated as described above (Table 1). Enough free DOX was added to a separate plasma sample to give a theoretical release of 1%. This was successfully detected using the same methods.

Results and Discussion

DOX release data for these substrates are summarized in table 1. Cathepsin B-mediated release of free DOX from representative examples lacking the PABC spacer (**2** and **3**) was not observed. Steric crowding around the target carbonyl carbon (darkened in the space-filling models in Figure 1) and/or the inability of the enzyme active site, which is located in a deep cleft,[4] to accommodate DOX are the most likely explanations. Enhanced leaving ability of the PABC anilide can be ruled out as the explanation, since cathepsin B released Gly-DOX from a similar substrate containing Val-Cit-Gly-DOX with $t_{1/2}$ = 2h.

Figure 1



Z-Phe-Lys-PABC-DOX **Z-Phe-Lys-DOX**

Space-filling models showing the Lys CO-carbons in black.

Several trends are evident in the cathepsin B half-lives. At the P_1 site a basic amino acid (Lys) that is protonated at pH 5 is more readily attacked than one that can only form hydrogen bonds (Cit). Substrates containing nitro- and tosyl-protected Arg (**19** and **20**) are only very slowly cleaved. The preference at the P_2 site is for a hydrophobic residue. Cathepsin B also accepts basic amino acids at P_2 but these are likely to be targets of other proteases in vivo.[4] In the P_1-Cit series significant differences in release rates are seen within a series of hydrophobic amino acids at P_2 where Val < Leu < Phe < Ile < Trp. With Lys at P_1 there is no measurable rate difference between Phe and Val at P_2 (although DOX release is so fast with these substrates, it is unlikely that a difference would have been seen). This P_2 selectivity in the Cit series may reflect different binding modes for Cit and Lys at P_1 resulting in different P_2 binding geometries. When the size of P_2 is reduced in Z-Ala-Lys-PABC-DOX **23**, release is noticeably slower.

Table 1. Half-lives of DOX Release at 37 °C

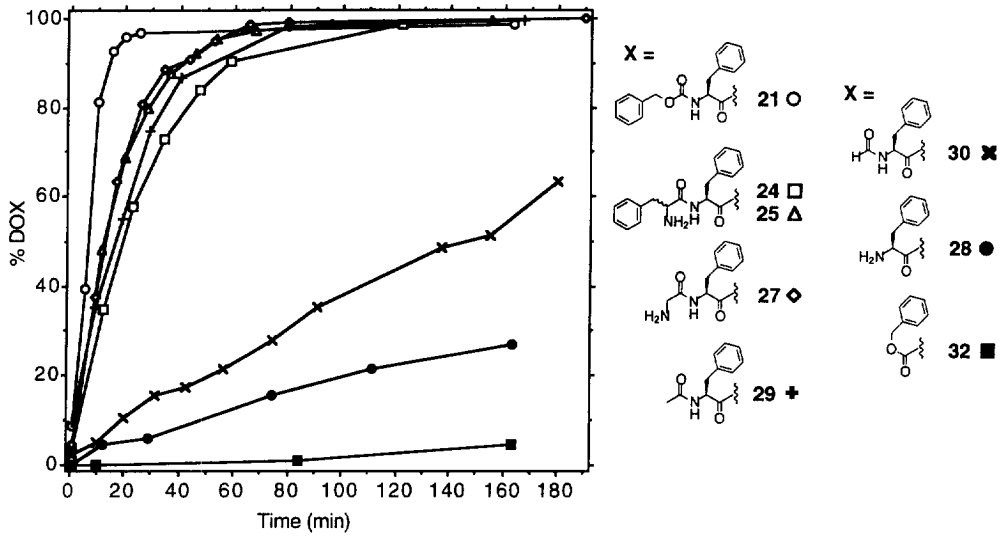
Substrate	#	Cathepsin B	Human Plasma
Z-Phe-Lys-PABC-DOX	**21**	8 min	NH*
Z-Phe-Lys-DOX	**3**	NH	
Z-Val-Lys-PABC-DOX	**22**	9 min	NH
Z-Ala-Lys-PABC-DOX	**23**	60 min	NH
Phe-Phe-Lys-PABC-DOX	**24**	19 min	50 min
D-Phe-Phe-Lys-PABC-DOX	**25**	13 min	NH
D-Ala-Phe-Lys-PABC-DOX	**26**	15 min	NH
Gly-Phe-Lys-PABC-DOX	**27**	13 min	4.5 h
Ac-Phe-Lys-PABC-DOX	**29**	16 min	NH
HCO-Phe-Lys-PABC-DOX	**30**	2.5 h	NH
Phe-Lys-PABC-DOX	**28**	3.7 h	
Z-Lys-PABC-DOX	**32**	32 h	
Z-Val-Cit-PABC-DOX	**14**	4 h	NH
Z-Val-Cit-DOX	**2**	NH	
Z-Phe-Cit-PABC-DOX	**15**	10.5 h	
Z-Leu-Cit-PABC-DOX	**16**	8.8 h	
Z-Ile-Cit-PABC-DOX	**17**	15.3 h	
Z-Trp-Cit-PABC-DOX	**18**	34 h	
Z-Phe-Arg(NO_2)-PABC-DOX	**19**	33 h	
Z-Phe-Arg(Ts)-PABC-DOX	**20**	33 h	

* NH = not hydrolyzed (no observed change over 6 - 7 h).

In a series of X-Lys-PABC-DOX substrates in which X was systematically varied from Z (**32**) to (D/L)-Phe-Phe (**25** and **24**) we found that the rate of drug release by cathepsin B increased with increasing chain length up to the α-carbon of P_3 (Figure 2).[14] The largest change occured when the size of the N-terminal protecting group HCO (**30**, $t_{1/2}$ = 2.5 h) was increased by a single methyl group to give Ac-Phe-Lys-PABC-DOX (**29**, $t_{1/2}$ = 16 min). Taralp and coworkers have suggested that the S_3 subsite of cathepsin B is primarily of a hydrophobic character[15] and, therefore, better binding of the Ac methyl group of **29** in the S_3 pocket may explain this large

effect. Thereafter, replacement of Ac with Gly (**27**) or D/L-Phe (**25** and **24**) made no real difference, but a further noticeable rate increase was seen when Phe is replaced by the nearly-isosteric Z (**21**, $t_{1/2}$ = 8 min). These results are not easy to interpret given the complexity of the active site of cathepsin B, with 7 dissociable residues able to influence activity at various pH values, and without more detailed kinetic parameters.

Figure 2. Effect of structural changes on cathepsin B-mediated release of DOX from X-Lys-PABC-DOX by at 37°C, pH 5.0.



Substrates containing the N-terminal protecting groups Z (**21**, **22**, and **14**), Ac (**29**) and HCO(**30**) or the unnatural amino acids D-Phe (**25**) and D-Ala (**26**) were not degraded by freshly drawn human plasma at 37 °C over 6 - 7 h, demonstrating the stability of the Phe-Lys dipeptide to plasma proteases and the PABC spacer to proteases and esterases. As expected, both the L-Phe and Gly-terminated substrates were digested, presumably by plasma aminopeptidases as evidenced in each case by a cluster of peaks seen by HPLC (at 495 nm) that ultimately resolved into a single DOX peak. While the choice of N-terminal groups for macromolecularly-linked DOX is not an issue, it will likely be an important consideration for protease-sensitive, small-molecule prodrugs that are to target sites where cathepsin B or L are expressed extracellularly. Aqueous solubility, for example could be enhanced by attaching charged N-terminal groups such as D-amino acids, phosphonates or sulfonates, especially for very lipophilic drugs like paclitaxel. Unwanted passive cell diffusuon could also be discouraged by the addition of charged or hydrogen bonding functionalities. Our results using these peptide-PABC linkers for drug delivery will be reported in future publications.

In summary, we have prepared peptide-DOX substrates that contain a self-immolative PABC spacer and that are efficiently cleaved by cathepsin B to release free DOX but are very stable in human plasma. Cathepsin B is an attractive target for release of drugs from conjugates that are taken up by receptor-mediated endocytosis since it is ubiquitous and found in relatively high levels in mamallian lysosomes. In addition, several of these compounds release DOX on a time scale that may make them useful as prodrugs for metastatic or primary tumors that express extracellular cathepsin B.

References

‡ Abbreviations used: AA, amino acid; Ac, acetyl; Ala, L-alanine; Alloc, allyloxycarbonyl; Arg, L-arginine; Cit, L-citrulline; DIEA, diisopropylethylamine; DME, dimethoxyethane; DMF, dimethylformamide; DOX, doxorubicin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; Fmoc, 9-fluorenylmethyloxycarbonyl; Gly, glycine; HOSu, N-hydroxysuccinimide; HPLC, high performance liquid chromatography; Ile, L-isoleucine; Leu, L-leucine; Lys, L-lysine; PABC, p-aminobenzylcarbonyl; PABOH, p-aminobenzyl alcohol; Phe, phenylalanine; PNP, *p*-nitrophenyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Trp, L-tryptophan; Ts, *p*-toluenesulfonyl; Val, L-valine; Z, benzyloxycarbonyl.

1. Present address: Boehringer Ingelheim Pharmaceuticals Inc., 900 Ridgebury Rd., P.O. Box 368, Ridgefield, CT 06877-0368, U.S.A.
2. Trail, P. A.; Willner, D.; Lasch, S. J.; Henderson, A. J.; Hofstead, S.; Casazza, A. M.; Firestone, R. A.; Hellström, I.; Hellström, K. E. *Science* **1993**, *261*, 212-215; Willner, D.; Trail, P. A.; Hofstead, S. J.; King, H. D.; Lasch, S. J.; Braslawsky, G. R.; Greenfield, R. S.; Kaneko, T.; Firestone, R. A. *Bioconj. Chem.* **1993**, *4*, 521-527.
3. Dubowchik, G. M.; Radia, S., *Tetrahedron Lett.*, **1997**, *38*, 5261-5264; Dubowchik, G. M.; King, H. D.; Pham-Kaplita, K., *Tetrahedron Lett.*, **1997**, *38*, 5265-5268.
4. Kirschke, H.; Barrett, A. J.; Rawlings, N. D. In *Protein Profile - Proteinases 1: Lysosomal Cysteine Proteinases;* Sheterline, P., Ed.; Academic: London, 1995; Vol. 2, pp 1587-1643.
5. Elliott, E.; Sloane, B. F. *Perspect. Drug Discov. Design* **1996**, *6*, 12-32.
6. Kopecek, J.; Duncan, R. *J. Control. Release* **1987**, *6*, 315-327.
7. Trouet, A.; Masquelier, M.; Baurain, R.; Deprez-De Campaneere, D. *Proc. Natl. Acad. Sci. U.S.A.* **1982**,*79*, 626-629.
8. Barrett, A. J. *Biochem. J.* **1980**, *187*, 909-912.
9. Haney, D. N., Belgian Patent, 899, 539, 1984; *Chem. Abstr.* **1985**, *102*, 20376.
10. Carl, P. L.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Med. Chem.* **1981**, *24*, 479-480; Azoulay, M.; Florent, J.-C.; Monneret, C.; Gesson, J. P.; Jacquesy, J.-C.; Tillequin, F.; Koch, M.; Bosslet, Czech, J.; Hoffman, D. *Anti-Cancer Drug Design* **1995**, *10*, 441-450.
11. All new compounds gave satisfactory NMR, mass-spectral, microanalytical and/or high-resolution mass spectral results. Representative preparation for **21**: Z-Phe-Lys(alloc)-PABC-DOX (34.9 mg, 29.4 μmoles) and $(PPh_3)_2PdCl_2$ (0.6 mg, 3%) in dry THF (1 mL) under argon at rt were treated with acetic acid (3.5 μL, 2 equiv) and then with Bu_3SnH (10 μL, 1.2 equiv). The reaction was stirred at rt for 1.5 h and then treated with 1 M HCl in ether (60 μL, 2 equiv). The mixture was stored in the freezer for 1 h and then the crude orange solid was collected by filtration and washed repeatedly with ether. The solid was washed through the glass frit with 5:1 CH_2Cl_2/CH_3OH and then the filtrate was evaporated. The residue was sonicated for several minutes in CH_3OH (5 mL) and then filtered to remove insoluble by-products. The filtrate was evaporated to give an orange-red solid (25.1 mg, 75%). ^1H NMR ($CDCl_3/CD_3OD$) δ 1.18 (2H, d), 1.34, 1.65 and 1.73 (6H, m), 2.14 (2H, m), 2.81 (2H, m), 3.76 (1H, m), 3.98 (3H, s), 4.05 (1H, m), 4.38 and 4.45 (each 1H, m), 4.67 (2H, s), 4.85 (1H, m), 7.04 and 7.20 (10H, m), 7.14 and 7.43 (4H, m), 7.30, 7.69 and 7.92 (each 1H, m). HPLC (15 cm C-18 column, 4:1 CH_3OH/50 mM Et_3N-HCO_2H buffer (pH 2.8), 1 mL/min., λ = 495 nm): ret. time 7.1 min. MS (FAB): 1102 $(MH)^+$, 1124 $(M+Na)^+$. HRMS: calcd for $C_{58}H_{64}N_5O_{17}$: 1102.4297. Found: 1102.4290.
12. Dangles, O.; Guibe, F.; Balavoine, G.; Lavielle, S.; Marquet, A. *J. Org. Chem.* **1987**, *52*, 4984-4993.
13. Bajkowski, A. S.; Frankfater, A. *Anal. Biochem.* **1975**, *68*, 119-127; Barrett, A. J.; Kirschke, H. *Meth. Enzymol.* **1981**, *80 (Pt. C)*, 535-561.
14. Brömme, D.; Bescherer, K.; Kirschke, H.; Fittkau, S. *Biochem. J.* **1987**, *245*, 381-385.
15. Taralp, A.; Kaplan, H.; Sytwu, I.-I.; Vlattas, I.; Bohacek, R.; Knap, A. K.; Hirama, T.; Huber, C. P.; Hasnain, S. *J. Biol. Chem.* **1995**, *270*, 18036-18043.