Enzyme Inhibition

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Selective Inhibition of Glycosidases by Feedback Prodrugs**

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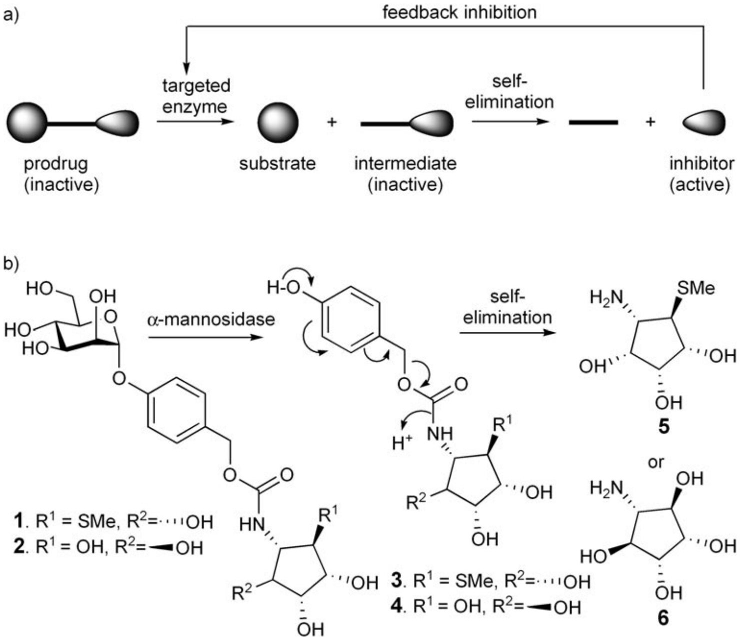
Protein- and lipid-linked oligosaccharides at the surface of eukaryotic cells are responsible for a wide range of biological processes impacting health and disease.[1–4] Examples of such processes include fertilization, embryogenesis, neuronal development, hormone activities, and the proliferation of cells and their organization into specific tissues. Remarkable changes in cell-surface carbohydrates occur with tumor progression, which appears to be intimately associated with the dreaded state of metastasis.[5–7] Furthermore, carbohydrates of host cells are often employed by pathogens for cell entry. Not surprisingly, compounds that can interfere in the biosynthesis of oligosaccharides are regarded as attractive leads for drug discovery for a wide range of diseases.[8–14] For example, inhibitors of pancreatic α-amylase, such as arcarbose, voglibose, and miglitol, have been introduced for the treatment of diabetes.[15] Furthermore, compounds such as zamamivir and oseltamivir (tamiflu) have been developed as inhibitors of neuramidase for the treatment of the flu.[16–20] Despite these successes, it has been difficult to develop safe and efficacious glycosidase inhibitors for the treatment of many other diseases. A major problem of many natural and synthetic glycosidase inhibitors is that they inhibit other glycosidases, which may lead to serious side effects. More-selective inhibitors may be obtained by carefully designed structure—activity-relationship studies[21–29] in combination with a better understanding of the mechanism of action of glycosidases. This approach is, however, complicated by the fact that of the approximate 100 mammalian glycosidases, only a small number have been cloned and overexpressed.

We report herein a general approach for the design and synthesis of more-selective glycosidase inhibitors based on feedback inhibition. Feedback inhibition is a mechanism in which a biosynthetic pathway regulates itself through inhibition of the first committed step in the pathway by a downstream or final product.[30] Although this type of inhibition is widely used in nature, it has not been exploited for the design and synthesis of selective therapeutics. The feedback-inhibition prodrugs described herein are composed of an inhibitor that is modified by a linker that renders them inactive (Scheme 1 a). The linker, in turn, is attached to a glycoside that can be hydrolyzed by a targeted glycosidase. The resulting intermediate is designed in such a way that it will undergo self-fragmentation to release the active inhibitor. Subsequently, the inhibitor inhibits the enzyme that initiated its release. The attraction of this approach is that the inhibitor will only be released at the site of the enzyme. Selectivity is achieved owing to the exquisite selectivity of glycosidases for their substrates. Furthermore, the release of the inhibitor is terminated when a sufficient quantity of the inhibitor is released. The remaining pool of prodrug will, however, be activated when the active enzyme reappears, thereby approximating conditions of continuous infusion.



Scheme 1. a) General strategy for the inhibition of enzymes by a feedback mechanism. b) A feedback inhibitor for mannosidases.

Compound **1** is a prototypic feedback inhibitor that is designed to selectively target α-mannosidases. It is composed of mannostatin A,[10] which is a potent inhibitor of α-mannosidases and has garnered attention as a lead compound for cancer-drug development (Scheme 1 b). The amino group of mannostatin A is modified by a 4-hydroxybenzyl carbamate, which in turn is linked to an α-mannoside. The acylation of the amino group of mannostatin A renders the compound inactive for inhibition of mannosidases.[31] However, an α-mannosidase can hydrolyze the glycosidic linkage resulting in the formation of intermediate **3**, which will self-fragment[32] to give mannostatin A (**5**). The active mannostatin A will then inhibit the α-mannosidase that initiated its release, therefore terminating the enzymatic reaction. On the other hand, the enzymatic hydrolysis of compound **2** will release aminocyclopentitetrol **6**, which is not an inhibitor of an α-mannosidase,[33] and therefore this reaction is expected to proceed to completion.

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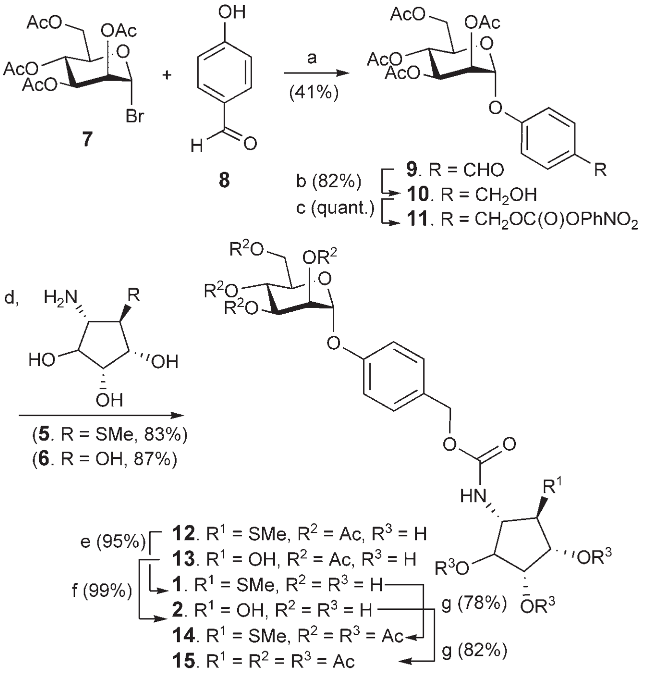
The synthesis of compounds **1** and **2** is summarized in Scheme 2. Coupling of 1-bromo-2,3,4,6-tetra-*O*-acetyl-α-D-mannose (**7**) with *p*-hydroxybenzaldehyde (**8**) by using silver(I)oxide as the promoter in refluxing acetonitrile gave **9** in a modest yield. The aldehyde moiety of **9** was reduced with NaBH$_4$ to give **10**, which was converted into activated intermediate **11** by reaction with 4-nitrophenyl chloroformate. This compound was immediately reacted with **5**[34] and **6**[35] to afford compounds **12** and **13**, respectively. The acetyl esters of **12** and **13** were cleaved by treatment with ammonia or hydrazine in methanol to give the targeted compounds **1** and **2**, respectively.



Scheme 2. Reagents and conditions: a) Ag$_2$O, CH$_3$CN, Δ, 1 h; b) NaBH$_4$, silica, CHCl$_3$, *i*PrOH; c) *p*-NO$_2$PhOCOCl, pyridine, DCM, 18 h; d) Et$_3$N, DMF, 18 h; e) NH$_3$ in MeOH; f) NH$_2$NH$_2$·H$_2$O, MeOH, 0 °C, 5 h; g) Ac$_2$O, pyridine. DCM = dichloromethane, DMF = *N*,*N*-dimethylformamide.

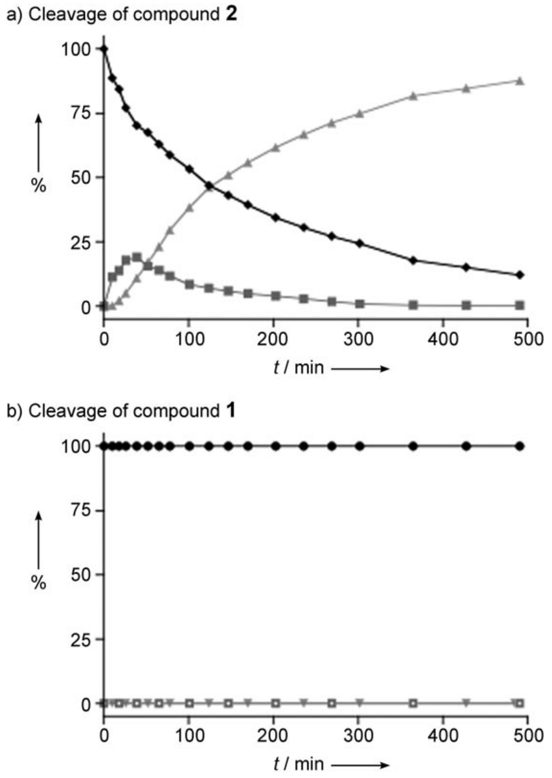


Figure 1. Release of prodrugs **1** and **2** by human lysosomal mannosidase. HLM (20 mU) was added to a solution of **2** (a) and **1** (b) (80 nmol) in deuterated sodium acetate buffer solution (100 mM, pH 5.6, 0.2 mL). The enzymatic cleavage of **1** (●) and **2** (◆) and the formation of the intermediates **3** (□) and **4** (■) and the final products **5** (▼) and **6** (▲), respectively, were monitored by ^{1}H NMR spectroscopy (600 MHz).

The enzymatic cleavage of **1** and **2** by human lysosomal mannosidase (HLM) was monitored by ^{1}H NMR spectroscopy (600 MHz).[36] HLM (20 mU) was added to a solution of **1** or **2** (80 nmol), which were dissolved in a deuterated sodium acetate buffer (100 mM, pH 5.6, 0.2 mL). The progress of the reaction was monitored by the integration of key signals of the starting material, the intermediate, and final product. As can be seen in Figure 1 a, HLM hydrolyzed **2** producing intermediate **4**, which self-fragmented to aminocyclopentitetrol **6**. Compound **6** is not an inhibitor of HLM and as a result the enzymatic conversion progressed to completion. On the other hand, virtually no hydrolysis was observed when **1** was treated with HLM (Figure 1 b). In this case, the released mannostatin A (**1**) inhibits HLM and terminates further hydrolysis of **1**. Mannostatin A is a potent inhibitor of retaining α-mannosidases ($K_i = 90$ nM, HLM)[37] and thus, the release of low concentrations of the active compound is sufficient to abolish enzyme activity. Indeed, the addition of 4-methylumbelliferyl-α-D-mannopyranoside did not result in the release of fluorescence methylumbelliferone, demonstrating that the mannosidase was inhibited by the released mannostatin A.

Compounds **1** and **2** were subjected to α-glucosidase, α-fucosidase, and α-galactosidase. As expected, these enzymes did not initiate the release of the compounds **5** and **6**, demonstrating that they have exquisite selectivity for α-mannosidases (data not shown).[41]

Next, the pH dependence of the self-fragmentation of intermediates **3** and **4** was investigated. It is well known that different organelles have different pH values. For example, lysosomes are significantly more acidic than the Golgi apparatus. Golgi mannosidase II has been identified as a target for antimetastatic therapy,[8,38] whereas inhibition of lysosomal mannosidase leads to the phenotype of lysosomal storage disease. It was expected that intermediates **3** and **4** would fragment significantly faster in neutral as opposed to acidic conditions, providing a basis for some selectivity for Golgi mannosidase over the lysosomal mannosidase. Thus, compound **2** (0.4 mM), in deuterated phosphate/citrate buffer solutions (0.2:0.1M) of different pH values, was treated with a large quantity (7 Units mL^{-1}) of Jack Bean α-mannosidase (JBM) to completely convert the prodrug into intermediate **4**.

Subsequently, the decomposition of **4** was monitored by ^{1}H NMR spectroscopy by integration of key signals of the intermediate and final product. As can be seen in Table 1, the decomposition of **4** is significantly faster at more-basic pH values and elevated temperatures. These results can easily be rationalized by assuming that at a higher pH value, a large proportion of the intermediate is in the phenoxide form, which decomposes significantly faster than the analogous phenol.

Table 1: pH dependence of half-lives upon the self-fragmentation of **2**.[a]

pH	4.0	4.5	5.0	5.5	6.0	6.5
37 °C (min)	480	270	100	45	16	9
25 °C (min)	2800	–	–	–	–	45

[a] Determined by incubating prodrug **2** (80 nmol) and Jack Bean mannosidase (1.4 U) in deuterated 0.2 M phosphate/0.1 M citrate buffer (0.2 mL) at 37 °C or 25 °C in a NMR tube and measuring ^{1}H NMR spectra over time on a 600 MHz spectrometer.

For many applications, compounds such as **1** need to be cell permeable to inhibit intracellular glycosidases. Analysis of fetal calf serum (FCS), which is often used for culturing cells, revealed that it contains a significant amount of mannosidase activity. Thus, these mannosidases can unmask mannostatin A, which can then pass through the cell membrane to inhibit intracellular mannosidases. It was anticipated that *O*-acetylation of **1** and **2** to give **14** and **15**, respectively, would considerably improve the metabolic stability of the prodrug. Furthermore, it has been shown that *O*-acetylation of saccharides can facilitate cellular uptake.[39] Subsequently, intracellular esterases can cleave the acetyl esters revealing the saccharide, which can then interfere in the biosynthesis of oligosaccharides. FCS exhibits only very low levels of esterase activity therefore improving extracellular stability of acetylated carbohydrates. Thus, it was anticipated that compound **14** would be easily taken up by cells, and subsequent deacetylation by intracellular esterases would reveal **1**, which can then be hydrolyzed by an α-manmosidase to initiate the release of mannostatin A. To investigate whether **14** possesses intracellular activity, it was subjected to MDAY-D2 cells and the disappearance of polylactosamine monitored. It is known that the metastatic MDAY-D2 lymphoma cell line overexpresses the enzyme *N*-acetyl glucosaminyltransferase V (GnTV).[8] This enzyme adds an additional β1-6-linked *N*-acetylglucosamine moiety to Asn-linked oligosaccharides, which are subsequently extended by a polylactosamine chain. This increased branching has been observed in human breast, colon, and skin carcinomas and has been correlated with invasive and metastatic potential. Inhibition of the mannose-trimming enzyme, human Golgi α-mannosidase II (HGMII), which acts late in the *N*-glycan processing pathway, provides one route to blocking additional branching of *N*-linked oligosaccharides.[8] In this case, the biosynthetic precursor for GnTV is not formed and hence the polylactosamine chain cannot be appended. Thus, to investigate whether the acetylated prodrugs can inhibit intracellular Golgi mannosidase II, MDAY-D2 lymphoma cells were cultured in the presence of different concentrations of **14** and **15** for two days. Subsequently, the presence of polylactosamine linked to proteins was determined by analyzing cell lysates by Western blotting with a fluorescein-labeled PHA lectin.[40] Interestingly, at a concentration of 1 μM of **14**, a 50 % reduction in fluorescein labeling was observed, whereas compound **15** did not display any inhibitory activity (Figure 2).

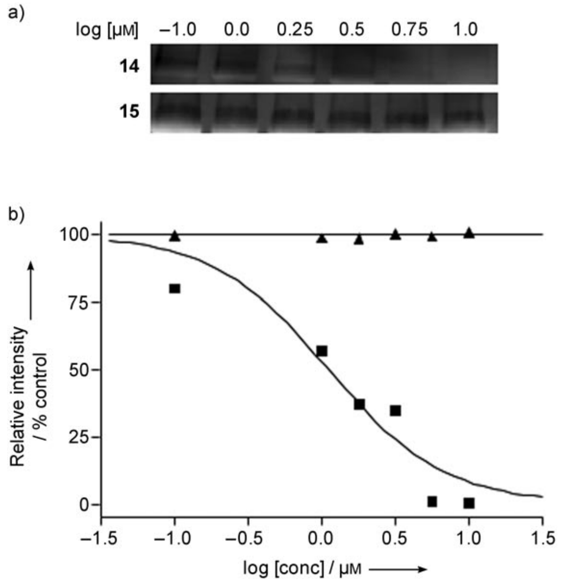


Figure 2. Inhibition of polylactosamine formation by compounds **14** and **15**. MDAY-D2 cells were cultured for 2 days in the absence (control) or presence of different concentrations of compounds **14** (■) or **15** (▲). a) Cellular proteins (10 μg) were subjected to SDS-PAGE and polylactosamine was visualized by using a fluorescein-labeled L-PHA lectin. b) Fluorescence bands were scanned by laser beam densitometry, and the relative intensity depicted.

In conclusion, a feedback prodrug approach in which a glycosidase inhibitor is released by the targeted glycosidase offers an attractive approach for enhancing the selectivity of a glycosidase inhibitor. Another advantage of this type of prodrug is that only the amount of compound required for complete inhibition of the targeted enzyme is released. It is to be expected that chemical modification of a lead compound in combination with a feedback prodrug formulation may make it easier to develop glycosidase inhibitors as safe therapeutics. Furthermore, efforts are underway to modify the linker region of the feedback prodrug, to increase the catalytic efficiency of enzymatic hydrolysis, and to increase the rate of self-fragmentation. Also, the possibility of using feedback prodrugs for other glycosidases, proteases, and kinases is being explored.

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[1] A. Varki, *Glycobiology* **1993**, *3*, 97.
[2] R. A. Dwek, *Chem. Rev.* **1996**, *96*, 683.
[3] T. Kolter, K. Sandhoff, *Angew. Chem.* **1999**, *111*, 1632; *Angew. Chem. Int. Ed.* **1999**, *38*, 1532.
[4] J. W. Dennis, M. Granovsky, C. E. Warren, *BioEssays* **1999**, *21*, 412.
[5] M. M. Fuster, J. D. Esko, *Nat. Rev. Cancer* **2005**, *5*, 526.
[6] D. H. Dube, C. R. Bertozzi, *Nat. Rev. Drug Discovery* **2005**, *4*, 477.
[7] J. W. Dennis, M. Granovsky, C. E. Warren, *Biochim. Biophys. Acta* **1999**, *1473*, 21.
[8] P. E. Goss, M. A. Baker, J. P. Carver, J. W. Dennis, *Clin. Cancer Res.* **1995**, *1*, 935.
[9] V. H. Lillelund, H. H. Jensen, X. F. Liang, M. Bols, *Chem. Rev.* **2002**, *102*, 515.
[10] A. Berecibar, C. Grandjean, A. Siriwardena, *Chem. Rev.* **1999**, *99*, 779.
[11] N. Asano, R. J. Nash, R. J. Molyneux, G. W. J. Fleet, *Tetrahedron: Asymmetry* **2000**, *11*, 1645.
[12] T. D. Heightman, A. T. Vasella, *Angew. Chem.* **1999**, *111*, 794; *Angew. Chem. Int. Ed.* **1999**, *38*, 750.
[13] M. vonItzstein, R. J. Thomson, *Curr. Med. Chem.* **1997**, *4*, 185.
[14] N. Asano, *Glycobiology* **2003**, *13*, 93R.
[15] R. Perfetti, P. S. Barnett, R. Mathur, J. M. Egan, *Diabetes/Metab. Rev.* **1998**, *14*, 207.
[16] M. Von Itzstein, W. Y. Wu, G. B. Kok, M. S. Pegg, J. C. Dyason, B. Jin, T. V. Phan, M. L. Smythe, H. F. White, S. W. Oliver, P. M. Colman, J. N. Varghese, D. M. Ryan, J. M. Woods, R. C. Bethell, V. J. Hotham, J. M. Cameron, C. R. Penn, *Nature* **1993**, *363*, 418.
[17] M. vonItzstein, J. C. Dyason, S. W. Oliver, H. F. White, W. Y. Wu, G. B. Kok, M. S. Pegg, *J. Med. Chem.* **1996**, *39*, 388.
[18] C. U. Kim, W. Lew, M. A. Williams, H. T. Liu, L. J. Zhang, S. Swaminathan, N. Bischofberger, M. S. Chen, D. B. Mendel, C. Y. Tai, W. G. Laver, R. C. Stevens, *J. Am. Chem. Soc.* **1997**, *119*, 681.
[19] W. X. Li, P. A. Escarpe, E. J. Eisenberg, K. C. Cundy, C. Sweet, K. J. Jakeman, J. Merson, W. Lew, M. Williams, L. J. Zhang, C. U. Kim, N. Bischofberger, M. S. Chen, D. B. Mendel, *Antimicrob. Agents Chemother.* **1998**, *42*, 647.
[20] P. Chand, P. L. Kotian, A. Dehghani, Y. El-Kattan, T. H. Lin, T. L. Hutchison, Y. S. Babu, S. Bantia, A. J. Elliott, J. A. Montgomery, *J. Med. Chem.* **2001**, *44*, 4379.
[21] G. Godin, P. Compain, O. R. Martin, K. Ikeda, L. Yu, N. Asano, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5991.
[22] F. M. Platt, G. R. Neises, R. A. Dwek, T. D. Butters, *J. Biol. Chem.* **1994**, *269*, 8362.
[23] F. M. Platt, G. R. Neises, G. B. Karlsson, R. A. Dwek, T. D. Butters, *J. Biol. Chem.* **1994**, *269*, 27108.
[24] H. S. Overkleeft, G. H. Renkema, J. Neele, P. Vianello, I. O. Hung, A. Strijland, A. M. van der Burg, G. J. Koomen, U. K. Pandit, J. Aerts, *J. Biol. Chem.* **1998**, *273*, 26522.
[25] C. Y. Wu, C. F. Chang, J. S. Y. Chen, C. H. Wong, C. H. Lin, *Angew. Chem.* **2003**, *115*, 5119; *Angew. Chem. Int. Ed.* **2003**, *42*, 4661.
[26] L. L. Johnson, T. A. Houston, *Tetrahedron Lett.* **2002**, *43*, 8905.
[27] I. Robina, A. J. Moreno-Vargas, J. G. Fernandez-Bolanos, J. Fuentes, R. Demange, P. Vogel, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2555.
[28] R. Lysek, C. Schutz, P. Vogel, *Helv. Chim. Acta* **2005**, *88*, 2788.
[29] T. D. Butters, R. A. Dwek, F. M. Platt, *Curr. Top. Med. Chem.* **2003**, *3*, 561.
[30] A. B. Pardee, G. P. V. Reddy, *Gene* **2003**, *321*, 17.
[31] S. B. King, B. Ganem, *J. Am. Chem. Soc.* **1994**, *116*, 562.
[32] F. M. H. de Groot, W. J. Loos, R. Koekkoek, L. W. A. van Berkom, G. F. Busscher, A. E. Seelen, C. Albrecht, P. de Bruijn, H. W. Scheeren, *J. Org. Chem.* **2001**, *66*, 8815.
[33] S. Ogawa, T. Morikawa, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1047.
[34] S. Ogawa, Y. M. Yu, *J. Chem. Soc. Chem. Commun.* **1991**, 890.
[35] T. Suami, Y. Sakota, K. Tadano, S. Nishiyam, *Bull. Chem. Soc. Jpn.* **1971**, *44*, 2222.
[36] J. C. Wilson, M. J. Kiefel, D. I. Angus, M. von Itzstein, *Org. Lett.* **1999**, *1*, 443.
[37] B. Li, S. P. Kawatkar, S. George, H. Strachan, R. J. Woods, A. Siriwardena, K. W. Moremen, G. J. Boons, *ChemBioChem* **2004**, *5*, 1220.
[38] A. D. Elbein, *FASEB J.* **1991**, *5*, 3055.
[39] A. K. Sarkar, T. A. Fritz, W. H. Taylor, J. D. Esko, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 3323.
[40] J. W. Dennis, K. Koch, S. Yousefi, I. Vanderelst, *Cancer Res.* **1990**, *50*, 1867.
[41] Treatment of compound **1** with cell lysate in the presence of the mannosidase inhibitors swainsonine and kifunensine did not lead to significant decomposition, therefore indicating that proteases or other enzymes do not decompose the prodrug.