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Abstract

The severe side effects associated with the use of anthracycline anticancer agents continues to limit their use. Herein we describe the synthesis and preliminary biological evaluation of three enzymatically activatable doxorubicin-oligosaccharide prodrugs. The synthetic protocol allows late stage variation of the carbohydrate and is compatible with the use of disaccharides such as lactose as well as more complex oligosaccharides such as xyloglucan oligomers. The enzymatic release of doxorubicin from the prodrugs by both protease (plasmin) and human carboxylesterases (hCE1 and 2) was demonstrated *in vitro* and the cytotoxic effect of the prodrugs were assayed on MCF-7 breast cancer cells.

Introduction

Despite continuing efforts in oncogenic drug development, surgery remains an important form of treatment for solid tumours. This approach generally includes a postoperative drug therapy to prevent reoccurrence of the tumour. Currently used drugs for such adjuvant treatment include the anthracycline antibiotic Doxorubicin (Dox).[1] While the details of the mechanism of action of anthracyclines are still under investigation,[2] the cytotoxic activity is suggested to be a result of intercalation with DNA[3] and inhibition of topoisomerase II.[4] Due to the unselective nature of the anthracyclines, treatments are often associated with severe side effects such as cardiac toxicity.[5] This has led to extensive efforts in reducing the systemic toxicity of Dox and other anticancer drugs by different types of prodrug approaches.[6] In these strategies, the drug is modified in a way that minimizes its off target activity while allowing activation at the intended site. Prodrug approaches for Dox include both non-covalent and covalent modifications. A notable example of noncovalent prodrugs of Dox is the inclusion of Dox in liposomes.[7] Lipid encapsulation of the drug contributes to decreasing toxic side effects, since liposomes can penetrate endothelial lesions found in the neovasculature of tumors but not in normal blood vessels. FDA approved pegylated and non-pegylated liposomal Dox formulations, namely Doxil® and Myocet® respectively for treating Aids related Kaposi's sarcoma, ovarian cancer and breast cancer.[8] The covalent approach typically involves modification either on the primary amine to form carbamates,[9, 10] or on the ketone functionality by forming hydrazones. The pH-sensitive hydrazone can be cleaved in the acidic microenvironment of the tumour[11] and the carbamate can be attached to an enzymatically hydrolyzable oligopeptide. Both approaches have been used to design cell-targeting prodrugs through the conjugation to antibodies.[12, 13]

The diverse functionalities of carbohydrates make them potential candidates for use in prodrugs. Amazingly, there are very few examples of glycosylated prodrugs reported in the literature to date. Carbohydrates have mainly been used as a triggering group for the drug release since they can be hydrolyzed by the glycosidases overexpressed by cancer cells. The

most prominent example is that of glucuronic acid[14, 15] that is degraded by β-glucuronidase which have been found at high levels in tumour tissue.[16] Assets of carbohydrates are not restricted to their biocompatibility and biodegradability. They also present a large range of remarkable biological and physico-chemical properties. Carbohydrate-protein interactions play an important role for regulating biochemical processes, such as cell differentiation, proliferation and adhesion, inflammation, and immune response. The feasibility of using carbohydrate ligands to target protein receptors, termed 'glycotargeting', was first demonstrated in 1971.[17] Since then the potential of using carbohydrates to design drug-delivering systems has been made clear. However, small molecule drugs, no matter how heavily glycosylated they are, have the potential to pass into the kidneys, through glomerular filtration, and be rapidly cleared. For this reason, much effort are devoted to designing macromolecular glycosylated carriers and there is little work on the development of such glycotargeting prodrugs.[18]

Carbohydrates also interact with other carbohydrates through the establishment of an extensive network of hydrogen bondings and of hydrophobic interactions. In particular, polysaccharide-polysaccharide interactions play an important role in the control of the architecture of animal and plant cells. Cellulose and xyloglucan (XG) assemble to form the cellulose/XG network, which is considered the dominant load-bearing structure in the growing cell walls of non-graminaceous land plants. In a biomimetic approach, Brumer et al. reported the efficient modification of cellulosic materials with different molecules including enzymes and the cell adhesion peptide RGD through anchorage of xyloglucan-based glycoconjugates at their surface.[19, 20] A particular advantage of such non-covalent modification is to circumvent the limited reactivity of insoluble cellulose and to preserve the fibre's integrity.

In the present work, we report the synthesis and preliminary biological evaluation of new enzymatically activated Dox glyco-prodrugs aimed at being administered *in situ* at the tumor site after resection. The carbohydrates, lactose and XGO, the oligosaccharide-repeating unit of xyloglucan, were chosen to address two specific roles. Lactose binds to galectins, a family of β-galactoside binding proteins associated with tumour malignancy.[21, 22] Interaction between lactose and galectins is expected to decrease diffusion of the glyco-prodrug and to hamper its rapid clearance thus allowing a sustained release of the Dox when administered in solution. On the other hand, the use of XGO could allow the immobilization of the Dox prodrug onto cellulosic wound dressing. The synthetic route consisted in the conjugation of furan-modified carbohydrates and maleimide-activacted Dox by a metal free Diels Alder cycloaddition (Figure 1). We have previously used thiol-maleimide Michael-type addition[23] coupling for conjugation of reducing-end functionalized carbohydrates. While being a metal-free reaction, this reaction requires handling sulfurylated molecules susceptible to oxidation. Another type of ligation reaction is the Diels-Alder cycloaddition.[24] The inverse electron demand Diels–Alder reaction (iEDDA) between 1,2,4,5-tetrazines and olefins has increasingly gained importance in chemical biology as a fast and metal-free bioorthogonal ligation reaction.[25, 26] Furan-maleimide cycloaddition is another type of popular Diels-Alder reaction having the advantage of using widely available reactants. This conjugation method recently allowed us to prepare glycosylated chromatographic matrices for the capture of carbohydrate-binding proteins.[27] This reaction allows catalysis-free introduction of the carbohydrate part in water or in organic solvent at a late stage, avoiding extensive carbohydrate protecting group strategies. The presence of a peptide- or ester-containing spacer arm between the maleimide group and the anthracycline should allow the enzymatic release of the Dox by endogenous peptidases or esterases.
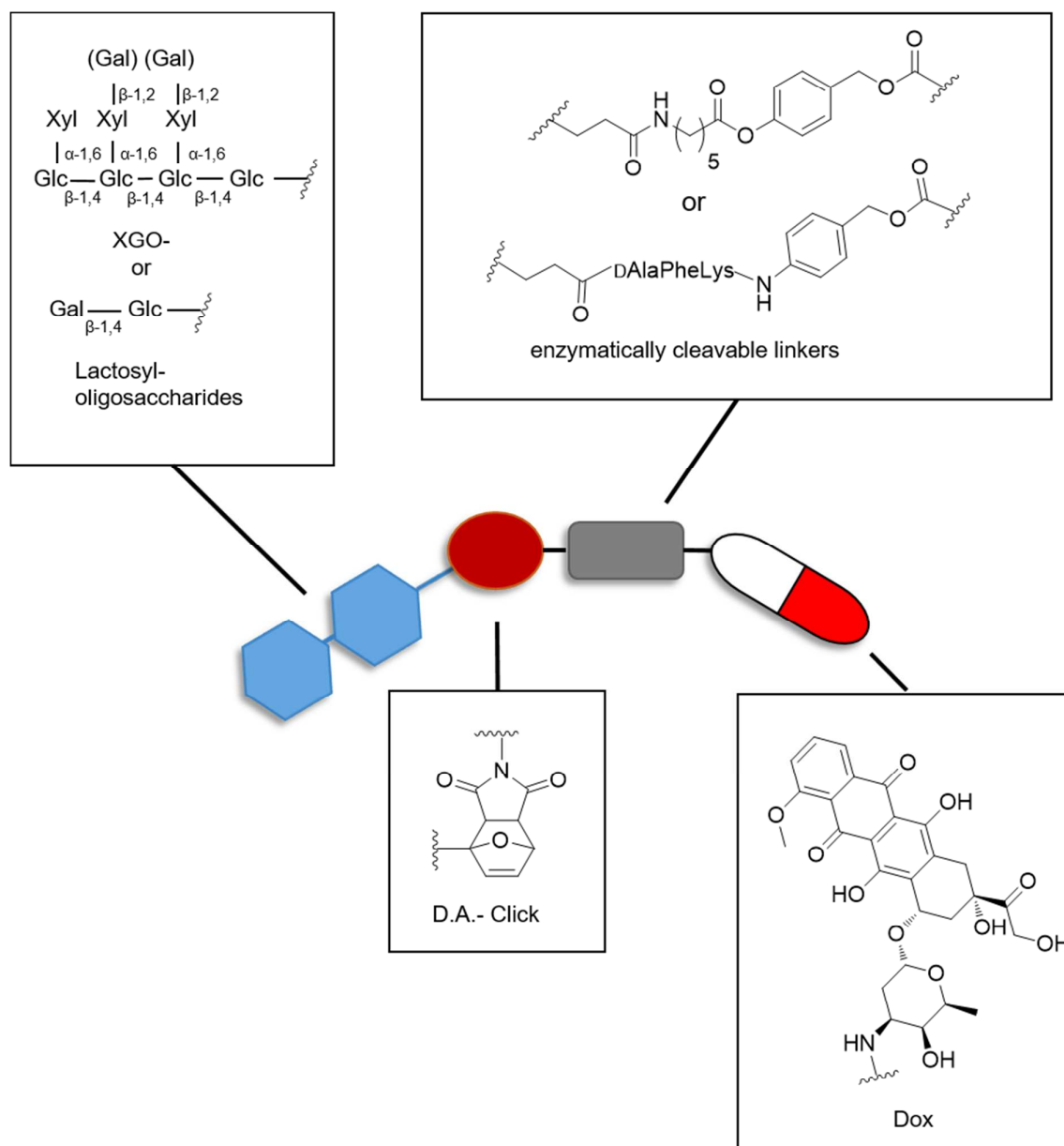
Figure 1. General scheme of the enzymatically activated glyco-prodrugs of Dox. D.A.=Diels–Alder.

Results and Discussion

Synthesis

We sought to obtain two types of glyco-prodrugs of Dox with different activating mechanisms. Our first strategy was to link the drug to the carbohydrate part through ester formation, permitting the release of the drug by esterases, particularly human carboxylesterase 2 that is commonly expressed in tumor tissues.[28] The ester functionality is linked to the primary amine of Dox by a *p*-aminobenzyl alcohol (PABA) spacer. Hydrolysis of the ester bond will initiate a cascade reaction of this so-called self-immolative spacer, first reported by Katzenellenbogen,[29] ultimately resulting in the release of Dox. Our second strategy sought to enable Dox release by specific proteases. To this goal, we chose a tripeptide linker (D-Ala-Phe-Lys)[30] known to be cleaved by plasmin, which has been identified as a potential target

protease for prodrug activation of cancer drugs.[31] This linker has previously been used in combination with the PABA self-immolative linker in the synthesis of Dox[32] and Dox derivative prodrugs.[33] The latter were shown to be stable in human plasma and poorly membrane permeable, thereby limiting activation to locally secreted proteolytic activity and reducing the likelihood of severe side effects.

Starting with the linker drug end of the prodrugs, the synthesis of the maleimidyl ester linked Dox (MalEsterDox **1**) was performed starting from ω-aminocapronic acid **2** (Scheme 1). The five-carbon spacer length provided by the use of ω-aminocapronic acid has previously been reported for enzymatically cleaved prodrugs of Dox.[34]
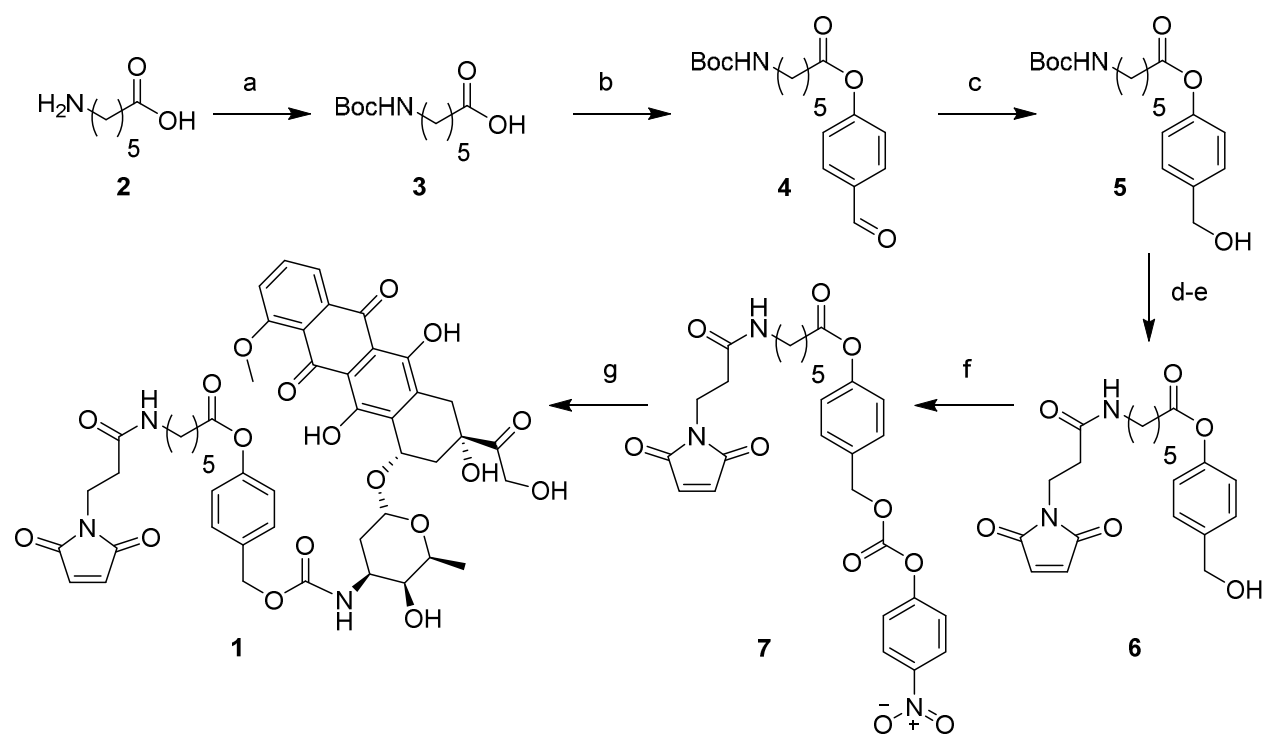

Scheme 1. Synthesis of **1**. a) (Boc)$_2$O (1.1 equiv.) in NaOH(aq.), 1,4-dioxane 1:2, 16 h, 89%. b) DCC (1 equiv.), DMAP (20 mol%), *p*-methoxybenzaldehyde (0.95 equiv.) in CH$_2$Cl$_2$, 0 □ to r.t., 21 h, 86%. c) NaBH$_4$ (0.95 equiv.) in CF$_3$CH$_2$OH, 0 □, 1 h 20 min, 90%. d) TFA: CH$_2$Cl$_2$ 1:1, r.t. 20 min, then e) 3-(*N*-maleimido)propionic acid (1.1 equiv.), DCC (1.1 equiv.), NMM (3 equiv.) in CH$_2$Cl$_2$, 0 □ to r.t., 17 h, 51% over 2 steps. f) Bis(4-nitrophenyl) carbonate (1.5 equiv.), DIPEA (3 equiv.) in CH$_2$Cl$_2$, r.t., 4 h, 70%. g) Dox*HCl (0.9 equiv.), Et$_3$N (2 equiv.) in DMF, r.t., 22h, 59%.

tert-Butyloxycarbonyl (*t*Boc) protection of the amine with di-(*tert*-butyl)dicarbonate in the presence of sodium hydroxide (NaOH) gave carboxylic acid **3** which was coupled with *p*-hydroxybenzaldehyde by Steglich esterification to give ester **4** in 86% yield. The next step was the reduction of the aldehyde to the corresponding alcohol. Performing the reduction with NaBH$_4$ in THF at 0°C resulted in multiple products as observed by TLC already after 15 min. Trifluoroethanol has previously been reported as a co-solvent for NaBH$_4$ reductions.[35, 36] Reduction of aldehyde **4** with NaBH$_4$ in trifluoroethanol at 0°C resulted in full conversion

after one hour and allowed us to isolate hydroxyl **5** in high yield (90%). Next, the maleimide (Mal) was introduced by *t*Boc deprotection with trifluoroacetic acid (TFA) followed by amidation with 3-maleimidopropionic acid in the presence of dicyclohexylcarbodiimide (DCC) and *N*-methylmorpholine (NMM). MalEsterOH **6** was isolated in 51% over two steps. To introduce the carbamate-linked Dox, the hydroxyl of **6** was converted to a mixed anhydride by reaction with bis *p*-nitrophenoxyanhydride in the presence of *N,N*-diisopropylethylamine (DIPEA) in dichloromethane (DCM) (70% yield). Stirring **7** in dimethylformamide (DMF) at room temperature (r.t.) with Dox in the presence of triethylamine resulted in MalEsterDox **1** in 59% yield.

The synthesis of the peptide-linked glyco-prodrug started with the synthesis of the D-Ala-Phe-Lys tripeptide. The synthesis of this tripeptide has previously been described both in solution[32] and on solid support.[33] Our synthetic strategy for the Dox tripeptide motif started from N_α-Fmoc-N_ε-Alloc-Lysine **8** which was activated by *iso*-butylchloroformate and functionalized with *p*-aminobenzylalcohol to give **9** in 72 % yield (Scheme 2).
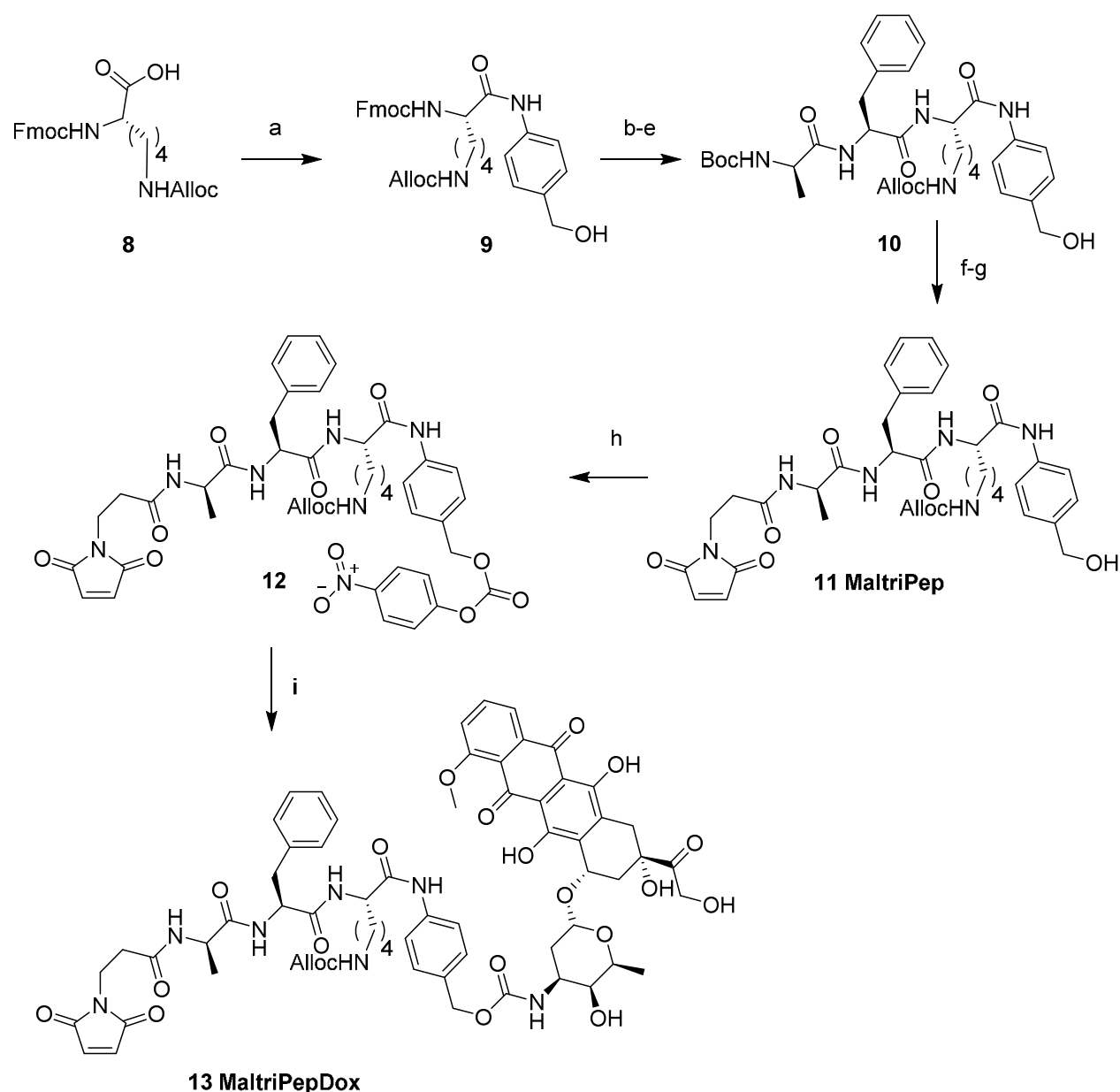
Scheme 2. Synthesis of **13**. a) *i*butylchloroformate (1.1 equiv.), *p*-aminobenzylalchohol (1.2 equiv.), NMM (2.3 equiv.) in THF, - 40 □, 72%. b) 1,4-dioxane:MeOH: 2M NaOH (aq.), r.t., 20 min, then c) DMTMM (1 equiv.), FmocPheOH (1 equiv.), NMM (1.1 equiv.) in CH₃CN, r.t., 19h, then d) 1,4-dioxane:MeOH: 2M NaOH (aq.), r.t., 20 min, then e) DMTMM (1 equiv.), Boc-D-AlaOH (1 equiv.), NMM (1.1 equiv.) in CH₃CN, r.t., 14 h, 47% over 4 steps. f) TFA: CH₂Cl₂ 0.8:1, r.t., 15 min, then g) DMTMMBF₄ (1.1 equiv.), 3-(*N*-maleimido)propionic acid (1.2 equiv.), NMM (1.1 equiv.) in CH₃CN, r.t., 22 h, 43% over two steps. h) bis(4-nitrophenyl) carbonate (5 equiv.), DIPEA (3 equiv.) in DMF, r.t., 46 h then i) Dox*HCl (0.9 equiv.), Et₃N (2 equiv.) in DMF, r.t., 24h, 75%.

The tripeptide linker was synthesized by solution phase peptide synthesis with *N*-Fmoc protected amino acids using 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMMBF₄)[37, 38] as coupling reagent followed by deprotection with NaOH (aq.). The tripeptide **10** was obtained in 47% yield over 4 steps. The maleimide was

introduced in a similar way as for the previously described linker by TFA deprotection of the *t*Boc followed by amidation to give MaltriPep **11** in 43% yield over two steps. The hygroscopic nature of the tripeptide in combination with the risk of hydrolyzing the maleimide made it necessary to thoroughly dry (high vacuum pump) and store all following intermediates below -15□. As for the ester-based linker described above, the hydroxyl function of **11** was activated as a mixed anhydride by reaction with five equivalents of bis *p*-nitrophenoxy carbonate in the presence of DIPEA. The mixed anhydride **12** was isolated by precipitation in diethyl ether and was used in the following substitution without further purification. Stirring **12** with Dox and Et$_3$N in DMF at room temperature gave MaltriPepDox **13** in 75% yield and high purity as confirmed by HPLC (see SI).

The next step was the conjugation of the drug-linker part with the carbohydrate moiety. The furfuryl-functionalized carbohydrates were obtained through a two steps method for synthesizing *N*-glycosylamides first introduced by Lokhoff et al.[39] and utilized in our group to synthesize a range of reducing-end functionalized oligosaccharides.[23, 27] The unprotected carbohydrate was stirred in neat furfurylamine for 72 hours to afford a transient glycosylamine that was made non-hydrolyzable by acylation of the nitrogen with acetic anhydride. The synthesis of furfuryl-lactose **14** was previously published for the production of conjugatable glycosaminoglycans.[40] The furfuryl-XGO **15** was synthesized for this study from the oligosaccharide-repeating unit of tamarind seed xyloglucan. First, the XGO repeating unit, which consists in a mixture of hepta-, octa- and nona-saccharides in a ratio of 15/35/50, was obtained quantitatively by enzymatic hydrolysis of the polysaccharide as reported previously.[41] The furfuryl-XGO was then obtained in 90% yield following the same protocol as for lactose. The conjugation of MalEsterDox **1** with furfuryl-lactose **14** in DMF and water (1:1) at 37 ºC gave LacEsterDox **16** in high yield (91%, Scheme 3). The XGOEsterDox prodrug **17** was prepared under similar reaction conditions but was isolated in 35% yield only after purification on reverse phase C$_{18}$ cartridges. The yield is lower than typically observed in this type of cycloaddition and is partly explained by extensive chromatographic purification. The reaction conditions and the purification procedure should be optimized but sufficient quantities for drug release and cell toxicity studies were isolated and no attempt to improve the yield have been done so far.
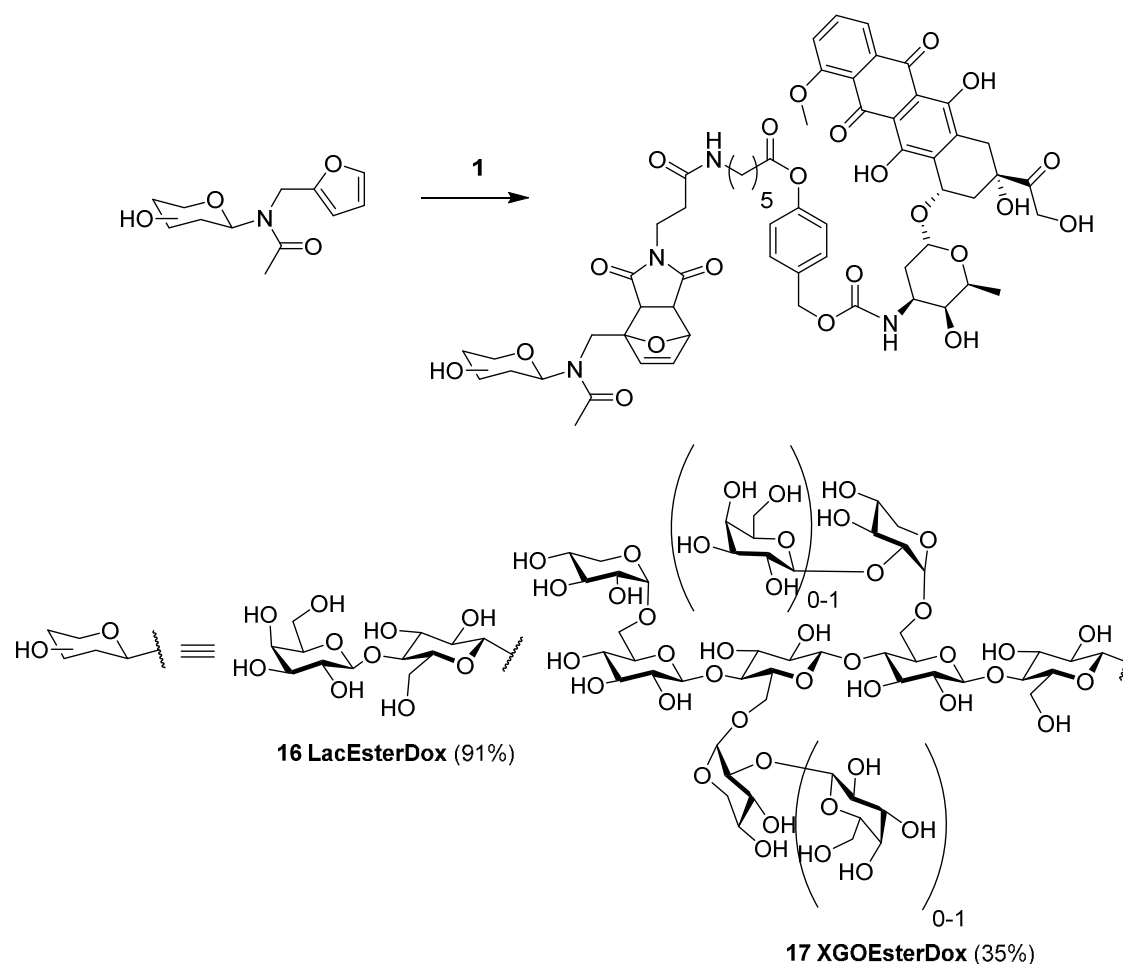
Scheme 3. Synthesis of **16** and **17**. DMF:H₂O 1:1, 37 ºC, 3 and 4 days for **16** and **17**, respectively.

To obtain the tripeptide linked glyco-prodrug (LactriPep(Alloc)Dox) (**18,** Scheme 4), **13** and furfuryl-lactose **14** were stirred in a mixture of DMF and H₂O (2:1) at 37□ for 7 days. **18** (57% yield) was isolated in high purity as confirmed by HPLC (see SI) after purification on silica eluting with 10% water in acetonitrile.
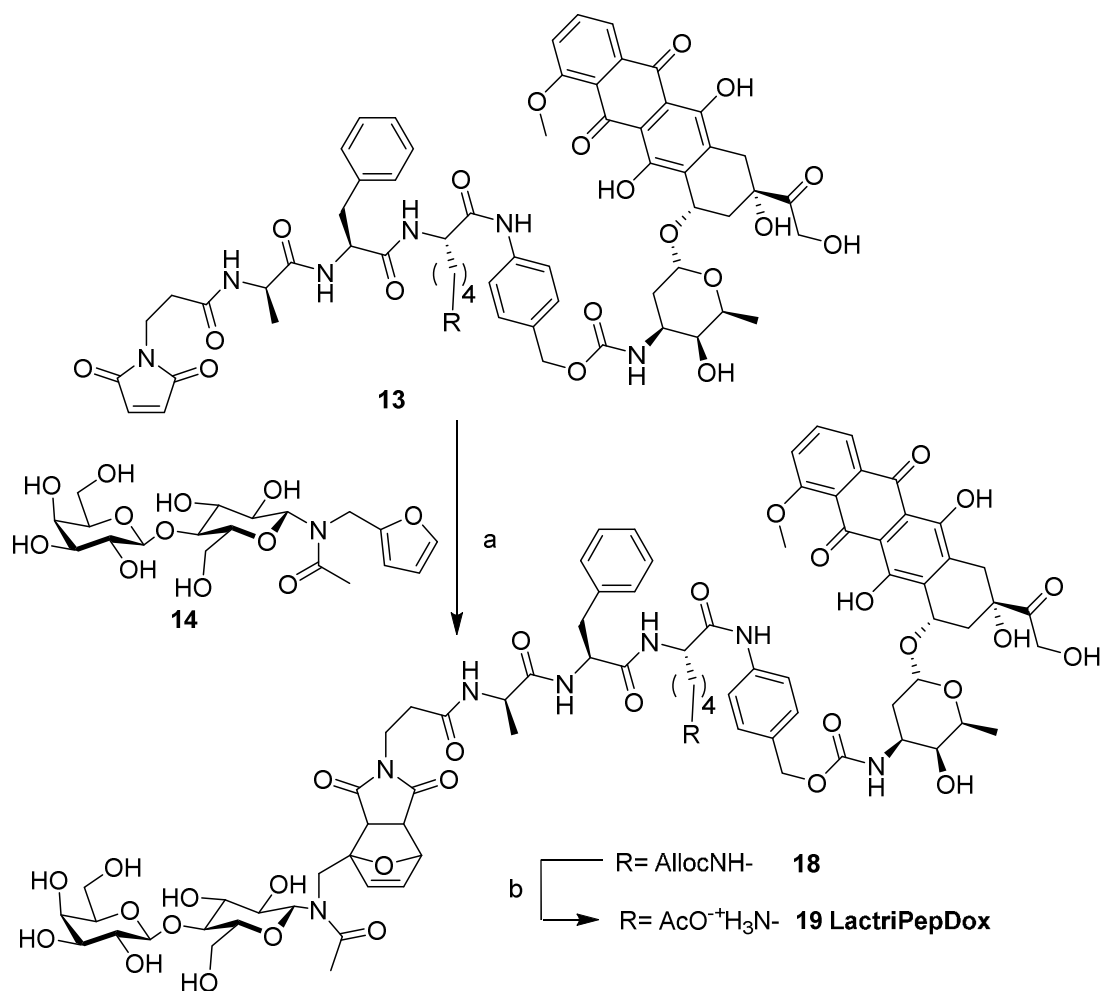
Scheme 4. Synthesis of **19.** a) DMF:H$_2$O 2:1, 37□, 7 days, 58%. b) Pd(PPh$_3$)$_4$ (1.2 equiv.), CH$_2$Cl$_2$:AcOH 5:1, r.t., 30 min, 59%.

The last step of removal of the Alloc protecting group from the lysine side chain was more tricky. Commonly used conditions for Alloc removal include catalytic amounts of Pd^0 in the presence of an allyl scavenger such as morpholine, tributyltin hydride or silane.[42] However, using catalytic amounts of Pd(PPh$_3$)$_4$ (20 mol%) and morpholine as allyl scavenger, we were unable to isolate the desired product, only observing degradation on TLC. Eliminating the scavenger and running the reaction with equimolar amount of Pd(PPh$_3$)$_4$ in a mixture of acetic acid and dichloromethane as described by Barthel et al.[33] gave the desired product **19** (LactriPepDox) in 59% yield after precipitation from diethyl ether.

Enzymatic release of Dox from glyco-prodrugs
To confirm the intended enzymatic release of Dox, the LactriPepDox **19** (100 µM) was incubated *in vitro* in the presence 15 µg/ml of human plasmin at 37 ºC in phosphate buffer (pH 7.5). The disappearance of prodrug and appearance of Dox was followed by HPLC (Figure 2). We first checked the stability of the prodrugs in these conditions in the absence of plasmin (no release after up to 25h at 37°C, data not shown). In the presence of plasmin, nearly full conversion of the prodrug into Dox was achieved after 2 h.
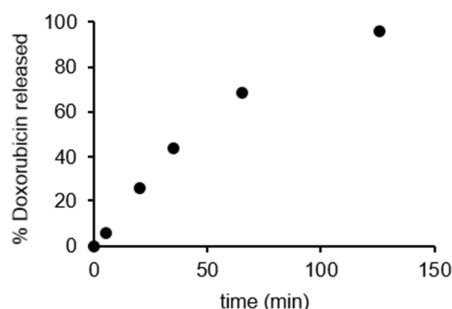
Figure 2. *In vitro* protease mediated release of Dox (•) from LactriPepDox **19** (100μM) using 15µg/ml plasmin in phosphate buffer (pH 7.5) at 37 ºC.

The release of Dox from LacEsterDox **16** was performed in 50 mM Tris buffer (pH 7.4). Incubating **16** (100µM) at 37 ºC in the presence of human carboxylesterase 1 (hCE1) (100 µg/ml) resulted in only minor (~5%) release of Dox after 22 h (Figure 3).
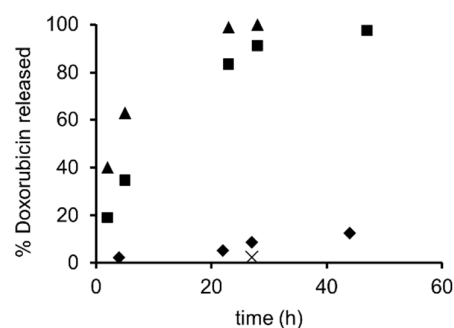
Figure 3. *In vitro* esterase mediated release of Dox using 100μg/ml esterase in 50 mM Tris buffer (pH 7.4) at 37 ºC from LacEsterDox **16** with hCE2(▲), from LacEsterDox **16** with hCE1(♦), from XGOEsterDox **17** with hCE2(■) and LacEsterDox **16** without enzyme (x).The values for hCE2 measurements are mean values of duplicate experiments.

In contrast, incubating **16** with human carboxylesterase 2 (hCE2) under identical conditions resulted in full release after 23h. The rate of hydrolysis for XGOEsterDox **17** was slightly slower than for **16** (83% after 23h), possibly owing to the steric effect of the larger oligosaccharide. Nevertheless, after 48h, 98% of **17** had been hydrolyzed. The difference in rate between hCE1 and hCE2 could be explained by the presence of a phenyl on the alcohol side since hCE2 is known to hydrolyze esters with bulkier alcohol groups at a higher rate than hCE1.[43] As was observed for the tripeptide prodrug **19**, incubating the ester prodrug (LacEsterDox) in the absence of esterase resulted in less than 2% Dox after 27h (data not shown). These results confirm that Dox is enzymatically released from prodrugs **16, 17** and **19** and that these three prodrugs are stable in the buffers in absence of enzyme.

Biological evaluation
As a preliminary test for verifying the biological interest of the prodrugs, the cytotoxicity of compounds **16, 17** and **19** was assayed on MCF-7 breast cancer cells and compared with that of free Dox (Table 1).

Compound	IC$_{50}$ (µM)
16	0.51 ± 0.07
17	0.31 ± 0.15
19	1.11 ± 0.11
Dox	0.53

Table 1. Inhibition of MCF-7 breast cancer cell growth by the glyco-prodrugs and free Dox as reference. IC$_{50}$ values were calculated as the concentration of compound needed to reduce cell growth by 50%. Values are reported as mean ± the standard deviation (SD) of triplicate measurements.

The plasmin-activated prodrug **19** displayed a slightly higher (~2 fold) IC$_{50}$ compared to Dox while the esterase activated prodrugs **16** and **17** showed IC$_{50}$ values similar to that of the parent drug. Since it has been previously shown that protection of the primary amine of Dox results in a significant decrease in activity,[44] these results are a clear indication of Dox release from prodrugs **16, 17** and **19** in presence of the MCF-7 cells. One would have probably expected a more pronounced difference between the IC$_{50}$ of the ester and amide prodrugs as plasmin (which is not over-expressed on MCF-7 cells) has not been added in the cell culture medium), and further experiments are now required to study the mode of action of the glyco-prodrugs. Nevertheless, the similar IC$_{50}$ obtained for XGOEsterDox **17** and LacEsterDox **16** are very encouraging since they show that the release of Dox is not dependent on the size and therefore steric hindrance of the oligosaccharide moiety. In a forthcoming work, we wish to implement the use xyloglucan glycoconjugates to the development of drug-functionalized cellulosic biomaterials.

Conclusion

We have synthesized a small series of enzymatically activatable glyco-prodrugs of Dox. We demonstrated the release of the drug by enzymatic cleavage *in vitro* and *in cellulo* on MCF-7 cancer cells, as the prodrugs and Dox itself display similar IC$_{50}$. The late stage metal free cycloaddition used to ligate the oligosaccharide and the drug allows this methodology to be used with different oligosaccharides and potentially with different drugs. Current work in our groups includes extending this ligation to synthesize prodrugs of other drug classes.

Experimental procedures

General considerations

All commercial chemicals were used without prior purification. ^{1}H- and ^{13}C-NMR spectra were obtained at 400 and 101 MHz respectively, using a Bruker Advance DRX 400 spectrometer. Solvent residual peaks were used as reference and calibrated as follows; CDCl$_3$: 7.26ppm; MeOD-d_4: 3.31ppm; DMSO-d_6: 2.50 ppm. Reactions were monitored by TLC (Merck Silica gel 60 F$_{254}$) and analyzed under UV (254 nm). Reactions involving carbohydrates were followed by staining TLC plates with H$_2$SO$_4$: MeOH : H$_2$O (3:45:45). Column chromatography was performed by manual flash chromatography (wet-packed silica, 0.04-0.063 mm) or by automated column chromatography on a Grace Reveleris instrument using prepacked silica columns. Analytical high performance liquid chromatography (HPLC) was performed using a Waters µBondapak C$_{18}$-column (3.9 x 300mm, 125Å pore size, 10 µm particle size) connected to a Waters photodiode array detector 996. HPLC method A: 10-

100% MeOH in water (phosphoric acid pH 2.5) over 5 min, 2 ml/min. HPLC method B: 0-100% MeOH in water (phosphoric acid pH 2.5) over 5 min, then 100% water for 5 min, 2 ml/min. Mass analyses (ESI) were recorded on a Bruker Daltonics Esquire 3000+ instrument and HRMS analyses were obtained on a Waters Xevo G2-S QTOF instrument.

6-((*tert*-Butoxycarbonyl)amino)hexanoic acid (**3**)

Following a published procedure,[45] NaOH (4.0 g, 0.1 mol) was dissolved in water (130 ml) and 1,4-dioxane (260 ml) was added. ω-Aminocapronic acid (13.1 g, 99.9 mmol) was added, allowed to dissolve and the solution was cooled to 0 °C. Di-*tert*-butyl dicarbonate (24.0 g, 110 mmol) was added in three portions, the reaction mixture was stirred at 0 °C for 30 min and then at r.t. for 16 h. The 1,4-dioxane was removed by rotary evaporation and the crude was diluted with water (400 ml) and washed with EtOAc (2 x 200 ml). The aqueous phase was acidified by addition of 1 M HCl (aq.) and extracted with EtOAc (3 x 300 ml). The organic phases from the second extraction were collected and dried over $MgSO_4$. Removal of the solvents gave the expected product **3** as an oil that crystallizes to light pink crystals (20.6 g, 89% yield). R_f: 0.20 (2% MeOH in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.85 (br s, 1H), 5.97 (br s, 0.3H, -CONH-), 4.63 (br s, 0.7H, -CONH-), 3.08 (br s, 2H), 2.32 (t, J = 7.4 Hz, 2H), 1.62 (p, J = 7.5 Hz, 2H), 1.53 – 1.18 (m, 13H) ; ESI-MS m/z [M+Na]$^+$: 254.2.

4-Formylphenyl-6-((tert-butoxycarbonyl)amino)hexanoate (**4**)

3 (10.0 g, 43.2 mmol) was dissolved in dry CH_2Cl_2 (43 ml) in a two necked flask under argon. DCC (8.9 g, 43.2 mmol) and DMAP (1.0 g, 8.2 mmol) were added, the reaction mixture was cooled to 0 °C and *p*-hydroxybenzaldehyde (5.0 g, 41.2 mmol) was immediately added. The reaction was stirred at 0 °C for 30 min and then at r.t. for 21 h. The white precipitate that formed was filtered off and washed twice with a small volume of CH_2Cl_2. The solvents were removed from the filtrate which gave an off-white solid that was redissolved in CH_2Cl_2 (200 ml), washed with 0.5 M HCl (50 ml), sat. $NaHCO_3$ (50 ml) and brine (50 ml). The organic phase was dried over $MgSO_4$ and filtered. Removal of the solvents by rotary evaporation gave the crude as an off white solid. Purification by column chromatography on silica eluting with 2% MeOH in CH_2Cl_2 gave **4** as a white solid (12.5 g, 86% yield). R_f: 0.36 (2% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.98 (s, 1H), 7.95-7.86 (m, 2H), 7.30-7.20 (m, 2H), 4.57 (br s, 1H), 3.14 (q, J = 6.7 Hz, 2H), 2.59 (t, J = 7.4 Hz, 2H), 1.77 (p, J = 7.5 Hz, 2H), 1.61 – 1.49 (m, 2H), 1.48 – 1.38 (m, 11H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 191.0, 171.4, 156.1, 155.5, 134.1, 131.3, 122.5, 79.3, 40.5, 34.3, 29.9, 28.5, 26.3, 24.5; MS (ESI) m/z: [M+Na]$^+$: 358.3 HRMS m/z [M+Na]$^+$ calculated for $C_{18}H_{25}NO_5Na$: 358.1630, found: 358.1627.

4-(Hydroxymethyl)phenyl-6-((tert-butoxycarbonyl)amino)hexanoate (**5**)

4 (1.00 g, 2.98 mmol) was dissolved in trifluoroethanol (6 ml) and the flask was cooled on ice. $NaBH_4$ (107 mg, 2.83 mmol) was added and the reaction was stirred on ice. Full consumption of **4** was observed after 1 h on TLC (2% MeOH in CH_2Cl_2). The reaction was kept on ice and quenched by slow addition of sat. NH_4Cl (7 ml) after 1h 20 min. The mixture was allowed to reach r.t. and stirred for 40 min. Water (5 ml) was added and the aqueous phase was extracted with EtOAc (3 x 30 ml). The organic phases were pooled, dried over Na_2SO_4 and filtered. Removal of the solvents gave a colorless oil, which was purified by flash column chromatography on silica eluting with 1% MeOH in CH_2Cl_2 to obtain **5** as a white solid (900 mg, 90% yield). R_f: 0.32 (2% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37-7.29 (m, 2H), 7.06-6.99 (m, 2H), 4.62 (s, 3H,-CH_2- and –CONH-), 3.10 (q, J = 6.6 Hz, 2H), 2.5 3 (t, J = 7.4 Hz, 2H), 2.49 (br s, 1H), 1.74 (p, J = 7.5 Hz, 2H), 1.56 – 1.46 (m, 2H), 1.45 – 1.35(m, 11H) ; ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.2, 156.1, 150.0, 138.7, 128.0,

121.6, 79.2, 64.6, 40.4, 34.3, 29.8, 28.5, 26.3, 24.6; MS (ESI) *m/z*: $[M+Na]^+$: 360.2; HRMS *m/z* $[M+Na]^+$ calculated for $C_{18}H_{27}NO_5Na$: 360.1787, found: 360.1786.

MalEsterOH (**6**)

5 (400 mg, 1.19 mmol) was dissolved in dry CH_2Cl_2 (1.5 ml) and TFA (1.5 ml) was added. The reaction was stirred at r.t. for 20 min. Full consumption of **5** was observed by TLC (10% MeOH in CH_2Cl_2). Solvents were removed and coevaporated four times with CH_2Cl_2. The resulting clear oil was redissolved in dry CH_2Cl_2 (2 ml) and basified (pH approx. 8) by addition of *N*-methylmorpholine (400 µl). 3-(Maleimidyl)propanoic acid (220 mg, 1.30 mmol) was added, the reaction was cooled on ice and DCC (268 mg, 1.30 mmol) was added. The ice bath was removed and the reaction was stirred at r.t. for 17 h. The white precipitate that had formed was filtered off and the filtrate was concentrated. The yellow oil that resulted was redissolved in CH_2Cl_2 (20 ml), washed with 0.5 M HCl (5 ml) and dried over $MgSO_4$ and filtered. Removal of the solvents gave a white solid, which was purified by flash column chromatography on silica eluting with 25% cyclohexane in EtOAc to obtain **6** as a white solid (236 mg, 51% yield). R_f: 0.52 (10% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41-7.34 (m, 2H), 7.09-7.02 (m, 2H), 6.68 (s,2H), 5.74 (br s, 1H), 4.67 (s, 2H), 3.81 (t, *J* = 7.2 Hz, 2H), 3.28-3.19 (m, 2H), 2.55 (t, *J* = 7.3 Hz, 2H), 2.48 (t, *J* = 7.2 Hz, 2H), 1.86 (br s, 1H), 1.75 (p, *J* = 7.4 Hz, 2H), 1.58 – 1.48 (m, 2H), 1.46 – 1.36 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ172.3, 170.7, 169.6, 150.2, 138.7, 134.3, 128.2, 121.8, 64.8, 39.4, 34.9, 34.4, 34.2, 29.2, 26.4, 24.5; MS (ESI) *m/z*: $[M+Na]^+$: 411.3; HRMS *m/z* $[M+H]^+$ calculated for $C_{20}H_{25}N_2O_6$: 389.1713, found: 389.1714.

MalEsterPNP (**7**)

6 (178 mg, 0.46 mmol**)** and bis(*p*-nitrophenyl)carbonate (210 mg, 0.69 mmol) were added to a dry flask filled with argon which was capped, evacuated and refilled with argon (repeated 3 times). Dry CH_2Cl_2 (4.6 ml) was added followed by diisoproylethylamine (245 µl, 1.38 mmol). The yellow reaction mixture was stirred at r.t. for 4 h in the dark. Full consumption of **6** was observed on TLC (2% MeOH in CH_2Cl_2). The reaction mixture was diluted with CH_2Cl_2 (25 ml) and washed with aq. citric acid (10 % w/v). The organic phase was dried over Na_2SO_4 and filtered. Removal of the solvents gave a yellow oil. DEE (25 ml) was added and brief sonication resulted in a white precipitate. The precipitate was filtered off, washed with DEE repeatedly, collected and redissolved in a minimum of CH_2Cl_2. The solution was added dropwise to stirred DEE (25 ml). The white precipitate that formed was collected by filtration and dried to give **7** as a white solid (178 mg, 70% yield). R_f: 0.28 (2% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31-8.23 (m, 2H), 7.50-7.43 (m, 2H), 7.41-7.35 (m, 2H), 7.16-7.09 (m, 2H), 6.70 (s, 2H), 5.64 (br s, 1H), 5.28 (s, 2H), 3.84 (t, *J* = 7.2 Hz, 1H), 3.31-3.20 (m, 2H), 2.58 (t, *J* = 7.4 Hz, 1H), 2.51 (t, *J* = 7.1 Hz, 1H), 1.82-1.72 (m, 2H), 1.58 – 1.50 (m, 2H), 1.49 – 1.38 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 170.7, 169.6, 155.6, 152.6, 151.3, 145.6, 134.4, 131.9, 130.2, 125.5, 122.2, 121.9, 70.4, 39.4, 34.9, 34.4, 34.3, 29.3, 26.4, 24.5; MS (ESI) *m/z*: $[M+H]^+$: 554.2 and $[M+Na]^+$: 576.2; HRMS *m/z* $[M+H]^+$ calculated for $C_{27}H_{28}N_3O_{10}$: 554.1775, found: 554.1776.

MalEsterDox (**1**)

7 (132 mg, 0.24 mmol) was dissolved in dry DMF (2.2 ml) and the solution was added to Doxorubicin hydrochloride (128 mg, 0.22 mmol). Et_3N (61 µl, 0.44 mmol) was added and the dark red reaction mixture was stirred under argon. After 22 h, the reaction mixture was added dropwise to stirred DEE (75 ml) resulting in a red precipitate which was collected by filtration and further purified by flash column chromatography eluting with 2-5% MeOH in CH_2Cl_2. **1** (124 mg, 59% yield) was isolated as a red foam. R_f: 0.29 (5% MeOH in CH_2Cl_2); ^{1}H NMR

(400 MHz, CDCl$_3$) δ 13.96 (s, 1H), 13.23 (s, 1H), 8.02 (dd, J = 7.7, 1.1 Hz, 1H), 7.82 – 7.73 (m, 1H), 7.39 (dd, J = 8.6, 1.1 Hz, 1H), 7.31 (d, J = 8.0 Hz, 2H), 7.01 (d, J = 8.0 Hz, 2H), 6.68 (s, 2H), 5.73 (s, 1H), 5.49 (d, J = 3.8 Hz, 1H), 5.28 (br s, 1H), 5.22 (d, J = 8.6 Hz, 1H), 5.08-4.91 (m, 2H), 4.75 (dd, J = 5.0, 1.4 Hz, 2H), 4.57 (s, 1H), 4.18 – 4.08 (m, 1H), 4.07 (s, 3H), 3.85 (br s, 1H), 3.81 (t, J = 7.2 Hz, 2H), 3.66 (d, J = 7.2 Hz, 1H), 3.32 – 3.16 (m, 3H), 3.08-2.94 (m, 2H), 2.53 (t, J = 7.3 Hz, 2H), 2.48 (t, J = 7.2 Hz, 2H), 2.39-2.27 (m, 1H), 2.25-2.10 (m, 2H), 1.91 – 1.68 (m, 4H), 1.52 (p, J = 7.1 Hz, 2H), 1.46-1.33 (m, 2H), 1.28 (d, J = 6.5 Hz, 3H); ^{13}C NMR (101 MHz, CDCl$_3$) δ 214.0, 187.2, 186.8, 172.2, 170.7, 169.7, 161.2, 156.3, 155.8, 155.6, 150.6, 135.9, 135.6, 134.3, 134.1, 133.7, 129.5, 121.8, 121.0, 120.0, 118.6, 111.7, 111.6, 110.1, 100.8, 76.8, 69.7, 69.6, 67.5, 66.2, 65.7, 56.8, 47.2, 39.4, 35.8, 34.9, 34.4, 34.2, 34.1, 30.3, 29.2, 26.4, 24.5, 17.0; MS (ESI) m/z: [M+Na]$^+$: 980.3; HRMS m/z [M+H]$^+$ calculated for C$_{48}$H$_{52}$N$_3$O$_{18}$: 958.3246, found: 958.3235.

FmocLys(Alloc)PABOH (**9**)
Following a published procedure,[32] FmocLys(Alloc)OH (1.44g, 3.18 mmol) was dissolved in dry THF (20 ml) under argon in a two-necked flask equipped with an addition funnel. The solution was cooled to -40 °C and *N*-methylmorpholine (0.39 ml, 3.54 mmol) and isobutyl chloroformate (0.45 ml, 3.47 mmol) were added. The turbid mixture was stirred at -40 °C for 3 h after which a solution of *p*-aminobenzylalcohol (470 mg, 3.82 mmol) and *N*-methylmorpholine (0.42 ml, 3.82 mmol) in dry THF (6 ml) was added dropwise over 10 min. The reaction mixture was stirred at -40 °C for 2 h and then at room temperatrure for 1 h. The solvents were removed and the yellow residue was taken up in CH$_2$Cl$_2$ (250 ml). The organic phase was washed with 0.5 M NaHCO$_3$ solution (100 ml), 0.5 M NaHSO$_4$ solution (100 ml) and brine (100 ml). The organic solvents were removed under reduced pressure and the yellow residue was purified by flash column chromatography on silica (2-10% methanol in CH$_2$Cl$_2$) to provide **9** as a white solid (1.28 g, 72% yield). ^{1}H NMR (400 MHz, dmso-d_6) δ 9.96 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.74 (dd, J = 7.5, 4.9 Hz, 2H), 7.62 (d, J = 7.9 Hz, 1H), 7.55 (d, J = 8.4 Hz, 2H), 7.41 (t, J = 7.3 Hz, 2H), 7.37-7.29 (m, 2H), 7.24 (d, J = 8.3 Hz, 2H), 7.19 (t, J = 5.8 Hz, 1H), 5.89 (ddt, J = 17.2, 10.5, 5.3 Hz, 1H), 5.30-5.20 (m, 1H), 5.18 – 5.12 (m, 1H),5.10 (t, J = 5.7 Hz, 1H) 4.52-4.39 (m, 4H), 4.34 – 4.06 (m, 4H), 3.07 – 2.91 (m, 2H), 1.75-1.56 (m, 2H), 1.51 – 1.21 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.0, 156.1, 155.9, 143.9, 143.8, 140.7, 137.5, 137.4, 133.8, 127.6, 127.0, 126.9, 125.3, 120.1, 118.9, 116.8, 65.6, 64.1, 62.6, 55.4, 46.7, 31.6, 29.1, 22.9. HRMS m/z [M+H]$^+$ calculated for C$_{32}$H$_{36}$N$_3$O$_6$: 558.2604, found: 558.2606.

Tripep (**10**)
Compound **9** (560 mg, 1.00 mmol) was dissolved in a premade mixture of 1,4-dioxane:MeOH:2M NaOH(aq.) (14:5:1, 20 ml).[46] After 30 min (TLC, CH$_2$Cl$_2$:MeOH 90:10), the solution was neutralized by addition of acetic acid. The solvents were removed by evaporation and coevaporated with 1,4-dioxane (2 x 20 ml). The oily solid residue was washed with DEE (3 x 10 ml) and then with CH$_2$Cl$_2$ (10 x 10 ml). The CH$_2$Cl$_2$ fractions were collected and the solvent was removed to yield the free amine as a pale yellow oil (326 mg) which was used in the next step without further purification.
A solution of DMTMMBF$_4$ (365 mg, 1.11 mmol), FmocPheOH (390 mg, 1.01 mmol) and *N*-methyl morpholine (121 μl, 1.10 mmol) in CH$_3$CN (5 ml) was stirred for 10 min and then added to a solution of the crude form the previous step (326 mg) in CH$_3$CN (5 ml). The reaction mixture was stirred at r.t for 19 h (TLC, CH$_2$Cl$_2$:MeOH 90:10). The solvents were removed by evaporation and CH$_2$Cl$_2$ (100 ml) was added and the organic phase was washed with water (25 ml), 0.5 M NaHCO$_3$ (25 ml), 0.5 M NaHSO$_4$ (3 x 25 ml) and water (50 ml).

The solvents were removed from the organic phase to give an off-white solid (665 mg) which was used in the next step without further purification.

A premade mixture of 1,4-dioxane:MeOH:2M NaOH(aq.) (14:5:1, 20 ml) was added to the crude from the previous step and the reaction mixture was stirred at r.t. for 50 min (TLC, CH_2Cl_2:MeOH 90:10). The reaction was neutralized by addition of acetic acid. The solvents were removed by evaporation and coevaporated with 1,4-dioxane (20 ml). The resulting white residue was washed with DEE (3 x 5 ml) and then with CH_2Cl_2 (10 x 20 ml). The white precipitate formed in the DEE phase was collected by filtration, dissolved in CH_2Cl_2 and pooled with the other CH_2Cl_2 fractions. The CH_2Cl_2 fractions were collected and the solvent was removed to yield the free amine as an off white solid (463 mg) which was used in the next step without further purification.

A solution of $DMTMMBF_4$ (360 mg, 1.10 mmol), Boc-D-AlaOH (190 mg, 1.00 mmol) and N-methyl morpholine (121 µl, 1.10 mmol) in CH_3CN (5 ml) was stirred for 10 min and then added to a solution of the crude from the previous step (463 mg) in CH_3CN (6 ml). The reaction mixture was stirred at r.t for 14 h (TLC, CH_2Cl_2:MeOH 90:10). The solvents were removed by evaporation and CH_2Cl_2 (70 ml) was added and the organic phase was washed with water (40 ml), 0.5 M $NaHSO_4$ (2 x 20 ml), 0.5 M $NaHCO_3$ (3 x 20 ml) and water (20 ml). The solvents were removed from the organic phase to give an off-white solid, which was purified by flash column chromatography on silica eluting with 2-5% methanol in CH_2Cl_2. The product was dryloaded on to the column after being dissolved in 10% methanol in CH_2Cl_2 and dried on to silica. Compound **10** was isolated as a white solid (309 mg, 47% over 4 steps). ^{1}H NMR (400 MHz, MeOD-d_4) δ 7.61 (d, J = 8.3 Hz, 2H), 7.30 (d, J = 8.5 Hz, 2H), 7.28 – 7.09 (m, 5H), 5.91 (ddt, J = 16.3, 10.7, 5.5 Hz, 1H), 5.28 (dd, J = 17.1, 1.7 Hz, 1H), 5.16 (d, J = 10.5 Hz, 1H), 4.60 (dd, J = 9.8, 4.5 Hz, 1H), 4.56 (s, 2H), 4.50 (d, J = 5.0 Hz, 2H), 4.43 (dd, J = 9.6, 4.9 Hz, 1H), 4.00 (q, J = 7.1 Hz, 1H), 3.30-3.23 (m, 1H), 3.11 (t, J = 6.7 Hz, 2H), 2.95 (dd, J = 14.2, 9.8 Hz, 1H), 2.02 – 1.78 (m, 2H),1.59-1.40 (m, 4H) 1.33 (s, 9H), 1.13 (d, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, MeOD-d_4) δ 176.9, 173.6, 172.2, 158.8, 157.9, 138.8, 138.5, 138.4, 134.5, 130.2, 129.5, 128.5, 127.8, 121.4, 117.4, 80.7, 66.3, 64.8, 56.4, 55.7, 51.8, 41.5, 37.9, 32.3, 30.5, 28.7, 24.5, 17.7. HRMS m/z [M+H]$^+$ calculated for $C_{34}H_{48}N_5O_8$: 654.3503, found: 654.3496.

MaltriPep (**11**)

10 (150 mg, 0.23 mmol) was suspended in dry CH_2Cl_2 (1ml) under argon, TFA (0.8 ml) was added and the solution was stirred for 15 min. Full consumption of **10** was confirmed by TLC (10% methanol in CH_2Cl_2). The solvents were removed and coevaporated with CH_2Cl_2 (3 x 1 ml). The resulting residue was taken up in CH_3CN (3.5 ml) and neutralized by addition of NMM (150 µl). $DMTMMBF_4$ (82 mg, 0.25 mmol), 3-(maleimidyl)propanoic acid (46 mg, 0.27 mmol) and NMM (27 µl, 0.25 mmol) were stirred in CH_3CN (1.5 ml) at r.t. until all material dissolved (~5 min). The solution of deprotected **10** was then added and the reaction mixture was stirred at r.t. for 22 h. The formed precipitate was filtered off and washed with ice cold CH_3CN and DEE. The filtrate was collected, diluted in CH_2Cl_2 (100 ml) and washed with 0.5 M $NaHSO_4$ (30 ml) and water (30 ml). The organic phases were dried over $MgSO_4$ and the solvents were then removed to give a white solid which was pooled with the precipitated material. The crude was purified by flash column chromatography on silica eluting with 5% methanol in CH_2Cl_2. The crude product was dryloaded on to the column after being dissolved in methanol and dried on to silica. The expected product **11** was isolated as a white solid (72 mg, 43% over 2 steps). ^{1}H NMR (400 MHz, MeOD-d_4) δ 7.60 (app d, 2H),7.29 (app d, 2H), 7.28-7.14 (m, 5H), 6.76 (s, 2H) 5.91 (ddt, J = 16.3, 10.6, 5.4 Hz, 1H), 5.28 (dd, J = 17.2, 1.8 Hz, 1H), 5.20 – 5.12 (m, 1H), 4.62 – 4.52 (m, 3H), 4.50 (dt, J = 5.6, 1.6 Hz, 2H), 4.40 (dd, J = 9.6, 5.0 Hz, 1H), 4.14 (q, J = 7.1 Hz, 1H), 3.73-3.57 (m, 2H), 3.30-3.25

(m, 1H, partly overlapping with solvent peak), 3.13 (t, J = 6.7 Hz, 2H), 2.95 (dd, J = 14.2, 10.0 Hz, 1H), 2.47-2.27 (m, 2H), 2.00 – 1.84 (m, 2H), 1.62 – 1.36 (m, 4H), 1.14 (d, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, MeOD-d_4) δ 176.2, 173.7, 173.2, 172.4, 172.1, 158.8, 138.8, 138.5, 135.4, 134.6, 130.2, 129.5, 128.5, 127.8, 121.4, 117.4, 112.9, 66.3, 64.8, 56.7, 55.8, 51.1, 41.5, 37.8, 35.2, 35.1, 32.3, 30.5, 24.5, 17.1. MS (ESI) m/z: [M+H]$^+$: 705.4 and [M+Na]$^+$: 727.4; HRMS m/z [M+H]$^+$ calculated for $C_{36}H_{45}N_6O_9$: 705.3248, found: 705.3253.

MaltriPepOPNP (12)

11 (65 mg, 0.09 mmol) and bis(p-nitrophenyl)carbonate (82 mg, 0.27 mmol) were dissolved in dry DMF (1.5 ml) under argon. The flask was capped, evacuated and refilled with argon several short turns before DIPEA (47 µl, 0.27 mmol) was added by syringe. The reaction mixture was stirred at r.t. for 23 h at which time a second portion of bis(p-nitrophenyl)carbonate (55 mg, 0.18mmol) was added. The reaction was stirred for an additional 23 h after which all **11** was consumed as verified by TLC (5% methanol in CH_2Cl_2). The reaction mixture was diluted in CH_2Cl_2 (40 ml), washed with citric acid solution (10 wt%, 2 x 15 ml) and dried over $MgSO_4$. The solvents were removed resulting in a yellow oil, which was dissolved in a small amount of CH_2Cl_2 and added dropwise to DEE (40 ml) while stirring. The product precipitated as a white solid (60 mg) containing ~15% p-nitrophenol (estimated from ^{1}H-NMR integrals). The identity of the expected product was confirmed by ^{1}H-NMR, ESI-MS and HRMS and the material was used without further purification in the following step. ^{1}H NMR (400 MHz, MeOD-d_4) δ 8.31 (app d, 2H), 7.69 (d, J = 8.6 Hz, 2H), 7.46 (app d, 2H), 7.41 (d, J = 8.6 Hz, 2H), 7.31-7.23 (m, 4H), 7.23-7.16 (m, 1H), 6.74 (s, 2H), 5.97-5.84 (m, 1H), 5.33-5.23 (m, 3H), 5.16 (d, J = 10.5 Hz, 1H), 4.56 (dd, J = 10.0, 4.4 Hz, 1H), 4.53 – 4.46 (m, 2H), 4.41 (dd, J = 9.6, 5.0 Hz, 1H), 4.14 (q, J = 7.1 Hz, 1H), 3.73 – 3.44 (m, 3H), 3.14 (t, J = 6.7 Hz, 2H), 2.95 (dd, J = 14.2, 10.1 Hz, 1H), 2.46-2.25 (m, 2H), 2.08 – 1.81 (m, 1H), 1.63 – 1.37 (m, 4H), 1.15 (d, J = 7.1 Hz, 3H). MS (ESI) m/z: [M+Na]$^+$: 892.3; HRMS m/z [M+H]$^+$ calculated for $C_{43}H_{48}N_7O_{13}$: 870.3310, found: 870.3318.

MaltriPepDox (13)

12 (53 mg, 0.061 mmol) was dissolved in dry DMF (1.7 ml) and the hydrochloride salt of doxorubicin (35 mg, 0.061 mmol) was added followed by Et_3N (18 µl, 0.12 mmol). The reaction mixture was stirred at r.t. and followed by TLC (5% methanol in CH_2Cl_2). After 24 h, the reaction mixture was added dropwise to DEE (35 ml) under stirring which resulted in a red precipitate. The precipitate was collected by filtration and isolated by washing the filter paper with a solution of 5% methanol in CH_2Cl_2. The solvents were removed to give a red solid, which was purified by flash column chromatography on silica eluting with 5% methanol in CH_2Cl_2. The product was dryloaded on to the column after being dissolved in methanol:CH_2Cl_2 mixture and dried on to silica. The expected product **13** was isolated as a red solid (59 mg, 75%). HPLC method A. MS (ESI) m/z: [M+Na]$^+$: 1296.5; HRMS m/z [M+Na]$^+$ calculated for $C_{64}H_{71}N_7O_{21}Na$: 1296.4601, found: 1296.4608. Characteristic peaks of maleimidyl (6.96 ppm, s, 2H), Vinyl proton from Alloc protecting group (5.86 ppm, m, 1H), methoxy methyl of doxorubicin (3.97 ppm, s, 3H), Alaninyl methyl (1.12 ppm, d, 3H) and doxorubicin glycon methyl (0.91ppm, d, 3H) with matching integrals found on ^{1}H-NMR (DMSO-d_6) (see SI).

N-Acetamido-N-furfuryl-XGO (15)

A solution of XGO[41] (1.015 g, 8.3 mmol based on the octasaccharide) in furfurylamine (4 mL) was stirred for 72h at 25°C. The solution was poured into ethyl acetate (25 mL), cooled to 0°C and centrifuged for 20 min at 6000 rpm. The solid was washed with ethyl acetate and centrifuged again. The crude solid of glycosylamine was then dissolved in methanol (15 mL)

and acetic anhydride (7 mL) was slowly added at 0°C to the solution. The reaction mixture was then allowed to warm up to room temperature and stirred a further 16h. The solution was then concentrated and co-evaporated with toluene. The residue was dissolved in 14% aqueous ammonia (50 mL), stirred 1h at room temperature to hydrolyze potential *O*-acetyl groups. After concentration of the solution to half of its initial volume under vacuum, the aqueous phase was extracted 3 times with ethyl acetate to remove free *N*-acetyl furfurylamine and concentrated. *N*-acetamido-*N*-furfuryl-XGO was isolated in 90% yield (998 mg) after purification by flash chromatography on silica gel (CH$_3$CN/H$_2$O 7:3 v/v). ^{1}H NMR (400 MHz, D$_2$O) δ 7.51 and 7.45 (2 x s, rotamers, 1H), 6.46-6.36 (m, 2H), 5.53 (d, $J =$ 9.0 Hz, 0.3H, H$_1^{GlcN}$ rotamer), 5.18 (m, 1H), 5.13 (d, $J =$ 9.0 Hz, 1H, H$_1^{GlcN}$ rotamer), 4.96 (m, 2H), 4.62-4.55 (m, 5H), 4.06-3.33 (m, 51H), 2.30 and 2.26 (2 x s, rotamers, 3H, NCOC*H$_3$*). The presence of the amide rotamers was confirmed by their disappearance at elevated temperature (323K) as observed by VT-NMR (see SI). (ESI) *m/z*: [M+Na]$^+$: 1206.4 (DP7), 1368.5 (DP8), 1530.5 (DP9). HR-MS *m/z* [M+Na]$^+$ calculated for DP7; C$_{46}$H$_{73}$NO$_{34}$Na; 1206.3912 Found 1206.3876, *m/z* [M+Na]$^+$ calculated for DP8; C$_{52}$H$_{83}$NO$_{39}$Na; 1368.4440 Found 1368.4401, *m/z* [M+Na]$^+$ calculated for DP9; C$_{58}$H$_{93}$NO$_{44}$Na; 1530.4968 Found 1530.4930.

LacEsterDox (16)
1 (21 mg, 0.022 mmol) was dissolved in DMF (60 μl) and *N*-acetamido-*N*-furfuryl-Lactose[40] (28 mg, 0.06 mmol) was added followed by water (40μl). The reaction mixture was stirred at 37 °C for 3 days. The solvents were removed by rotary evaporation and the crude material was dissolved in CH$_2$Cl$_2$/MeOH, loaded on to silica and purified by flash column chromatography eluting with 10-20% H$_2$O in CH$_3$CN. **16** was isolated as a red solid (30 mg, 91%) after lyophilization. HPLC method A (see SI); MS (ESI) *m/z*: [M+Na]$^+$: 1443.6; HR-MS *m/z* [M+Na]$^+$ calculated for C$_{67}$H$_{80}$N$_4$O$_{30}$Na 1443.4755; Found 1443.4757.

XGOEsterDox (17)
1 (10 mg, 0.01 mmol) was dissolved in DMF (60 μl) and *N*-acetamido-*N*-furfuryl-XGO **15** (66 mg, 0.05 mmol) was added followed by water (60μl). The reaction mixture was stirred at 37 °C for 4 days. The solvents were removed by rotary evaporation and the crude was purified by two consecutive C$_{18}$-columns (1g) eluting with a gradient of 0-100% MeOH in water. The expected product **17** was isolated as a red solid (8 mg, 35%) after lyophilization. HPLC method A (see SI); (ESI) *m/z*: [M-H]$^-$: 2140.9 (DP7) , 2302.9 (DP8), 2464.9 (DP9). HR-MS *m/z* [M+Na]$^+$ calculated for DP7; C$_{94}$H$_{124}$N$_4$O$_{52}$Na; 2163.7029 Found 2163.7079, *m/z* [M+Na]$^+$ calculated for DP8; C$_{100}$H$_{134}$N$_4$O$_{57}$Na; 2325.7607 Found 2325.7495, *m/z* [M+Na]$^+$ calculated for DP9; C$_{106}$H$_{144}$N$_4$O$_{62}$Na; 2487.8135 Found 2487.8136.

LactriPep(Alloc)Dox (18)
13 (15 mg, 0.012 mmol) was dissolved in DMF (80 μl) and *N*-acetamido-*N*-furfuryl-Lactose (16 mg, 0.035 mmol) was added followed by water (40μl). The reaction mixture was stirred at 37 °C for 7 days. The solvents were removed by rotary evaporation and the crude material was dissolved in CH$_2$Cl$_2$/MeOH, loaded on to silica and purified by flash column chromatography eluting with 10% H$_2$O in CH$_3$CN. The expected product **18** was isolated as a red solid (12 mg, 58%) after lyophilization. HPLC method A (see SI). MS (ESI) *m/z*: [M+Na]$^+$: 1759.7; HRMS *m/z* [M+Na]$^+$ calculated for C$_{83}$H$_{100}$N$_8$O$_{33}$Na: 1759.6290, found: 1759.6302.

LactriPepDox (19)
Following a modified procedure for Alloc deprotection,[33] **18** (7 mg, 4.0 μmol) was dissolved in degassed CH$_2$Cl$_2$:AcOH (5:1, 600 μl). Pd(PPh$_3$)$_4$ (6 mg, 5.2 μmol) was added. The reaction

was stirred at rt in the dark and followed by TLC (CH$_2$Cl$_2$:MeOH:NH$_4$OH 80:18:2). After 30 min, the CH$_2$Cl$_2$ was removed and DEE (1 ml) was added which resulted in a red precipitation which was washed with DEE (1 ml), EtOAc (2 x 1 ml) and then DEE (1 ml) again. Ultrasonication and quick centrifugation was used in each wash cycle. The resulting red pellet was dried in a dessicator under low pressure. The resulting red solid (4 mg, 59%) had a mass on ESI-MS and HRMS corresponding with the expected product **19** and consisted of one main compound with minor impurities on HPLC-UV. HPLC method A. MS (ESI) *m/z*: [M+H]$^+$: 1653.6 HRMS *m/z* [M+H]$^+$ calculated for C$_{79}$H$_{97}$N$_8$O$_{31}$: 1653.6260, found: 1653.6244.

Enzymatic release of Doxorubicin

Plasmin and human carboxylesterases were purchased from Sigma-Aldrich.

Plasmin
Following a protocol from the enzyme provider,[47] a 100 µM solution of **19** in phosphate buffer (pH 7.5) containing 100mM Lysine was incubated at 37 □ with plasmin from human plasma (15µg/ml). The reaction was followed by reversed phase HPLC (method B) by injecting 15µl aliquots from the reaction. The ratio of released doxorubicin over prodrug was calculated from integration of the peaks at 480 nm and plotted against time.

Carboxylesterase
A 100 µM solution of ester linked prodrug (**16** or **17**) in Tris buffer (50 mM, pH 7.4)[48] was incubated at 37 □ with either recombinant human carboxylesterase 1 isoform b (100µg/ml) or recombinant human carboxylesterase 2 (100µg/ml) expressed in baculovirus infected BTI insect cells . The reaction was followed by reversed phase HPLC (method B) by injecting 15 µl aliquots from the reaction. The ratio of released doxorubicin over prodrug was calculated from integration of the peaks at 480 nm and plotted against time.

Growth inhibition assay
The breast cancer cells MCF-7 were seeded at a density of 2×10^3 cells per well in 96-well plates. After 24 h, cells were treated with an increasing concentration of compounds, from 0 to 100 µM. Cells were incubated for 72 h, and then, 20 µL of MTS (Promega) was added per well for one hour at 37 °C. The absorbance was read at 492 nm using a spectrophotometer (SPECTRAmax, Molecular Devices). IC$_{50}$ values were obtained from the cytotoxicity curves using SOFTmax PRO software.

Supporting Information description
The SI contains NMR spectra and HPLC chromatograms.

Acknowledgments

Abbreviations
Doxorubicin, Dox; Xyloglucan, XG; trifluoroacetic acid, TFA; dicyclohexylcarbodiimide, DCC; *N*-methylmorpholine, NMM; *N,N*-diisopropylethylamine, DIPEA;

(dimethoxy)triazinylmethylmorpholonium   tetrafluoroborate,   DMTMMBF$_4$;   human
carboxylesterase 2, hCE2; *p*-aminobenzyl alcohol, PABA; maleimidyl ester linked
Doxorubicin, MalEsterDox; maleimidyl tripeptidyl linked Doxorubicin, MaltriPepDox

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