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Synthesis and evaluation of cardiac glycoside mimics as potential anticancer drugs

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ABSTRACT

The cardiac glycoside digitoxin, consisting of a steroid core linked to a labile trisaccharide, has been used for centuries for the treatment of congestive heart failure. The well known pharmacological effect is a result of the ability of cardiac glycosides to inhibit the Na^+, K^+-ATPase. Within recent years cardiac glycosides have furthermore been suggested to possess valuable anticancer activity. To mimic the labile trisaccharide of digitoxin with a stabile carbohydrate surrogate, we have used sulfur linked ethylene glycol moieties of varying length (mono-, di-, tri- or tetra-ethylene glycol), and furthermore used these linkers as handles for the synthesis of bivalent steroids. The prepared compounds were evaluated for their potencies to inhibit the Na^+, K^+-ATPase and for their cytotoxic effect on cancerous MCF-7 cells. A clear trend is observed in both inhibition and cytotoxic effect, where the bioactivity decreases as the size increases. The most potent Na^+, K^+-ATPase inhibitors are the compounds with the shortest ethylene glycol chain (K_{app} 0.48 μM) and thiodigitoxigenin (K_{app} 0.42 μM), which both are comparable with digitoxigenin (K_{app} 0.52 μM). For the cancer cell viability assay the shortest mimics were found to have highest efficacy, with the best ligand having a monoethylene glycol unit (IC_{50} 0.24 μM), which was slightly better than digitoxigenin (IC_{50} 0.64 μM), while none of the novel cardiac glycoside mimics display an in vitro effect as high as digitoxin (IC_{50} 0.02 μM).

1. Introduction

Cardiac glycosides constitute a class of naturally occurring toxins that are able to bind to and thereby inhibit the Na^+, K^+-ATPase.[1] Among these are compounds like digitoxin and digoxin, which can be extracted from the plants *Digitalis purpurea* and *Digitalis lanata*. They are considered important medicinal agents and have been applied for the treatment of congestive heart failure for over 200 years,[2] and were in 2005 used for 1.7 million patients in the USA.[3] The positive inotropic effect obtained by the treatment with, for example, digitoxin has been explained by the 'Na^+ pump lag hypotheses', which proposes that the digitoxin effect on the heart is associated with inhibition of the Na^+, K^+-ATPase.[4] Besides being an inhibitor of Na^+, K^+-ATPase ion pump, digitoxin is also believed to serve as a signaling compound and its binding induces conformational changes of the Na^+, K^+-ATPase that activate cascade reactions leading to a variety of cellular responses.[5] Activations of the signaling cascades appear to occur at extremely low concentrations, that is, involving only a few ATPase molecules without affecting bulk Na^+, K^+-ATPase activity in the cell.

Cardiac glycosides seem to be beneficial for the treatment of disorders like polyglutamine based diseases[6] and cystic fibrosis,[7] and their application in cancer therapy represents a new perspective. In 1979, Stenkvist et al.[8] reported that 28 patients with breast cancer and digitalis treatment had better survival rate and less cancer recurrence than a control group of 114 patients. A 20 year follow-up study showed that the death rate for digitalis treated patients was 6% compared to 34% in control group.[9] Additionally, several publications on epidemiological data indicate lower mortality in cancer patients who received digitalis therapy compared to cancer patients not receiving digitalis. These key preliminary data have paved the way for numerous published studies, which establish the anticancer effect of cardiac glycosides.[10]

The glycon moiety of cardiac glycosides like digitoxin is known to influence Na^+, K^+-ATPase inhibition.[11] Comparable inhibitory potencies are observed for digitoxin (**1**) and the aglycon digitoxigenin (**4**), while the di- and monoglycosylated steroids **2** and **3** exhibit a three- to four-fold increased potency compared to **1** and **4**.[12] The binding affinities of the glycons **1**, **2** and **3** are likewise analogous, while **4** exhibit a sixfold decrease in affinity.[12]

López-Lázaro et al.[13] have studied the effect of digitoxin (**1**) and derivatives for growth inhibition activity in three human cancer cell lines. A promising anticancer effect was observed for digitoxin (**1**), and the effect was 20-fold lower for digitoxigenin (**4**).

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Synthetic efforts directed towards manipulation on the carbohydrate fragment of cardiac glycosides have been conducted only to a very small extent. With the focus of digitoxin, Rathore et al. have studied the effect of carbohydrate stereochemistry on the Na^+, K^+-ATPase inhibition.[11] Additionally, non-natural and potentially unstable glycosylated N,O-substituted hydroxylamines being analogues of digitoxin have been evaluated as anticancer compounds by Langenhan et al.[14]

As medicinal agents, cardiac glycosides like digitoxin (**1**) possess a labile glycon moiety, and the digitoxoside of digitoxin is especially reactive (Fig. 1), due to it being 2,6-dideoxy glycoside possessing an axial 3-OH.[15] The metabolism of digitoxin produces three major metabolites: digitoxigenin bisdigitoxoside **2**, digitoxigenin monodigitoxoside **3** and the aglycon digitoxigenin (**4**), which all have high binding affinities and inhibitory potencies of the Na^+, K^+-ATPase. However, the pharmacokinetic profile strongly depends on the presence of glycon moiety with the half life significantly decreasing with loss of each digitoxoxide. The short half lives and fast clearances of monodigitoxoside **3** and digitoxigenin (**4**) have despite their potent Na^+, K^+-ATPase inhibition rendered these two metabolites unsuitable for clinical use.[16]

With monodigitoxoside being the most potent Na^+, K^+-ATPase inhibitor among the digitoxigenin dervatives with varying degrees of glycosylation, we speculate whether it will also have a superior cytotoxic effect against cancer cells as suggested by Iyer et al.[17]

Given the short half life and high clearance of the cardiac steroids with low degree of glycosylation we decided to study the possibility of mimicking the glycon moiety with stable ethylene glycol chains of varying length. The use of ethylene glycols as carbohydrate mimics has previously been reported by Silva et al.[18] Polyethylene glycol chains are often used to increase protein serum half life[19] mimicking one of the many effects of protein glycosylation.[20] The length of tetra ethylene glycol is modeled to be 14.3 Å (O-3 to terminal oxygen of tetraethylene glycol), which is comparable to the length of tridigitoxoside modeled to be 15.2 Å (O-3 to O-4 of terminal digitoxoside).[21]

We have therefore synthesized several digitoxin analogues (or digitoxigenin derivatives), where the digitoxosides are replaced by ethylene glycol moieties of varying lengths (Fig. 2). Furthermore we have compared the biological activity of these compounds together with their bivalent analogues as Na^+, K^+-ATPase inhibitors and as anticancer agents against the human breast cancer cell line MCF-7.

2. Results and discussion

2.1. Chemistry

Using the chemistry developed by Langenhan et al.[14a], Sawlewic et al.[22] and Gobbini et al.[23] we decided to attach a sulfur atom to facilitate easy linking of the ethylene glycol units (Scheme 1). Initially, treatment of digitoxin (**1**) under Jones oxidation conditions resulted in glycoside cleavage and alcohol oxidation affording digitoxigenon (**13**) in 75% yield. Subsequent reaction of ketone **13** with sodium borohydride at low temperature gave 3-*epi*-digitoxigenin **14** in 77% yield along with 5% of the undesired epimer, digitoxigenin (**4**). Although very selective, this reduction never proceeded with complete stereocontrol contrary to previous

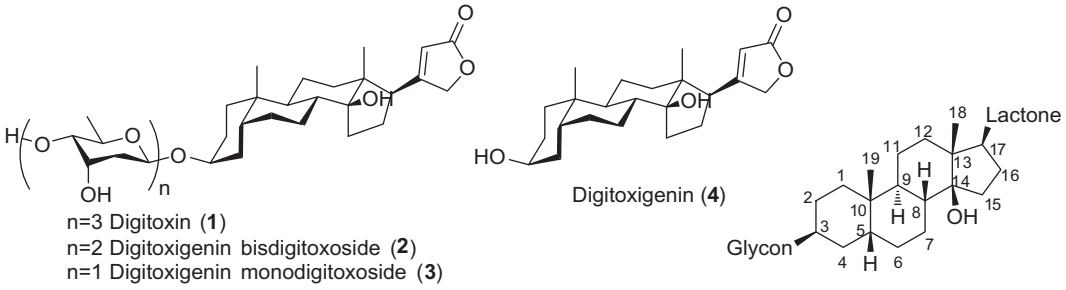


Figure 1. Structures of digitoxin (**1**), and metabolites **2**, **3** and **4**. A general structure of cardiac glycosides is shown to the right.

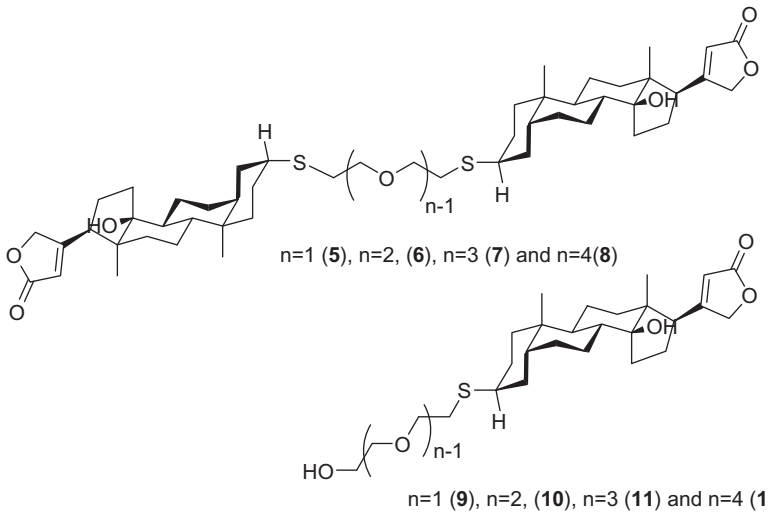
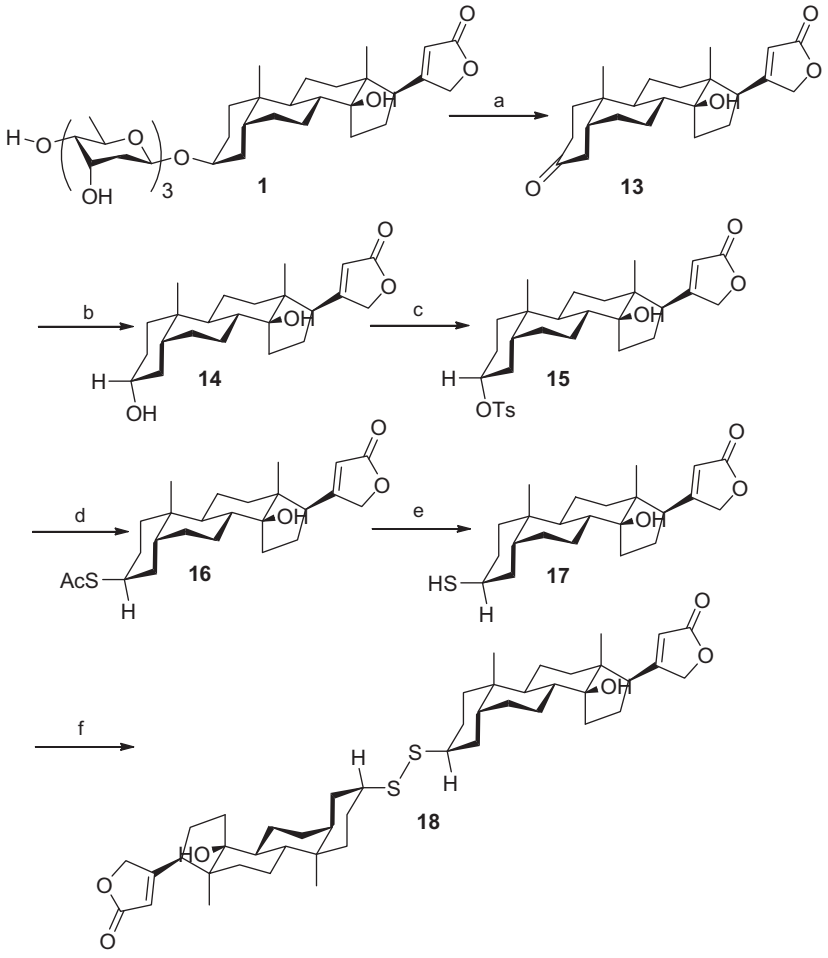


Figure 2. Structures of the prepared novel cardiac glycoside mimics, the mono-valent (**9**–**12**) and bivalent steroids (**5**–**8**).



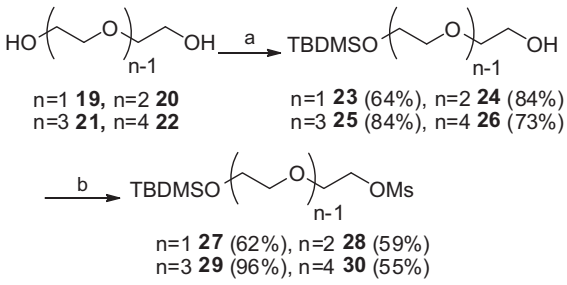
Scheme 1. Reagents and conditions: (a) CrO_3, H_2SO_4/H_2O, acetone, rt, 3 h, 75%; (b) $NaBH_4$, dioxane/H_2O, 0 °C, 30 min, 77%; (c) Ts_2O, DMAP, pyridine, rt, o/n, 97%; (d) KSAc, DMF, 60 °C, o/n, 69%; (e) NH_3, MeOH/THF, rt, 2 h; (f) column chromatography, 46%.

reports.[22,23] Tosylation of secondary alcohol **14** gave tosylate **15** in near quantitative yield, which subsequently was substituted by potassium thioacetate to afford the thioacetyl steroid **16** in 69% yields. Thiodigitoxigenin **17** was obtained from **16** as described by Gobbini et al.[23] using NH_3 in MeOH/THF (1:1). The free thiol **17** was found to be easily oxidized to disteroid disulfide **18** during column chromatography (Scheme 1).

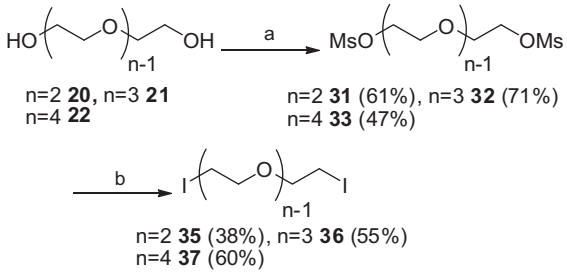
Ethylene glycol derivatives (**27**–**30**) bearing one TBDMS-protected hydroxyl functionality and another as a mesylated nucleofuge, were reacted with crude thiol **17** for the preparation of mono-valent cardiac steroid mimics (**9**–**12**). The main limitation in the choice of protecting group on the ethylene glycol unit was the deprotection conditions, which was required to be neutral since basic conditions (TBAF) is known to lead to epimerisation at position 17,[24] while acidic conditions can cause elimination of the tertiary alcohol in position 14. Accordingly, the *tert*-butyldimethylsilyl protecting group was deprotected with a near neutral fluoride ion source (Et_3N/3HF) in good yields (Schemes 4 and 5).

For the preparation of ethylene glycol (vide supra), an excess amount of ethylene glycol **19**, **20**, **21** or **22** was treated with TBDMS-Cl in the presence of 4-(dimethylamino)pyridine (DMAP) to afford mono TBDMS-protected **23**, **24**,[25] **25**[25] or **26**, respectively. Mesylation of the remaining alcohol afforded **27**[26], **28**, **29** or **30**[27] (Scheme 2).

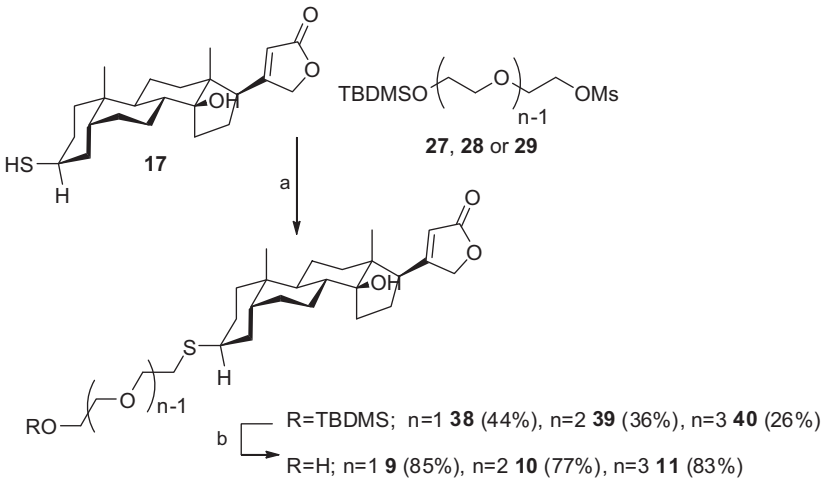
For the synthesis of bivalent steroids dihalide ethylene glycol derivatives were used. These (**35**–**37**) were prepared from their corresponding ethylene glycols **20**, **21** or **22** via their dimesylated inter-



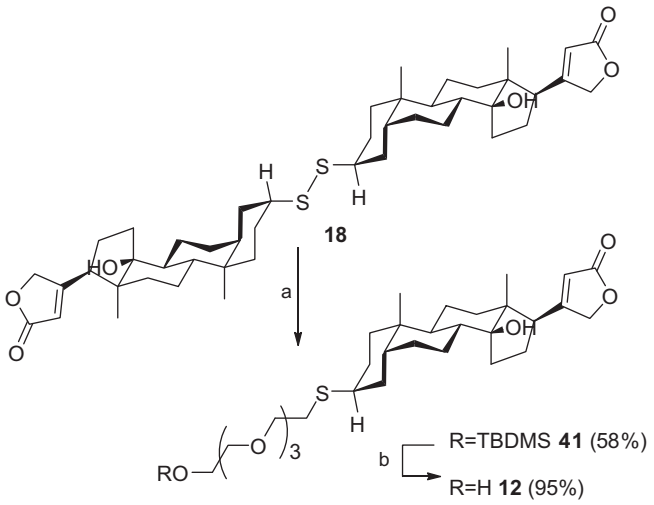
Scheme 2. Reagents and conditions: (a) TBDMS-Cl, DMAP, pyridine, rt, o/n; (b) MsCl, Et_3N, Et_2O, 0 °C, 1 h.



Scheme 3. Reagents and conditions: (a) MsCl, Et_3N, Et_2O, 0 °C to rt, 4 h; (b) NaI, acetone, reflux, o/n.



Scheme 4. Reagents and conditions: (a) NaH, DMF, rt, 2 h, 26–44% over two steps; (b) $Et_3N{:}3HF$, CH_2Cl_2, rt, o/n)

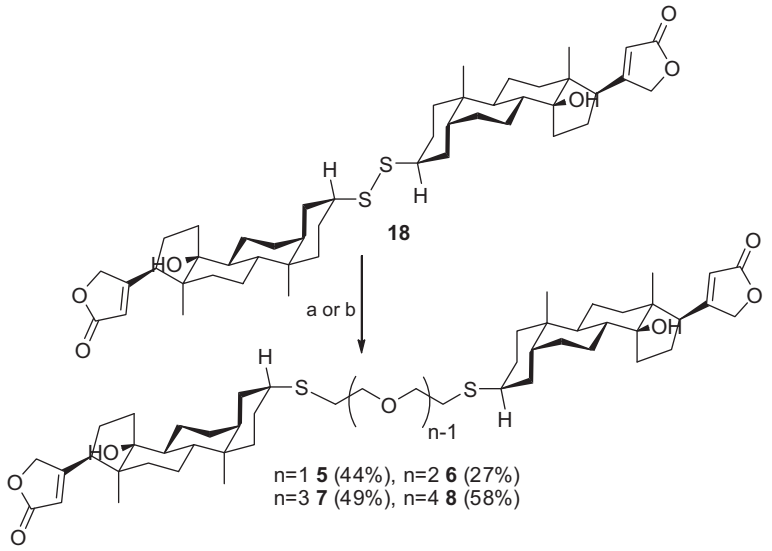


Scheme 5. Reagents and conditions: (a) tetraethylene glycol linker **30**, $NaBH_4$, absolute EtOH, rt, 20 h; (b) $Et_3N{:}3HF$, CH_2Cl_2, rt, o/n.

mediates (**31**–**33**).[27] 1,2-dibromo ethane (n = 1, **34**) was used to prepare the shortest bivalently linked cardiac steroids (Scheme 3).

Crude thiol **17** was reacted with the TBDMS-protected and mesylated ethylene glycol linkers **27**–**30** in the presence of sodium hydride to obtain the corresponding TBDMS-protected monovalent steroids **38**–**40** in moderate yields (Scheme 4). Desilylation under neutral conditions uneventfully afforded the desired monovalent steroids **9**, **10** and **11** in good yields. Attempts to synthesize the mono-valently displaying target having the tetraethylene glycol unit (**12**) using the thiol **17** proved unsuccessful in accordance with dropping yields as the linker size increased. The tetraethylene glycol linked steroid (**12**), was, however, successfully prepared via the intermediate **41** by reduction of the disulfide **18** in the presence of **30** (Scheme 5).

Difficulties arose in the formation of bivalent steroids (**5**–**8**, Scheme 6). Despite full consumption of the crude thiol **17** was observed, no product could be isolated. As a result, the crude thiol **17** was purified by column chromatography, which afforded the disteroid disulfide derivative **18**. The bivalent steroids were successfully obtained from the disulfide **18** under reductive condition in the presence of a dihalide ethylene glycol linker (Scheme 6).



Scheme 6. Reagents and conditions: (a) dihalide ethylene glycol linker **34**, **36** or **37**, $NaBH_4$, absolute EtOH, rt, 3–17 h. Reagents and conditions for the formation of **6** (b) diiodide diethylene glycol **35**, $NaBH_4$, dry DMF, rt, 1 h.

2.2. Inhibition of Na^+, K^+-ATPase

The cardiac glycoside mimics were tested for inhibition of Na^+, K^+-ATPase activity (Figs. 3 and 4) and the apparent inhibitory constants (K_{app}) are listed in Table 1. The synthesized compounds exhibit a clear trend; as the length of the ethylene glycol linker increases the inhibitory power decreases. The bivalent steroids **5**–**8** display a significantly reduced inhibitory potency compared to digitoxin (**1**), digitoxigenin (**4**) and the mono-valent steroids (**9**–**12**). The impaired inhibitory potency of the bivalent cardiac glycoside mimics could be due to the steric bulk of the extra steroid.

The highest apparent inhibitory potency is observed for **9** (0.48 μM), which is close to the K_{app} value 0.52 μM of digitoxigenin (**4**). The potency of **10** lies in the interval between digitoxin (**1**) and digitoxigenin (**4**), while the largest mono-valent steroid **12** shows a twofold decrease in potency compared to digitoxin (**1**). These observations are consistent with the published data from Paula et al.[12], where the inhibition potency was found to increase when trimming of the γ- and β-glycons and decreases again when no glycon is present. For cardiac glycosides including digitoxin (**1**), α- and β-glycon moieties are important for favorable interactions and the inhibition potency, while the γ-glycone bear little significance for inhibition.[12] The attached ethylene glycol moieties are suggested to occupy a sterically restricted site, which have favorable polar interactions to the α-saccharide unit.

The disulfide **18** was unstable and reduced to 3-mercapto digitoxigenin **17**. The presented K_{app} value for free thiol **17** is estimated to 0.42 μM.

2.3. Antiproliferation effect on cancer cells

The compounds were also evaluated for their effect on the viability of MCF-7 breast cancer cells. It was found that the inhibitory trend for cell viability resembles the trend for Na^+, K^+-ATPase inhibition activity, with a few noteworthy exceptions (Table 1 and Fig. 3). In the activity assay a slightly higher inhibitory potency is observed for digitoxigenin **4** (0.52 μM) compared to digitoxin **1** (0.99 μM), whereas the relationship is reversed in the viability assay, showing that digitoxin (**1**) reduces cell viability very potently (IC_{50} 0.02 μM vs 0.64 μM for digitoxigenin). The interpretation of the results of the viability essays rely on the effective concentrations of the compounds in the incubation media. Most of the compounds, including digitoxin, are not able to penetrate the cell membranes and will remain in the extracellular media. Digitoxigenin, however, will distribute between the intracellular and extracellular media, thus, its effective concentration on the extracellular side (relevant for binding to ATPase) will be lower. The apparently decreased inhibitory potency of the digitoxigenin will depend on the ratio of the extracellular to intracellular volumes and intracellular protein concentration, since proteins are able to non-specifically adsorb cardiotonic steroids.[28] The ability of digitoxigenin to readily permeate membranes is widely used in studies of sided membrane preparations.[29]

Generally, the bivalent steroids are significantly less effective than mono-valent steroids. In the activity assay a high inhibitory potency was observed for the smallest mimic **9** compared to digitoxigenin (**4**). No improvement in the viability assay was observed

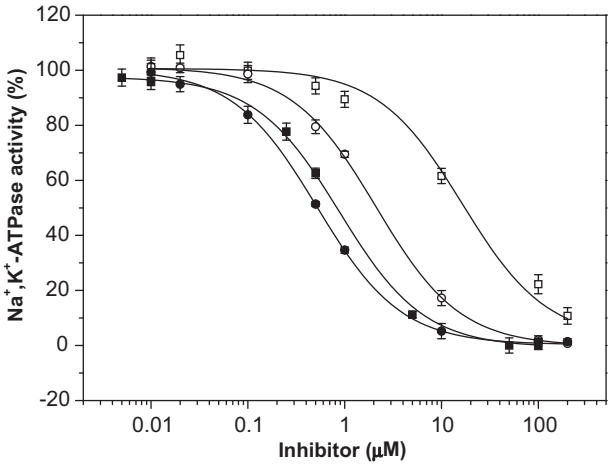


Figure 4. Inhibition of the Na^+,K^+-ATPase activity by digitoxigenin (filled circles), digitoxin (filled squares), compound **12** (open circles) and compound **6** (open squares). Residual activity is expressed in percent of that in the absence of inhibitor. Na^+, K^+-ATPase is defined as ouabain-sensitive ATPase activity.

Table 1
Mean K_{app} values (μM) of cardiac glycoside mimics against renal pig Na^+, K^+-ATPase. Mean IC_{50} values (μM) for cardiac glycoside mimics against the human breast cancer cell lines MCF-7. The mean values are from at least three determinations

	Compound	Inhibition K_{app} (μM)	IC_{50} Values (μM) in MCF-7
Digitoxin (**DT**)	**1**	0.99	0.02 ± 0.0005
Digitoxigenin (**DTG**)	**4**	0.52	0.6 ± 0.1
Mono-valent Steroids	**9**	0.48	0.2 ± 0.02
	10	0.73	0.4 ± 0.1
	11	1.27	0.8 ± 0.2
	12	2.1	1.4 ± 0.3
Thiol	**17**	0.42	0.5 ± 0.02
Bivalent Steroids	**5**	9.9	7.0 ± 0.5
	6	17.2	8.6 ± 1.9
	7	20.5	6.6 ± 1.7
	8	>53	13 ± 6

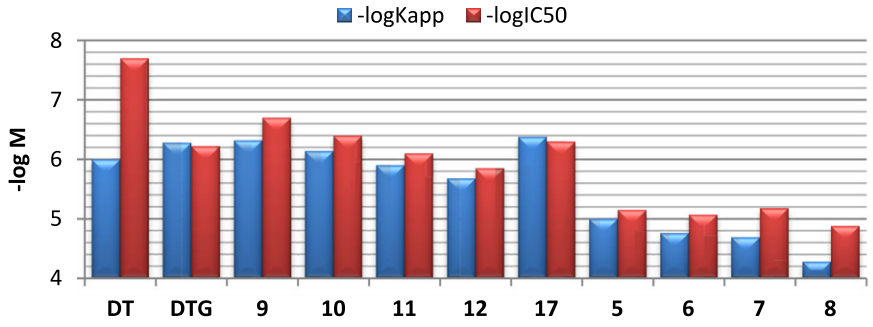


Figure 3. The blue columns illustrates −log K_{app} (M) of cardiac glycoside mimics against renal pig Na^+, K^+-ATPase, while the red columns illustrates −log IC_{50} (M) for cardiac glycoside mimics against the human breast cancer cell lines MCF-7. Digitoxin (**1**, **DT**), digitoxigenin (**4**, **DTG**), thiol **17**, mimics **9**–**12** are mono-valent mimics in increasing size, mimics **5**–**8** are bivalent mimics in increasing size.

for any of the mimics compared to digitoxin (**1**). However, the three smallest mimics **9**, **10** and **11** exhibit IC_{50} values in the interval between digitoxin (**1**) and digitoxigenin (**4**). The remaining compounds **5**–**8**, **12** and **17** have considerable lower anticancer effects.

3. Conclusion

Novel digitoxin mimics had been synthesized by replacing of the labile trisaccharide moiety with stable ethylene glycol chains of different lengths. The compounds preserved their ability to inhibit Na^+, K^+-ATPase, demonstrating that glycosylation is not a prerequisite for their binding to the enzyme. In actual fact, monoethylene glycol **9** (K_{app} 0.42 μM) was found to be slightly more potent than digitoxin (K_{app} 0.99 μM). We furthermore reconfirmed that cardiac glycoside mimics are potent anti-cancer compounds, though the compounds prepared in this study were less powerful than digitoxin regarding the effects on the viability of breast cancer cells.

In addition, using ethylene glycol linkers of varying lengths, we have also prepared four compounds having a bivalent display of the cardiotonic steroid core. These compounds were found to have between 20 and 110-fold decreased inhibitory potency against the Na^+, K^+-ATPase.

The monovalent cardiac glycoside mimics prepared in this study could in principle lead to compounds with improved pharmacokinetic properties optimizing their effect on cardiac contractility. The results from this study also suggest that ethylene glycol linked cardiac steroids could be used as biochemical tools for immobilisation and thereby purification of the Na^+, K^+-ATPase.

4. Experimental section

4.1. Na^+, K^+-ATPase activity assay

Relative inhibitory potency of the compounds was estimated under the steady-state conditions of ATPase reaction. Briefly, the purified Na^+, K^+-ATPase from pig kidney (specific activity of 25–30 U/mg protein)[30] was incubated for 2 h with various concentrations of the inhibitor in the reaction media containing 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 30 mM histidine buffer, pH 7.4, 20 °C. Then 3 mM ATP was added and the reaction was allowed to proceed for 5 min. The amount of inorganic phosphate released was measured and plotted as function of inhibitor concentration. The ouabain-insensitive ATPase activity was measured in a separate experiment in the presence of 1 mM ouabain and subtracted from each number. The inhibitory potency was evaluated by fitting of the data to the hyperbolic function. K_{app} value reflects both the affinity of the compound to the enzyme and the velocity of its interaction with the enzyme.

4.2. Viability assay

MCF-7 breast cancer cells were cultured in DMEM Glutamax (Gibco) supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere with 5% CO_2 at 37 °C.

For the toxicity screen, 3000 cells per well were seeded in 96-well format as triplets and cultured overnight. Afterwards, the medium was replaced with medium containing a dilution series of the compounds. An equivalent dilution series of the solvent DMSO served for normalization. The cells were exposed to the compounds for 48 h followed by 72 h culture in fresh medium without the compounds prior to the viability assay. Relative numbers of viable cells were determined with the fluorometric CellTiter-Blue reagent (Promega) according to manufacturer's instructions and the fluorescence signal was measured at the wavelengths $560_{Ex}/590_{Em}$ nm with a Victor3 multilabel counter (Perkin–Elmer).

4.3. Chemistry

All reagents, unless otherwise stated, were used as purchased without further purification. Solvents were dried according to standard procedures. Columns for flash chromatography were packed with silica gel (60 mesh). TLC plates (Kieselgel 60 F_{254}) were visualized by use of PMA (10 g phosphomolybdic acid in 100 mL. absolute EtOH) and heated until spots appeared or by UV-irradiation. ^1H and ^{13}C NMR experiments were recorded on a Varian Mercury 400 NMR instrument. Mass spectral data were carried out as electrospray experiments on a Micromass LC-TOF instrument. GC–MS analyses were carried out on a Hewlett-Packard 5890A gas chromatograph equipped with a 5971A MSD mass-selective detector. The GC column was an HP5 25 m with an internal diameter of 0.25 mm, the injection temperature was 250 °C, the He-flow set to 1.0 mL/min, temperature program 40 °C for 5 min to 230 °C, rate 15 °C/min. Characterization of steroid NMR-data was performed on the basis of published data of digitoxin and digitoxigenin from Drakenberg et al.[31]

4.4. General procedure for the synthesis of TBDMS-protected and mesylated ethylene glycol linkers

To a solution of ethylene glycol (**19**, **20**, **21** or **22**) (8–10 equiv) and DMAP (cat.) in dry pyridine (5 mL) was added TBDMS-Cl (1 equiv). The reaction mixture was stirred at room temperature overnight and then diluted with CH_2Cl_2 before the organic phase was washed twice with 1 M HCl, once with H_2O and then satd $NaHCO_3$. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The TBDMS-protected ethylene glycols (**23**, **24**, **25** or **26**) were used without further purification.

To a cooled solution (0 °C) of TBDMS-protected ethylene glycol (**23**, **24**, **25** or **26**) (1 equiv) in dry Et_2O (5–7 mL) was added dry Et_3N (2 equiv) followed by dropwise addition of MsCl (1.5 equiv). The reaction mixture was stirred at 0 °C to room temperature for 1 h and then diluted with CH_2Cl_2. The organic phase was washed with mildly acidic H_2O, H_2O and satd $NaCO_3$, and dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography.

4.4.1. Monoethylene glycol linker (27)

Monoethylene glycol **19** (2.94 g, 47 mmol) was reacted with TBDMS-Cl (699 mg, 4.64 mmol). The product **23** was obtained as a clear oil without further purification (525 mg, 64%): R_f: 0.45 (EtOAc/pentane 1:9); ^1H NMR (400 MHz, $CDCl_3$): δ_H 2.68 (m, 2H, H2), 2.62 (m, 2H), 2.21 (br s, 1H, OH), 0.89 (m, 9H, Si-C(CH_3)$_3$), 0.06 (m, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 64.3, 63.8, 26.0 (Si-C(CH_3)$_3$), 18.4 (Si-*C*(CH_3)$_3$), −5.2 (Si-CH_3); LRMS (ES): Calcd for $C_8H_{20}O_2SiNa$ 199.1, found 199.1.

Compound **23** (411 mg, 2.35 mmol) was reacted with Et_3N (0.58 mL, 4.18 mmol) and MsCl (0.24 mL, 3.09 mmol). The crude product was purified by column chromatography (EtOAc/pentane 1:9) to afford **27** as yellow oil (372 mg, 62%): R_f: 0.33 (EtOAc/pentane 1:9); ^1H NMR (400 MHz, $CDCl_3$): δ_H 4.26 (t, 2H, *J* 4.8 Hz), 3.86 (t, 2H, *J* 4.8 Hz), 3.02 (s, 3H, S-CH_3), 0.88 (s, 9H, Si-C(CH_3)$_3$), 0.07 (s, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 71.4, 61.4, 37.8 (S-CH_3), 25.9 (Si-C(CH_3)$_3$), 18.4 (Si-*C*(CH_3)$_3$), −5.3 (Si-CH_3); HRMS (ES): Calcd for $C_9H_{20}O_4SSiNa$ 277.0906, found 277.0903. NMR spectral values for **27** are in accordance with reference.[26]

4.4.2. Diethylene glycol linker (28)

Diethylene glycol **20** (3.43 g, 32 mmol) was reacted with TBDMS-Cl (622 mg, 4.13 mmol). The product **24** (765 mg, 84%) as clear oil was obtained without further purification: R_f: 0.33 (EtOAc/pentane 1:4); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.72 (m, 2H), 3.66 (m, 2H), 3.54 (m, 4H), 2.85 (br s, 1H, OH), 0.85 (m, 9H, Si-C(CH_3)$_3$), 0.03 (m, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 72.6, 72.5, 62.9, 61.8, 25.9 (Si-C(CH_3)$_3$), 18.4 (Si-*C*(CH_3)$_3$), −5.3 (Si-CH_3); LRMS (ES): Calcd for $C_{10}H_{24}O_3SiNa$ 243.1392, found 243.1389. NMR spectral values for **24** are in accordance with reference.[25]

Compound **24** (765 mg, 3.47 mmol) was reacted with Et_3N (0.87 mL, 6.3 mmol) and MsCl (0.35 mL, 6.3 mmol). The crude product was purified by column chromatography (15% EtOAc in pentane) to afford **28** as clear oil (605 mg, 59%): R_f: 0.33 (EtOAc/pentane 1:9); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.34 (m, 2H), 3.74 (m, 4H), 3.55 (m, 2H), 3.03 (s, 3H, S-CH_3), 0.86 (s, 9H, Si-C(CH_3)$_3$), 0.03 (s, 6H, Si-CH_3); HRMS (ES): Calcd for $C_{11}H_{26}OSSiNa$ 321.1168, found 321.1165.

4.4.3. Triethylene glycol linker (29)

Triethylene glycol **21** (4.95 g, 33 mmol) was reacted with TBDMS-Cl (741 mg, 4.92 mmol). The product **25** (1.096 g, 84%) was obtained as clear oil and used without further purification: R_f: 0.36 (EtOAc/pentane 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.74 (t, 2H, *J* 5.4 Hz), 3.70–3.57 (m, 8H), 3.54 (t, 2H, *J* 5.2 Hz) 2.66 (br s, 1H, OH), 0.87 (s, 9H, Si-*C*(CH_3)$_3$), 0.04 (s, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 72.8, 72.6, 70.9, 70.6, 62.8, 61.8, 26.0 (Si-C(*C*H_3)$_3$), 18.4 (Si-*C*(CH_3)$_3$), −5.2 (Si-CH_3); LRMS (ES): Calcd for $C_{12}H_{28}O_4SiNa$ 287.1655, found 287.0. NMR spectral values for **25** are in accordance with reference.[25]

Compound **25** (1.082 g, 4.09 mmol) was reacted with Et_3N (1.03 mL, 7.43 mmol) and MsCl (0.41 mL, 4.19 mmol). The product **29** (1.339 g, 96%) was obtained as pale yellow oil and used without further purification: R_f: 0.51 (EtOAc/pentane 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.36 (m, 2H), 3.75 (m, 4H), 3.64 (s, 4H), 3.53 (t, 2H, *J* 5.4 Hz), 3.05 (s, 3H, S-CH_3), 0.88 (s, 9H, Si-C(CH_3)$_3$), 0.05 (s, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 72.8, 70.8, 70.8, 69.4, 69.1, 62.8, 37.8 (S-CH_3), 26.0 (Si-C(*C*H_3)$_3$), 18.5 (Si-*C*(CH_3)$_3$), −5.2 (Si-*C*H_3); HRMS (ES): Calcd for $C_{13}H_{30}O_6SSiNa$ 365.1430, found 365.1431.

4.4.4. Tetraethylene glycol linker (30)

Tetraethylene glycol **22** (6.144 g, 32 mmol) was reacted with TBDMS-Cl (623 mg, 4.13 mmol). The product **26** (931 mg, 73%) was obtained as pale yellow oil and used without further purification: R_f: 0.48 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.66 (m, 2H), 3.61 (m, 2H), 3.56 (m, 8H), 3.50 (m, 2H), 3.46 (m, 2H), 3.11 (br s, 1H, OH), 0.80 (m, 9H, Si-C(CH_3)$_3$), −0.03 (m, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 72.6, 72.5, 70.6, 70.6, 70.5, 70.3, 62.6, 61.5, 25.9 (Si-C(*C*H_3)$_3$), 18.3 (Si-*C*(CH_3)$_3$), −5.3 (Si-*C*H_3); HRMS (ES): Calcd for $C_{14}H_{32}O_5SiNa$ 331.1917, found 331.1916.

Compound **26** (931 mg, 3.02 mmol) was reacted with Et_3N (0.75 mL, 5.43 mmol) and MsCl (0.31 mL, 3.99 mmol). The crude product was purified by column chromatography (EtOAc/pentane 1:2 → 1:1) to afford **30** as clear oil (644 mg, 55%): R_f: 0.39 (EtOAc/pentane 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.37 (m, 2H), 3.74 (m, 4H), 3.63 (m, 8H), 3.53 (t, 2H, *J* 5.4 Hz), 3.06 (d, 3H, *J* 1.2 Hz, S-CH_3), 0.87 (d, 9H, *J* 0.8 Hz, Si-C(CH_3)$_3$), 0.05 (d, 6H, *J* 0.8 Hz, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 72.8, 70.8, 70.7, 70.6, 69.4, 69.1, 62.8, 37.8 (S-*C*H_3), 26.0 (Si-C(*C*H_3)$_3$), 18.5 (Si-*C*(CH_3)$_3$, -5.2 (Si-CH_3); HRMS (ES): Calcd for $C_{15}H_{34}O_7SSiNa$ 409.692, found 409.1689. NMR spectral values for **30** are in accordance with reference.[32]

4.5. General procedure for the synthesis of di-iodo ethylene glycol linkers (35–37)

A solution of ethylene glycol (**20**, **21** or **22**) (1 equiv) in dry Et_2O (4–10 mL) was cooled to 0 °C and added dry Et_3N (5 equiv) followed by drop wise addition of MsCl (2.5 equiv). The reaction mixture was stirred at 0 °C to room temperature for 4 h and then diluted with CH_2Cl_2. The organic phase was washed with mildly acidic H_2O, H_2O, satd $NaHCO_3$ and dried over $MgSO_4$, filtered and concentrated under reduced pressure. The dimesylated ethylene glycol (**31**, **32** or **33**) was used without further purification.

To a stirred solution of dimesylated ethylene glycol (**31**, **32** or **33**) (1 equiv) in dry acetone (5 mL) was added NaI (4 equiv). The reaction mixture was stirred at reflux overnight before cooled to room temperature and concentrated under reduced pressure. To the residue was added CH_2Cl_2 and H_2O, and the aqueous phase was extracted four times with CH_2Cl_2. The combined organic phases were washed with satd NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by column chromatography to afford **35**, **36** or **37**.

4.5.1. Diethylene glycol linker (35)

Diethylene glycol **20** (213 mg, 2.01 mmol) was reacted with Et_3N (1.4 mL, 10.02 mmol) and MsCl (0.4 mL, 5.16 mmol). The product **31** was obtained without further purification as yellow oil (319 mg, 61%): R_f: 0.57 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.36 (m, 4H), 3.78 (m, 4H), 3.05 (s, 6H, S-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 69.1, 68.9, 37.7 (S-CH_3); LRMS (ES): Calcd for $C_6H_{14}O_7S_2Na$ 285.0, found 285.0.

Compound **31** (101 mg, 0.39 mmol) was reacted with NaI (231 mg, 1.54 mmol). The crude product was purified by column chromatography (1:4 CH_2Cl_2/pentane → CH_2Cl_2 → EtOAc) to afford **35** as clear oil (48 mg, 38%): R_f: 0.43 (CH_2Cl_2/pentane 1:4); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.77 (t, 4H, *J* 6.8 Hz), 3.26 (t, 4H, *J* 6.8 Hz): ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 71.5, 2.6; GC–MS (EI): Calcd for $C_4H_8I_2O$ 326, found 326. NMR spectral values for **31** and **35** are in accordance with reference.[27]

4.5.2. Triethylene glycol linker (36)

Triethylene glycol **21** (295 mg, 1.96 mmol) was reacted with Et_3N (1.4 mL, 10.0 mmol) and MsCl (0.4 mL, 5.16 mmol). The product **32** appeared as a yellow oil and was used without further purification (424 mg, 71%): R_f: 0.46 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.35 (m, 4H), 3.75 (m, 4H), 3.66 (s, 4H), 3.05 (s, 6H, S-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 70.6, 69.2, 69.1, 37.7 (S-CH_3); LRMS (ES): Calcd for $C_8H_{18}O_8S_2Na$ 329.0, found 328.9.

Compound **32** (72 mg, 0.24 mmol) was reacted with NaI (140 mg, 0.93 mmol). The crude product was purified by column chromatography (CH_2Cl_2) to afford **36** as pale yellow oil (49 mg, 55%): R_f: 0.55 (CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.77 (t, 4H, *J* 6.8 Hz), 3.67 (s, 4H), 3.26 (t, 4H, *J* 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 72.1, 70.3, 3.0; LRMS (ES): Calcd for $C_6H_{12}I_2O_2Na$ 392.9, found 392.9. NMR spectral values for **32** and **36** are in accordance with reference.[27]

4.5.3. Tetraethylene glycol linker (37)

Tetraethylene glycol **22** (1.081 g, 5.56 mmol) was reacted with Et_3N (3.9 mL, 27.9 mmol) and MsCl (1.1 mL, 14.2 mmol). The product **33** was obtained as a yellow oil and used without further purification (907 mg, 47%): R_f: 0.38 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.34 (m, 4H), 3.74 (m, 4H), 3.62 (m, 8H), 3.04 (s, 6H, S-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 70.7, 70.5, 69.3, 69.0, 37.7 (S-CH_3); HRMS (ES): Calcd for $C_{10}H_{22}O_9S_2Na$ 373.0603, found 373.0601.

Compound **33** (376 mg, 1.07 mmol) was reacted with NaI (642 mg, 4.28 mmol). The crude product was purified by column chromatography (CH_2Cl_2 → 2.5% Et_2O in CH_2Cl_2) to afford **37** as

clear oil (267 mg, 60%): R_f: 0.25 (EtOAc/pentane 1:9); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.74 (t, 4H, *J* 6.8 Hz), 3.65 (s, 8H), 3.25 (t, 4H, *J* 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 72.0, 70.8, 70.3, 3.1; HRMS (ES): Calcd for $C_8H_{16}I_2O_3Na$ 436.9087, found 436.9084. NMR spectral values for **33** and **37** are in accordance with reference.[27]

4.5.4. Digitoxigenon (13)

Jones reagent (CrO_3 (2.03 g, 20.3 mmol) in H_2O/H_2SO_4 (8 mL/2 mL)) was slowly added to a suspension of digitoxin **1** (994 mg, 1.29 mmol) in acetone (40 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h and added MeOH (5 mL) and stirred further 20 min. The mixture was diluted with H_2O and extracted four times with CH_2Cl_2. The combined organic phases were washed once with satd $NaHCO_3$ and twice with H_2O before dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford **13** (361 mg, 75%) as white foam. The product obtained was used without further purification: R_f: 0.40 (pentane/EtOAc 1:3); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.83 (s, 1H, H22), 4.97 (dd, 1H, $J_{17,21a}$ 1.8 Hz, J_{gem} 18.2 Hz, H21a), 4.78 (dd, 1H, $J_{17,21b}$ 1.4 Hz, J_{gem} 18.2 Hz, H21b), 2.77 (m, 1H, H17), 2.59 (t, 1H, *J* 14.4 Hz), 2.31 (dt, 1H, *J* 5.2 Hz, 14.4 Hz), 2.16–1.20 (m, 19H), 0.98 (s, 3H, CH_3), 0.86 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 212.9 (C3), 174.9 (C20 or C23), 174.7 (C23 or C20), 117.6 (C22), 85.1 (C14), 73.6 (C21), 50.8, 49.7 (C13, C17), 43.7, 42.1, 41.5, 39.7, 37.11, 36.7, 36.6, 35.2, 33.0, 26.9, 26.5, 22.5, 21.2, 21.0, 15.8; LRMS (ES): Calcd for $C_{23}H_{32}O_4Na$ 395.2, found 395.4. NMR spectral values are in accordance with reference.[31]

4.5.5. 3-*epi*-digitoxigenin (14)

To a solution of digitoxigenon **13** (361 mg, 0.969 mmol) in H_2O/dioxane (1:7, 24 mL) at 0 °C was added $NaBH_4$ (92 mg, 2.4 mmol). The reaction mixture was stirred at 0 °C for 1 h and diluted with CH_2Cl_2. The mixture was washed once with H_2O before the aqueous phase was extracted twice with CH_2Cl_2. The combined organic phases were washed with satd NaCl, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc/pentane 1:1 → EtOAc) to afford **14** as white solid (280 mg, 77%): R_f: 0.48 (EtOAc); ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 5.91 (s, 1H, H22), 4.97 (dd, 1H, $J_{17,21a}$ 1.8 Hz, J_{gem} 18.4 Hz, H21a), 4.88 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.4 Hz, H21b), 4.43 (br s, 1H, OH), 4.08 (s, 1H, H3), 3.38 (br s, 1H, H3), 3.31 (s, 1H, OH), 2.74 (m, 1H, H17), 2.05 (m, 2H), 1.83–1.05 (m, 18H), 0.92 (dt, *J* 3.2 Hz, *J* 14.0 Hz, 1H) 0.85 (s, 3H, CH_3), 0.77 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 176.3, 173.8 (C20, C23), 116.2 (C22), 83.7 (C14), 73.1 (C21), 69.8, 50.2, 49.4, 41.3, 41.0, 36.2, 35.5, 34.9, 34.5, 32.2, 30.5, 26.9, 26.4, 23.2, 21.2, 20.6 (CH_3), 15.7 (CH_3); LRMS (ES): Calcd for $C_{23}H_{34}O_4Na$ 397.2, found 397.3. NMR spectral values are in accordance with reference.[31]

4.5.6. 3-*epi*-*O*-*p*-toluenesulfonyl digitoxigenin (15)

To a solution of 3-*epi*-digitoxigenin **14** (269 mg, 0.718 mmol) in dry pyridine (15 mL) at 0 °C was added Ts_2O (592 mg, 1.81 mmol) and DMAP (cat.) The reaction mixture was stirred at room temperature overnight before quenched with H_2O (10 mL). The solution was stirred for 10 min and diluted with CH_2Cl_2 and washed twice with satd $NaHCO_3$. The aqueous phases were extracted thrice with CH_2Cl_2 and the combined organic layers dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc/pentane 2:5) to afford **15** as white foam (369 mg, 97%): R_f: 0.38 (EtOAc/pentane 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 7.79 (d, 2H, J_{ortho} 8.2 Hz, ArH), 7.33 (d, 2H, J_{ortho} 8.2 Hz, ArH), 5.87 (s, 1H, H22), 4.97 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.40 Hz, H21a), 4.79 (dd, 1H, $J_{17,21b}$ 1.8 Hz, J_{gem} 18.2 Hz, H21b), 4.45 (sp, 1H, *J* 5.2 Hz, H3), 2.77 (m, 1H, H17), 2.45 (s, 3H, $SO_2C_6H_4CH_3$), 2.15 (m, 2H), 1.96–1.16 (m, 18H), 0.99 (m, 1H), 0.88 (s, 3H, CH_3), 0.85 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 175.0, 174.7 (C20, C23), 144.6 (Ar), 134.5 (Ar), 129.8 (Ar), 127.6 (Ar), 117.6 (C22), 85.2 (C14), 82.8 (C3), 73.6 (C21), 50.9 (C13 or C17), 49.6 (C17 or C13), 41.7, 41.6, 39.8, 36.1, 34.7, 34.6, 33.1, 33.0, 27.7, 26.9, 26.7, 23.0, 21.7, 21.3, 20.9, 15.8; LRMS (ES): Calcd for $C_{30}H_{40}O_6SNa$ 551.2, found 551.3. NMR spectral values are in accordance with reference.[31]

4.5.7. 3-Deoxy-3-mercaptoacetyl-digitoxigenin (16)

To a solution of **15** (369 mg, 0.698 mmol) in dry DMF (10 mL) was added potassium thioacetate (202 mg, 177 mmol). The reaction mixture was stirred at 50 °C for 22 h before cooled to room temperature and diluted with EtOAc and washed four times with H_2O and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure before the crude product was purified by column chromatography (EtOAc/pentane 1:3 → 1:2) to afford **16** as white solid (209 mg, 69%): R_f: 0.31 (EtOAc/pentane 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.84 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.6 Hz, J_{gem} 18.0 Hz, H21a), 4.79 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 4.03 (br s, 1H, H3), 2.75 (m, 1H, H17), 2.29 (s, 3H, $SCOCH_3$), 2.13 (m, 2H), 1.89–1.14 (m, 19H), 0.92 (s, 3H, CH_3), 0.85 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 195.7 (SCO), 174.8, 174.7 (C20, C23), 117.7 (C22), 85.5 (C14), 73.6 (C21), 50.9, 49.7 (C13, C17), 42.5, 41.9, 40.0, 39.1, 36.1, 35.6, 33.2, 32.8, 31.9, 31.1, 26.9, 26.8, 26.5, 23.8, 21.6, 21.2, 15.9; HRMS (ES): Calcd for $C_{25}H_{36}O_4SNa$ 455.2232, found 455.2249. NMR spectral values are in accordance with reference.[31]

4.5.8. 3-Mercapto digitoxigenin (17)

Thioacetate **16** (270 mg, 0.624 mmol) was dissolved in dry MeOH/THF (1:1, 15 mL) and the mixture was degassed with N_2. Ammonia was then gently bubbled through the reaction mixture at room temperature for 2 h. The reaction mixture was concentrated under reduced pressure to afford crude **17** (303 mg, 124%), which was sufficiently pure for further reactions. R_f: 0.30 (EtOAc/pentane 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.85 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.0 Hz, H21a), 4.79 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.58 (br s, 1H, H3), 2.76 (m, 1H, H17), 2.14 (m, 2H), 1.92–1.15 (m, 19H), 0.96 (s, 3H, CH_3), 0.85 (s, 3H, CH_3); HRMS (ES): Calcd for $C_{23}H_{34}O_3SNa$ 413.2126, found 413.2132 NMR spectral values are in accordance with reference.[31]

4.5.9. 1,2-Bis(3-digitoxigenin)disulfane (18)

The crude **17** was purified by column chromatography (EtOAc/pentane 1:3 → 1:2) to afford **18** as white solid (226 mg, 46%): R_f: 0.30 (EtOAc/pentane 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.85 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.0 Hz, H21a), 4.79 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.58 (br s, 1H, H3), 2.76 (m, 1H, H17), 2.14 (m, 2H), 1.92–1.15 (m, 18H), 0.96 (s, 3H, CH_3), 0.85 (s, 3H, CH_3).

4.6. General synthesis of TBDMS-protected 3-(ethylene glycol)-digitoxigenin-monomer (*n* = 1, 2 or 3)

Sodium hydride (60%, 1.5 equiv) was added to a solution of crude thiol **17** (50–75 mg, 1 equiv) and mesylate **27**, **28** or **29** (3 equiv) in dry DMF (2 mL) under inert atmosphere. The mixture stirred at room temperature for 1–2 h before transferred to a separation funnel containing ice water and CH_2Cl_2, and the aqueous phase was extracted four times with CH_2Cl_2 before the combined organic phases were concentrated under reduced pressure and the residue was then dissolved in EtOAc and washed five times with H_2O. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography.

4.6.1. *tert*-Butyldimehylsilyl 2-(digitoxigenin-3-ylthio)ethanol (38)

Compound **17** (52 mg) was reacted with **27** (89 mg, 0.349 mmol) in dry DMF (2 mL) in the presence of NaH (7 mg, 60%, 0.175 mmol). The crude product was purified by column chromatography (EtOAc/pentane 1:4 → 1:1) to afford 27 mg of **38** as thick clear oil (0.049 mmol, 44% over 2 steps): R_f: 0.26 (EtOAc/pentane 1:3); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.87 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.6 Hz, J_{gem} 18.0 Hz, H21a), 4.80 (dd, 1H, $J_{17,21b}$ 2.0 Hz, J_{gem} 18.0 Hz, H21b), 3.73 (t, 2H, $J_{24,25}$ 7.2 Hz, H25), 3.25 (br s, 1H, H3), 2.78 (m, 1H, H17), 2.60 (t, 2H, $J_{24,25}$ 7.2 Hz, H24), 2.08 (m, 2H), 1.90–1.17 (m, 19H), 0.94 (s, 3H, CH_3), 0.90 (s, 9H, Si-C(CH_3)$_3$), 0.86 (s, 3H, CH_3), 0.06 (s, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.7 (C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 63.6 (C25), 51.1, 49.7 (C13, C17), 43.9, 42.1, 40.2, 37.1, 36.1, 35.8, 34.1, 33.3, 31.9, 30.9, 27.0, 26.9, 26.2, 26.1 (Si-C(CH_3)$_3$), 23.8, 21.7, 21.2, 18.5, 15.9, −5.1 (Si-CH_3); HRMS (ES): Calcd for $C_{31}H_{52}O_4SSiNa$ 571.3253, found 571.3289.

4.6.2. *tert*-Butyldimehylsilyl 4-(digitoxigenin-3-ylthio) diethylene glycol (39)

Compound **17** (75 mg) was treated with **28** (181 mg, 0.573 mmol) in the presence of NaH (12 mg, 60%, 0.300 mmol). The crude product was purified by column chromatography (EtOAc/pentane 1:4) to afford **39** as thick clear oil (28 mg, 36% over 2 steps): R_f: 0.20 (EtOAc/pentane 1:3); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.87 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.0 Hz, H21a), 4.80 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.75 (t, 2H, $J_{26,27}$ 5.2 Hz, H27), 3.63 (t, 2H, $J_{24,25}$ 7.2 Hz, H25), 3.53 (t, 2H, $J_{26,27}$ 5.2 Hz, H26), 3.25 (br s, 1H, H3), 2.78 (m, 1H, H17), 2.67 (t, 2H, $J_{24,25}$ 7.2 Hz, H24), 2.04 (m, 2H), 1.90–1.18 (m, 19H), 0.94 (s, 3H, CH_3), 0.89 (s, 9H, Si-C(CH_3)$_3$), 0.86 (s, 3H, CH_3), 0.06 (s, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.7(C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 72.6, 71.4 (C25, C26), 62.9 (C27), 51.1, 49.7 (C13, C17), 43.9, 42.1, 40.2, 37.2, 36.1, 35.7, 33.3, 31.8, 31.3, 30.9, 27.0, 26.9, 26.2, 26.1 (Si-C(CH_3)$_3$), 23.8, 21.7, 21.2, 18.5, 15.9, −5.1(Si-CH_3); HRMS (ES): Calcd for $C_{33}H_{56}O_5SSiNa$ 615.3515, found 615.3524.

4.6.3. *tert*-Butyldimehylsilyl 6-(digitoxigenin-3-ylthio) triethylene glycol (40)

Compound **17** (51 mg) was reacted with **29** (155 mg, 0.453 mmol) in the presence of NaH (7 mg, 60%, 0.175 mmol). The crude product was purified by column chromatography (EtOAc/pentane 1:3) to afford **40** as thick clear oil (19 mg, 26% over 2 steps): R_f: 0.20 (EtOAc/pentane 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.87 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.6 Hz, J_{gem} 18.0 Hz, H21a), 4.80 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.76 (t, 2H, $J_{28,29}$ 5.2 Hz, H29), 3.64 (m, 6H, H25, H26, H27), 3.56 (t, 2H, J 5.2 Hz, H28), 3.25 (br s, 1H, H3), 2.78 (m, 1H, H17), 2.68 (t, 2H, $J_{24,25}$ 7.2 Hz, H24), 2.12 (m, 2H), 1.91–1.19 (m, 19H), 0.94 (s, 3H, CH_3), 0.89 (s, 9H, Si-C(CH_3)$_3$), 0.87 (s, 3H, CH_3), 0.06 (s, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.6 (C20, C23), 117.9 (C22), 85.7 (C14), 73.6 (C21), 72.9, 71.3, 70.9, 70.6 (C25, C26, C27, C28), 62.9 (C29), 51.1, 49.7 (C13, C17), 43.9, 42.1, 40.2, 37.2, 36.2, 35.8, 33.3, 31.9, 31.2, 30.9, 27.0, 26.9, 26.2, 26.1 (Si-C(CH_3)$_3$), 23.8, 21.7, 21.2, 18.5, 15.9, −5.1(Si-CH_3); HRMS (ES): Calcd for $C_{35}H_{60}O_6SSiNa$ 659.3778, found 659.3760.

4.6.4. *tert*-Butyldimehylsilyl 8-(digitoxigenin-3-ylthio) tetraethylene glycol (41)

To a solution of **18** (25 mg, 0.032 mmol) in absolute EtOH (1 mL) was added $NaBH_4$ (5 mg, 0.132 mmol). The mixture was stirred at room temperature for 10 min, followed by addition of a solution of **30** (34 mg, 0.088 mmol) in absolute EtOH (0.5 mL). The reaction mixture was stirred at room temperature for 20 h before diluted with brine. The aqueous phase was extracted four times with CH_2Cl_2 and the combined organic phases were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc/CH_2Cl_2 1:9) and afforded **41** as clear oil (25 mg, 58%): R_f: 0.19 (EtOAc/CH_2Cl_2 1:4); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.86 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.6 Hz, J_{gem} 18.4 Hz, H21a), 4.79 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.75 (t, 2H, $J_{30,31}$ 5.6 Hz, H31), 3.64 (m, 10H, H25, H26, H27, H28, H29), 3.54 (t, 2H, J 5.6 Hz, H30), 3.23 (br s, 1H, H3), 2.77 (m, 1H, H17), 2.67 (t, 2H, $J_{24,25}$ 7.0 Hz, H24), 2.13 (m, 2H), 1.90–1.15 (m, 19H), 0.93 (s, 3H, CH_3), 0.88 (s, 9H, (Si-C(CH_3)$_3$), 0.86 (s, 3H, CH_3), 0.05 (s, 6H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.7 (C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 72.8, 71.2, 70.8, 70.8, 70.7, 70.4 (C25, C26, C27, C28, C29, C30), 62.8 (C31), 51.0, 49.7 (C13, C17), 43.9, 42.0, 40.1, 37.1, 36.1, 35.7, 33.3, 31.8, 31.1, 30.9, 26.9, 26.8, 26.2, 26.1 (Si-C(CH_3)$_3$), 23.8, 21.7, 21.2, 18.5, 15.9, -5.1 (Si-(CH_3)$_2$); HRMS (ES): Calcd for $C_{37}H_{64}O_7SSiNa$ 703.4040, found 703.4057.

4.7. General procedure for TBDMS-deprotection (*n* = 1, 2, 3 or 4)

Silylether **38**, **39**, **40** or **41** (1 equiv) was dissolved in dry CH_2Cl_2 (2–3 mL), cooled to 0 °C and added Et_3N:3HF (5–8 equiv). The reaction was stirred at room temperature for 20–24 h before diluted with toluene and concentrated under reduced pressure. The crude product was purified by column chromatography.

4.7.1. 2-(Digitoxigenin-3-ylthio)ethanol (9)

Compound **38** (27 mg, 0.049 mmol) was reacted with Et_3N:3HF (48 μL, 0.295 mmol). The crude product was purified by column chromatography (pentane/EtOAc 1:2) to afford **9** as clear oil (18 mg, 85%): R_f: 0.25 (EtOAc/pentane 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.87 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.6 Hz, J_{gem} 18.0 Hz, H21a), 4.80 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.70 (t, 2H, $J_{24,25}$ 6.0 Hz, H25), 3.23 (br s, 1H, H3), 2.77 (m, 1H, H17), 2.71 (t, 2H, $J_{24,25}$ 6.0 Hz, H24), 2.14 (m, 2H), 1.92–1.17 (m, 19H), 0.95 (s, 3H, CH_3), 0.87 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.6 (C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 60.6 (C25), 51.0, 49.7 (C13, C17), 43.3, 42.1, 40.1, 37.3, 36.1, 35.8, 35.1, 33.3, 31.9, 31.0, 27.0, 26.9, 26.3, 23.8, 21.7, 21.2, 15.9; HRMS (ES): Calcd for $C_{25}H_{38}O_4SNa$ 457.2389, found 457.2389.

4.7.2. 4-(Digitoxigenin-3-ylthio)diethylene glycol (10)

Compound **39** (28 mg, 0.047 mmol) was treated with Et_3N:3HF (55 μL, 0.337 mmol). The crude product was purified by column chromatography (pentane/EtOAc 1:2) to afford **10** as clear oil (17 mg, 77%): R_f: 0.45 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.87 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.0 Hz, H21a), 4.80 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.73 (t, 2H, $J_{26,27}$ 4.6 Hz, H27), 3.65 (t, 2H, $J_{24,25}$ 6.6 Hz, H25), 3.58 (t, 2H, $J_{26,27}$ 4.6 Hz, H26), 3.26 (br s, 1H, H3), 2.78 (m, 1H, H17), 2.70 (t, 2H, $J_{24,25}$ 6.6 Hz, H24), 2.14 (m, 2H), 1.91–1.18 (m, 19H), 0.94 (s, 3H, CH_3), 0.87 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.6 (C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 72.1, 70.8 (C25, C26), 61.9 (C27), 51.1, 49.7 (C13, C17), 44.1, 42.1, 40.2, 37.2, 36.1, 35.8, 33.3, 31.8, 31.5, 30.9, 27.0, 26.9, 26.2, 23.8, 21.7, 21.2, 15.9; HRMS (ES): Calcd for $C_{27}H_{42}O_5SNa$ 501.2651, found 501.2648.

4.7.3. 6-(Digitoxigenin-3-ylthio)triethylene glycol (11)

Compound **40** (19 mg, 0.030 mmol) was reacted with Et_3N:3HF (40 μL, 0.245 mmol). The crude product was purified by column chromatography (MeOH/CH_2Cl_2, 1:19) and afforded **11** as clear oil (13 mg, 83%): R_f: 0.51 (MeOH/CH_2Cl_2 1:19); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.87 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.0 Hz, H21a), 4.80 (dd, 1H, $J_{17,21b}$ 0.8 Hz, J_{gem}

18.0 Hz, H21b), 3.74 (m, 2H, H29), 3.64 (m, 8H, H25, H26, H27, H28), 3.25 (br s, 1H, H3), 2.78 (m, 1H, H17), 2.69 (t, 2H, $J_{24,25}$ 7.0 Hz, H24), 2.38 (br s, 1H, $C_{29}OH$), 2.13 (m, 2H), 1.91–1.18 (m, 19H), 0.94 (s, 3H, CH_3), 0.87 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.6 (C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 72.6, 71.2, 70.5, 70.5 (C25, C26, C27, C28), 61.9 (C29), 51.1, 49.7 (C13, C17), 44.0, 42.1, 40.2, 37.2, 36.1, 35.8, 33.3, 31.8, 31.2, 30.9, 27.0, 26.9, 26.2, 23.8, 21.7, 21.2, 15.9; HRMS (ES): Calcd for $C_{29}H_{46}O_6SNa$ 545.2913, found 545.2922.

4.7.4. 8-(Digitoxigenin-3-ylthio)tetra ethylene glycol (12)

Compound **41** (25 mg, 0.037 mmol) was treated with $Et_3N{:}3HF$ (29 μL, 0.181 mmol). The crude product was purified by column chromatography (MeOH/CH_2Cl_2 1:19) to afford **12** as clear oil (20 mg, 95%): R_f: 0.46 (MeOH/CH_2Cl_2 1:19); ^1H NMR (400 MHz, $CDCl_3$): δ_H 5.86 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.2 Hz, H21a), 4.79 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.2 Hz, H21b), 3.72 (m, 2H, H31), 3.63 (m, 12H, H25, H26, H27, H28, H29, H30), 3.24 (br s, 1H, H3), 2.77 (m, 1H, H17), 2.68 (t, 2H, $J_{24,25}$ 7.2 Hz, H24), 2.19–2.07 (m, 3H), 1.89–1.25 (m, 19H), 0.93 (s, 3H, CH_3), 0.86 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.7 (C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 72.6, 71.2, 70.7, 70.8, 70.5, 70.4 (C25, C26, C27, C28, C29, C30), 61.9 (C31), 51.0, 49.7 (C13, C17), 43.9, 42.0, 40.2, 37.2, 36.1, 35.7, 33.3, 31.8, 31.1, 30.9, 29.8, 27.0, 26.8, 26.2, 23.8, 21.7, 21.2, 15.9; HRMS (ES): Calcd for $C_{31}H_{50}O_7SNa$ 566.3277, found 566.3281.

4.8. General procedure for the synthesis of bivalent steroids (5, 7 and 8)

To a solution of **18** (1.1 equiv) in absolute EtOH (2 mL) was added $NaBH_4$ (4 equiv). The mixture was stirred at room temperature for 10 min, followed by addition of dihalide linker (1 equiv). The reaction mixture was stirred at room temperature for 1–17 h and diluted with brine. The aqueous phase was extracted four times with CH_2Cl_2 and the combined organic phases were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography.

4.8.1. 1,2-Bis(digitoxigenin-3-ylthio)ethane (5)

Compound **18** (59 mg, 0.076 mmol) was treated with $NaBH_4$ (11 mg, 0.29 mmol) in absolute EtOH (2 mL). 1,2-Dibromoethane (**34**) (6 μL, 0.070 mmol) was added after 10 min and the reaction mixture was stirred for 16 h. The crude product was purified by column chromatography (EtOAc/CH_2Cl_2 1:2) to afford **5** as white solid (26 mg, 46%): R_f: 0.30 (EtOAc/CH_2Cl_2 1:2); ^1H NMR (400 MHz, $CDCl_3$): δ_H 5.86 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.0 Hz, H21a), 4.79 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.24 (br s, 1H, H3), 2.77 (m, 1H, H17), 2.67 (m, 2H, H24), 2.13 (m, 2H), 1.90–1.18 (m, 19H), 0.93 (s, 3H, CH_3), 0.86 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.7, 174.6 (C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 51.0, 49.7 (C13, C17), 43.8, 42.0, 40.1, 37.2, 36.1, 35.7, 33.3, 32.1, 31.8, 30.9, 27.0, 26.8, 26.2, 23.8, 21.7, 21.2, 15.9; HRMS (ES): Calcd for $C_{48}H_{70}O_6S_2Na$ 829.4512, found 829.4517.

4.8.2. 1,4-(1,4-Dimercapto-1,4-dideoxy-diethylene glycol)bis(digitoxigenin-3-ylsulfane) (6)

Disulfide **18** (82 mg, 0.105 mmol) was treated with $NaBH_4$ (15 mg, 0.39 mmol) in dry DMF (1 mL) for 10 min before a solution of **35** (34 mg, 0.104 mmol) in dry DMF (1 mL) was added. The reaction mixture was stirred at room temperature for 1 h and then diluted with mildly acidic H_2O. The aqueous phase was extracted four times with CH_2Cl_2 before the combined organic phases were concentrated in vacuo. The residue was dissolved in EtOAc and washed five times with H_2O. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc/CH_2Cl_2 1:2) to afford **6** as white solid (24 mg, 27%): R_f: 0.24 (EtOAc/CH_2Cl_2 1:2); ^1H NMR (400 MHz, $CDCl_3$): δ_H 5.87 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.6 Hz, J_{gem} 18.0 Hz, H21a), 4.80 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.60 (t, 2H, $J_{24,25}$ 7.0 Hz, H25), 3.25 (br s, 1H, H3), 2.77 (m, 1H, H17), 2.67 (t, 2H, $J_{24,25}$ 7.0 Hz, H24), 2.12 (m, 2H), 1.90–1.17 (m, 19H), 0.94 (s, 3H, CH_3), 0.86 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.7 (C20, C23), 117.8 (C22), 85.7 (C14), 73.6 (C21), 70.9 (C25), 51.0, 49.7 (C13, C17), 44.0, 42.0, 40.1, 37.2, 36.1, 35.7, 33.3, 31.8, 31.3, 30.9, 27.0, 26.9, 26.2, 23.8, 21.7, 21.2, 15.9; HRMS (ES): Calcd for $C_{50}H_{74}O_7S_2Na$ 873.4773, found 873.4814.

4.8.3. 1,6-(1,6-Dimercapto-1,6-dideoxy-triethylene glycol)bis(digitoxigenin-3-ylsulfane) (7)

Disulfide **18** (18 mg, 0.023 mmol) was treated with $NaBH_4$ (3 mg, 0.079 mmol) in absolute EtOH (1 mL) for 10 min before a solution of **36** (12 mg, 0.032 mmol) in absolute EtOH (0.5 mL) was added. The reaction mixture was stirred for 3 h at room temperature before the crude product was purified by column chromatography (EtOAc/pentane 1:2) to afford **7** as white solid (10 mg, 49%): R_f: 0.25 (pentane/EtOAc 1:2); ^1H NMR (400 MHz, $CDCl_3$): δ_H 5.87 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.0 Hz, H21a), 4.80 (dd, 1H, $J_{17,21b}$ 1.6 Hz, J_{gem} 18.0 Hz, H21b), 3.62 (m, 4H, H25, H26), 3.25 (br s, 1H, H3), 2.78 (m, 1H, H17), 2.69 (t, 2H, $J_{24,25}$ 7.0 Hz, H24), 2.19 (m, 2H), 1.91–1.17 (m, 19H), 0.94 (s, 3H, CH_3), 0.87 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.5 (C20, C23), 117.9 (C22), 85.7 (C14), 73.6 (C21), 71.3, 70.5 (C25, C26), 51.1, 49.7 (C13, C17), 43.9, 42.1, 40.2, 37.2, 36.2, 35.8, 33.3, 31.9, 31.2, 30.9, 27.0, 26.9, 26.2, 23.8, 21.7, 21.2, 15.9; HRMS (ES): Calcd for $C_{52}H_{78}O_8S_2Na$ 917.5036, found 917.5029.

4.8.4. 1,8-(1,8-Dimercapto-1,8-dideoxy-tetraethylene glycol)bis(digitoxigenin-3-ylsulfane) (8)

Disulfide **18** (52 mg, 0.067 mmol) was treated with $NaBH_4$ (17 mg, 0.449 mmol) in absolute EtOH (2 mL) for 10 min before a solution of **37** (30 mg, 0.072 mmol) in absolute EtOH (1 mL) was added. The reaction mixture was stirred for 17 h and the crude product was purified by column chromatography (EtOAc/CH_2Cl_2 1:3) to afford **8** as white solid (39 mg, 58%): R_f: 0.26 (EtOAc/CH_2Cl_2 1:1); ^1H NMR (400 MHz, $CDCl_3$): δ_H 5.86 (s, 1H, H22), 4.98 (dd, 1H, $J_{17,21a}$ 1.2 Hz, J_{gem} 18.0 Hz, H21a), 4.79 (dd, 1H, $J_{17,21b}$ 1.2 Hz, J_{gem} 18.0 Hz, H21b), 3.61 (m, 6H, H25, H26, H27), 3.23 (br s, 1H, H3), 2.77 (m, 1H, H17), 2.67 (t, 2H, $J_{24,25}$ 7.0 Hz, H24), 2.13 (m, 2H), 1.90–1.16 (m, 19H), 0.94 (s, 3H, CH_3), 0.86 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.7, 174.7 (C20, C23), 117.8 (C22), 85.6 (C14), 73.6 (C21), 71.2, 70.7, 70.4 (C25, C26, C27), 51.0, 49.7 (C13, C17), 43.9, 41.9, 40.1, 37.1, 36.1, 35.7, 33.2, 31.8, 31.1, 30.9, 27.0, 26.8, 26.1, 23.8, 21.7, 21.2, 15.9; HRMS (ES): Calcd for $C_{54}H_{82}O_9S_2Na$ 961.5298, found 961.5291.

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