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Synthesis of the first spacer containing prodrug of a duocarmycin analogue and determination of its biological activity†‡

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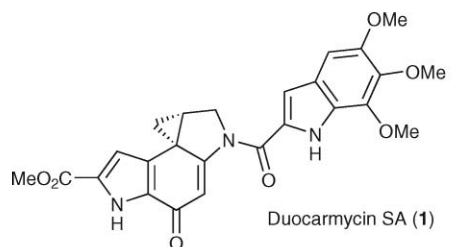
The synthesis of the first spacer containing, duocarmycin analogue prodrug **11** was realised, its biological properties evaluated and compared to its counterpart prodrug **2** without a spacer unit. The synthesis comprises the manufacture of the new acetylated derivatives **19** and **20b** of two double spacer systems, their activation and coupling to the pharmacophoric *seco*-drug (+)-**3**. Unprecedented biological results were found as the new prodrug **11** showed a fairly low QIC_{50} value of 20, but on the other hand a high stability and very low DNA alkylation efficiency. These findings indicate a changed cytostatic mode of action induced by the self-immolative spacer moiety which was employed.

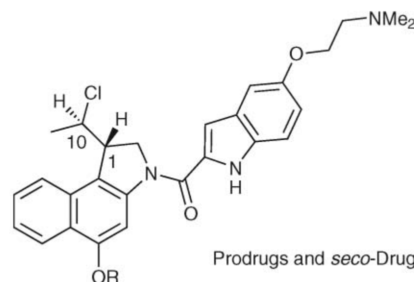
Introduction

One of the major goals in modern chemotherapy of malignant tumours is the development of more selective anticancer drugs to circumvent severe side effects caused by an insufficient differentiation between normal and tumour cells. A promising concept to overcome this problem is the antibody-directed enzyme prodrug therapy, which was first described by Bagshawe and is commonly referred to as ADEPT.[1,2] Selectivity in this approach is achieved by the use of conjugates of an enzyme which is capable of activating the prodrug and a monoclonal antibody which selectively binds to tumour associated antigens. In that way it is possible to restrict the release of the active drug predominantly to the tumour tissue.

The highly potent natural antibiotic duocarmycin SA (**1**, Fig. 1) with an IC_{50} value of 10 pM (L1210)[3] formed the basis of glycosidic prodrugs which were introduced by our group for a use in ADEPT.[4] *Seco*-analogues of **1** such as **3** were successfully detoxified by transforming their phenolic hydroxyl group into glycosides as *e.g.* in the β-galactoside **2** (Fig. 1).[5] By enzymatic cleavage of the glycosidic bond in **2** *seco*-structure **3** is released, which under physiological conditions subsequently undergoes a fast Winstein-cyclisation under loss of HCl. The active drug which is formed in such a cyclisation reaction contains a DNA alkylating spiro-cyclopropylcyclohexadienone moiety as in **1**.

A necessary requirement for a successful application is a sufficient difference in cytotoxicity of the prodrugs and the corresponding drugs which we have defined as QIC_{50} (QIC_{50} = IC_{50} of prodrug/IC_{50} of prodrug in the presence of the cleaving enzyme). Minimum requirements for suitable prodrugs are a QIC_{50} value of > 1000 combined with an IC_{50} value of the liberated drugs of < 10 nm.[6]





(+)-(1*S*,10*R*)-**2**: R = β-Gal IC_{50} = 3600 nM
IC_{50} = 0.75 nM (plus enzyme)
⟹ QIC_{50} = 4800

(+)-(1*S*,10*R*)-**3**: R = H·HCl IC_{50} = 0.75 nM

(+)-(1*S*,10*R*)-**4**: R = β-GlcA IC_{50} = 610 nM
IC_{50} = 0.90 nM (plus enzyme)
⟹ QIC_{50} = 700

Fig. 1 Duocarmycin SA (**1**), prodrugs **2** and **4** for ADEPT and/or PMT as well as *seco*-drug **3**.

For prodrug **2**, a QIC_{50} value of 4800 and an IC_{50} value of the corresponding drug **3** of 750 pm was achieved. This is to date the highest QIC_{50} value reported for a prodrug suitable for ADEPT. However, we recently described a related prodrug that revealed a detoxification which was similarly effective (QIC_{50} = 3500), but whose corresponding *seco*-drug showed a

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† Dedicated to Professor Saverio Florio on the occasion of his 70th birthday

‡ Electronic supplementary information (ESI) available: Synthesis of compound **14** and ^{1}H and ^{13}C NMR spectra of all compounds. See DOI: 10.1039/b925070k

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30–40 times increased cytotoxicity (IC_{50} = 16–26 pm) as compared to **3**.[7]

Further, we have also reported the synthesis and biological evaluation of the glucuronic acid derivative **4** that is not only applicable in ADEPT but also in prodrug monotherapy (PMT),[8] since the activating enzyme β-D-glucuronidase occurs in elevated concentrations in the extra-cellular space of solid tumours.

An undesired consequence of the direct linkage of a carbohydrate moiety to sterically demanding pharmacophores could be a reduced enzyme accessibility of the carbohydrates resulting in a lower rate of enzymatic hydrolysis. Thus, in some cases, *e.g.* using β-D-glucosides, the cytoxicity of the prodrug in the presence of the enzyme β-D-glucosidase was reduced which indicates an incomplete cleavage of the glycosidic bond.[9] One possibility to overcome this problem is the introduction of a spacer between the *seco*-drug or the active drug and the detoxifying unit. The work in the field of tumour-selective prodrugs which contain spacer units was pioneered by Scheeren *et al.* and Monneret *et al.* who introduced and evaluated various self-immolative spacer systems **5–10** in combination with analogues of the cytostatic antitumour drug paclitaxel (Fig. 2).[10] Despite recent advances in linker-equipped cytostatics with protease cleavable peptide-linkers by Senter *et al.*,[11] we focused on self-immolative spacers.

Important general requirements for prodrugs which contain spacer units are a swift enzymatic cleavage of the blocking group and a successive complete self-immolation to release the drug. Although good results for these parameters were achieved, the resulting QIC_{50} of prodrugs **5–10** range only from 1 to 722,[12] which might not be sufficient for a selective treatment of cancer as we pointed out.[6]

Essential issues in this approach are 1) the sufficient stability of the prodrug containing spacer units, 2) the improvement of the rate of the enzymatic cleavage, 3) an increased cytotoxicity of the prodrug in the presence of the enzyme and 4) the dramatic reduction of the cytotoxicity of such prodrugs as compared to the corresponding drugs. These issues have so far not been addressed and since the galactosidic prodrug **2** has thoroughly been investigated we aimed at the synthesis of the spacer analogues **11** and **12** for comparison (Fig. 3). As a spacer we chose the one in prodrug **6** and a combination of **6** and **7** (Fig. 2) with a β-D-galactose moiety as blocking group and a nitro group at the aromatic system in *ortho*-position to the glycosidic bond. The nitro group decreases the pK_a of the phenol formed after the enzymatic cleavage of the phenyl glycoside and hence improves the rate of self-immolation. Furthermore, good results for the stability of this spacer unit were reported.[13] However, Schmidt *et al.* pointed out that an *ortho*-nitro group in the spacer might facilitate decomposition after enzymatic sugar cleavage.[12a]

Results and discussion

Synthesis

For the synthesis of **11** and **12** acetobromogalactose was coupled with commercially available 4-hydroxy-3-nitro-benzaldehyde (**13**, Scheme 1). Best results were obtained using a phase-transfer Michael glycosidation[14] leading to **14** in 80% yield after crystallisation. The reduction of **14** using sodium borohydride to give the alcohol **15** according to the procedure of Farquhar *et al.*[15] required some optimisation as the intermediately formed alcoholate caused a partial migration of the acetyl groups. However, by addition of silica gel and acidic ion exchange resin a reproducible yield of 98% could be obtained. The transformation of the benzyl alcohol **15** into the reactive but at room temperature stable carbonate **16** was achieved using *para*-nitrochloroformate (*p*-NCF). Compound **16** was then coupled with the mono protected diamine **18**[10e,16] in the presence of catalytic amounts of 4-dimethylamino pyridine (DMAP) to afford the carbamate **20a** in 88% yield (based on **15**:

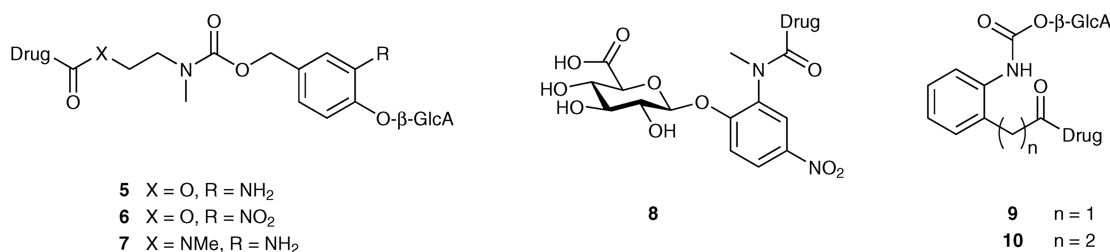

Fig. 2 Paclitaxel prodrugs with self-immolative spacer units by Monneret *et al.* and Scheeren *et al.* (Drug = Paclitaxel based drugs).

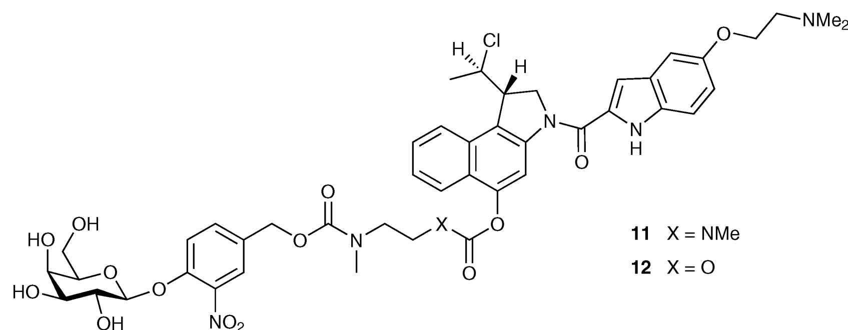

Fig. 3 Prodrugs **11** and **12** as Duocarmycin SA analogues with the glycosidic spacers.

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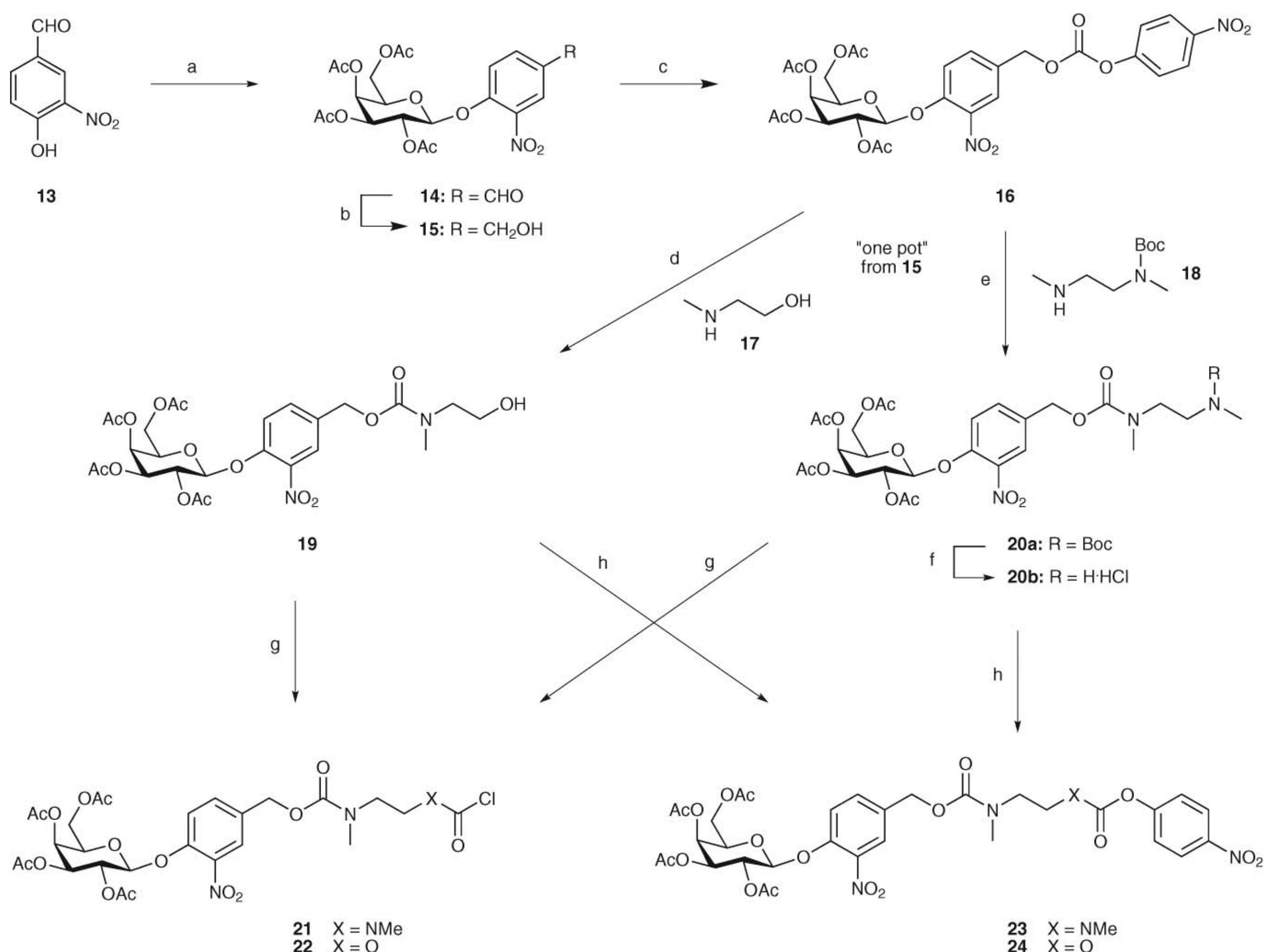

Scheme 1 Formation of the activated carbamates with the synthesis of **21–24**. *Reagents conditions*: a) α-D-Acetobromogalactose, $BnEt_3NBr$, NaOH, H_2O/CH_3Cl, reflux, 3 h, 80%; b) $NaBH_4$, IR120 H^+, silica gel, CH_3Cl/*i*-PrOH (4 : 1), 0 °C, 1.5 h, 98%; c) *para*-nitrophenylchloroformate (*p*-NCF), pyridine, CH_2Cl_2, 0 °C, 2 h, (91% when isolated); d) *N*-methyl-2-aminoethanol (**17**), DMAP, CH_2Cl_2, 0 → 25 °C, 2 h, 72%; e) *N*-Boc-*N*,*N*-dimethylaminoethane (18), DMAP, CH_2Cl_2, 0 → 25 °C, 4.5 h, 88%; f) 3M HCl in EtOAc, 0 → 25 °C, 30 min, 98%; g) Phosgene, Et_3N, CH_2Cl_2, 0 °C, 1 h, **21**: 97%, **22**: 85%; *p*-NCF, pyridine, CH_2Cl_2, 0 →25 °C, 30 min, 92%; h) *p*-NCF, pyridine, CH_2Cl_2, 0 → 25 °C, **23**: 6 h, 98%, **24**: 30 min, 92%.

81% yield) (Scheme 1). Alternatively, a one pot procedure starting from **15** gave **20a** in a slightly increased yield of 88% and cleavage of the *tert*-butyloxycarbonyl moiety in **20a** with anhydrous 3 M HCl in ethyl acetate led to the hydrochloride **20b** in almost quantitative yield.[12b] Similarly, coupling of **16** with the amino alcohol **17** gave **19** in 79% yield (based on **15**: 75%). The one pot procedure starting from **15** led to **19** in a slightly decreased yield of 72% yield compared to the two step transformation.

For the formation of the prodrugs **11** and **12** containing either a carbamate or a carbonate moiety, compounds **20b** and **19** were transformed into the activated chlorides **21** and **22** and the activated *p*-nitrophenylcarbamate **23** and *p*-nitrophenylcarbonate **24**. In the first case, **20b** and **19** were treated with phosgene in the presence of triethylamine to give **21** and **22** in 97% and 85% yield, respectively. In the second case, reaction of **20b** and **19** with *para*-nitrochloroformate gave **23** in 98% yield and **24** in 92% yield.

The structure determination of all compounds by NMR spectroscopy was straight forward; however, it should be mentioned that especially the NMR spectra of **21** and **22** indicate the existence of four conformational isomers even at elevated temperatures.

The coupling of compounds **21–24** with the enantiopure *anti*-methyl-*seco*-CBI-DMAI (+)-(1*S*,10*R*)-**3**, whose enantiopure synthesis has recently been described by us,[17] were performed in DMF under DMAP catalysis (Scheme 2). The reaction of the carbamoyl chloride **21** with **3** led to **25** in 38% yield; contrary, using the *p*-nitrocarbamate **23**, the desired compound could not be obtained despite several variations of the reaction conditions. On the other hand, using the *p*-nitrocarbonate **24**, product **26** was obtained in 58% yield, whereas in this case the corresponding chloride **22** led to **26** in only 27% yield. The final deacetylation step of **25** using Zemplén conditions led readily to the desired prodrug **11** in 79% yield after purification. Unfortunately, we were not able to perform the deacetylation of **26** using the same conditions as well as other methods, *e.g.* 1% HCl in MeOH.[18] In all attempts either a decomposition or no conversion was observed.

Surprisingly, besides a hydrolysis of the carbonate moiety in the case of the decomposition, a cleavage of the normally stable amide bond between the dimethylaminoethoxyindole carboxylic acid moiety and the *anti*-methyl-*seco*-CBI-unit was observed.

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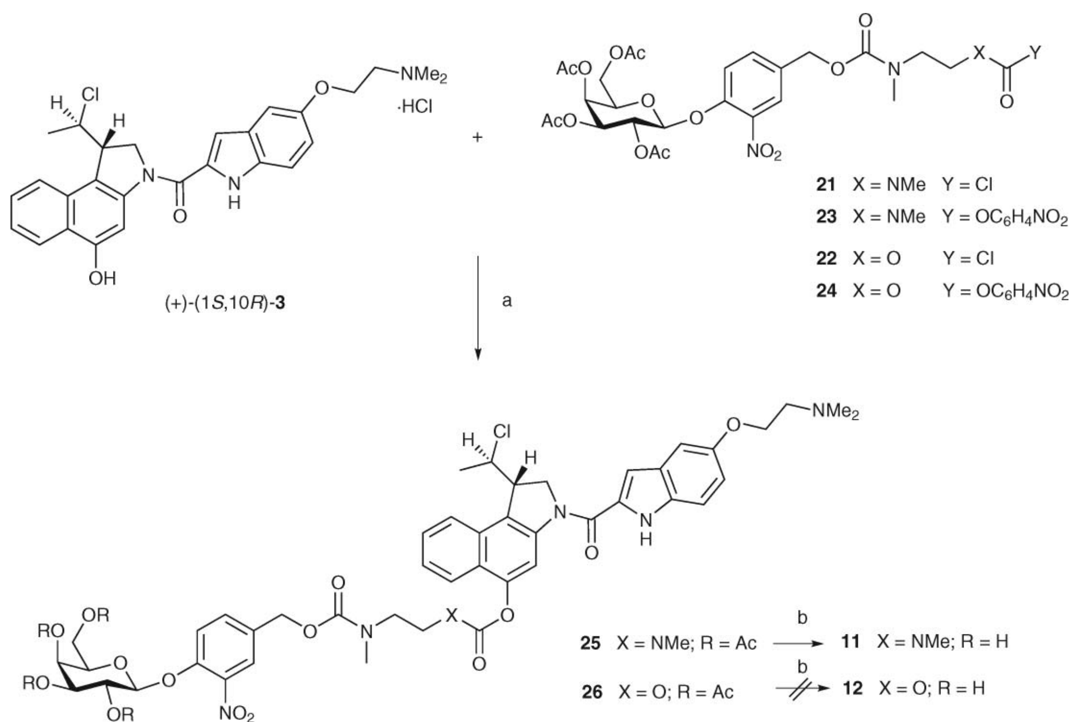

Scheme 2 Coupling of **21–24** with the *seco*-drug **25**. *Reagents and conditions*: a) DMAP, DMF, 0 → 25 °C, 4 h, **27**: 38%, **28**: 58%; b) NaOMe, MeOH, 0 → 25 °C, 3 h, **11**: 79%.

Biological evaluation

Stability. The stability of prodrug **11** in UltraCulture™ cell culture medium was determined using HPLC-MS. Prodrug **11** was stable over 24 h at pH 7.4 and 37 °C and no cleavage of any of the carbamate bonds and thus no generation of the cytotoxic drug was observed.

Cytotoxicity. The cytotoxicity of prodrug **11** in the presence and in the absence of β-D-galactosidase was determined using a human tumour colony forming ability (HTCFA)-assay that reflects the proliferation capacity of single cells and human bronchial carcinoma cells of line A549.

For prodrug **11** an IC_{50} value of 29 nM was determined in the absence of the enzyme, whereas in the presence of β-D-galactosidase a slight increase of the cytotoxicity of **11** with an IC_{50} value of 1.3 nM was found. From these data, a QIC_{50} value of approximately 20 results. Since the IC_{50} of the prodrug in the presence of the enzyme is almost identical to the IC_{50} of the *seco*-drug **3** (0.75 nM), an efficient cleavage of the glycosidic bond and a self-immolation of the spacer moiety can be assumed. Furthermore, an inhibition of the enzyme by the formed drug and the products from the spacer in a suicide mechanism can be excluded.

In comparison to prodrug **2** the QIC_{50} of **11** is surprisingly low. As **11** was shown to be perfectly stable over 24 h in cell culture medium and no free *seco*-drug and hence no drug was generated, we assume that the spacer moiety somehow facilitates the cellular uptake of the prodrug. The uptake is then followed by an intracellular cleavage of the glycosidic bond going along with an alkylation of cellular DNA by the drug which is released. On the other hand the high cytotoxicity of prodrug **11** could also be explained by a direct alkylation of the cellular DNA by the prodrug. To exclude the last possibility we performed additional experiments determining the DNA alkylation efficiency of prodrug **11** in comparison to the corresponding *seco*-drug **3** and also of prodrug **2**.

DNA alkylation studies. For the DNA alkylation studies of **11**, **2** and **3**, synthetic double-stranded oligonucleotides in combination with high-resolution electrospray mass spectrometry was used, a method which was among others recently established in our group in order to investigate these compounds interaction with DNA.[19] For this purpose, the compounds were incubated together with DNA for 24 h in a 1 : 1 and 3 : 1 molecular ratio in water as solvent. The subsequent electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) measurements were carried out directly without chromatographic purification of the reaction mixture and enrichment of the alkylated DNA.[20] Whereas *seco*-drug **3** showed a very high alkylation efficiency towards *N*-3 of adenine in one of the two double strands of the oligonucleotide already at a ratio of 1 : 1, only a small alkylation tendency could be observed for prodrug **2**, and no alkylation at all was found for prodrug **11**. Furthermore, even at a higher prodrug to DNA ratio of 3 : 1 prodrug **11** did not alkylate the DNA. Since prodrug **11** is more toxic than prodrug **2** but at the same time shows a decreased reactivity against DNA, a direct alkylation of DNA by **11** can be excluded as reason for its higher cytotoxicity as compared to **2**. Besides the already mentioned possibility of an intramolecular activation of prodrug **11**, a cell membrane disintegration effect or a general toxicity of **11** caused by its structure are also possible.

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Conclusion

We have prepared the novel prodrug **11** containing a spacer unit with a galactoside moiety as blocking group, which is based on the *seco*-form of an analogue of the highly cytotoxic antibiotic duocarmycin SA. It was shown that the introduction of the self-immolative spacer has no negative influence on the prodrug stability or its enzyme accessibility. However, the compound has a quite high cytotoxicity which is reflected by its low QIC_{50} value of only 20, thus, in the low range of the earlier reported ones of this type. Investigations of the alkylation efficiency of **11** using oligonucleotides attempting to explain these findings show a very low tendency for a direct DNA alkylation. The comparable high cytoxicity of **11** is therefore considered to either result from a intracellular activation after penetration of the compound through the cell membrane, a disintegration of the cell membrane or a general toxicity of **11** due to a changed biological mechanism. The results clearly indicate that in the future development of spacer units and prodrugs containing spacers, a careful investigation of the influence of the spacer unit on the bioactivity will be necessary.

Experimental

General

All reactions were performed in flame-dried glassware under an atmosphere of argon. Solvents were dried and purified according to the method defined by Perrin and Armarego. Commercial reagents were used without further purification. Thin-layer chromatography (TLC) was carried out on precoated Alugram SIL G/UV254 (0.25 mm) plates from Macherey-Nagel & Co. Column chromatography (CC) was carried out on silica gel 60 from Merck with particle size 0.063–0.200 mm for normal pressure and 0.020–0.063 mm for flash chromatography. IR spectra were determined on a Bruker Vektor 22 as KBr-pellets, UV-vis spectra on a Perkin-Elmer Lambda 2, and mass spectra on a Bruker Apex IV Fourier transform ion cyclotron resonance mass spectrometer for ESI-HRMS. ^{1}H-NMR spectra were recorded either on a Varian UNITY-300 MHz, Varian Inova 500 MHz, or Varian Inova 600 MHz. ^{13}C NMR spectra were recorded at 75, 125, or 150 MHz. Spectra were taken at room temperature (except stated otherwise) in deuterated solvents as indicated using the solvent peak as internal standard. The spectra of compounds **14–16**, **19–24** and the prodrug **11** and the new procedure for **14** can be found in the ESI.‡

For stability measurements by HPLC-MS the used column was a Phenomenex Synergi Max-RP C12 (150 mm × 2 mm, particle size 4 μm).

Synthesis of the spacer basic unit

2,3,4,6-Tetra-*O*-acetyl-[2-nitro-4-(hydroxymethyl)phenyl]-β-D-galactopyranoside (15). Benzaldehyde **14** (335 mg, 0.67 mmol, 1.0 equiv.) was dissolved in degassed $CHCl_3$ (5.0 mL) and *i*PrOH (1.1 mL), silica gel (42–60 mesh, 800 mg) and ion exchanger IR-120 H^+-form (~10 mg) added and the mixture cooled to 0 °C. Within 30 min freshly powdered $NaBH_4$ (50.1 mg, 1.35 mmol, 2.0 equiv.) was added portionwise and stirring continued at 0 °C for further 1.5 h. The reaction was quenched by addition of ice-cold sat. NH_4Cl solution (10 mL) and transferred to a separatory funnel with CH_2Cl_2–pentane (1 : 2, 15 mL). After phase separation, the aqueous layer was extracted again with CH_2Cl_2–pentane (1 : 2, 15 mL), the combined organic layers were washed with ice-water (10 mL) and brine (10 mL), filtered over cotton wool and the solvents were removed. Purification by column chromatography on silica (toluene–MeOH = 6 : 1) gave the benzyl alcohol **15** as colourless solid (327 mg, 0.66 mmol, 97%). R_f 0.37 (toluene–MeOH = 6 : 1), 0.15 (EtOAc–pentane = 1 : 1); $[\alpha]^{23}_{D}$ = +54.8 (*c* = 1.0, $CHCl_3$); λ_{max} (CH_3CN)/nm = 213.5, 257.5 and 314.5 (lg ε 1.2078, 0.4980 and 0.2546); $\tilde{\nu}_{max}$/cm^{-1} 3584, 3492, 2888, 1745, 1624, 1580, 1533, 1499, 1437, 1371, 1240, 1129, 1073, 1045, 952, 913, 837, 713, 595; ^{1}H-NMR (599.7 MHz, $CDCl_3$): δ = 1.98, 2.04, 2.09, 2.15 (4 × s, zus. 12 H, 4 × $COCH_3$), 2.22 (s_{br}, 1 H, OH), 4.04 (dt, *J* = 7.0, 6.2, 2.0 Hz, 1 H, H-5), 4.13 (dd, *J* = 11.5, 6.2 Hz, 1 H, H-6_a), 4.22 (dd, *J* = 11.5, 7.0 Hz, 1 H, H-6_b), 4.69 (s, 2 H, $ArCH_2OH$), 5.02 (d, *J* = 7.9 Hz, 1 H, H-1), 5.07 (dd, *J* = 10.5, 3.1 Hz, 1 H, H-3), 5.43 (dd, *J* = 3.3, 1.0 Hz, 1 H, H-4), 5.49 (dd, *J* = 10.5, 8.0 Hz, 1 H, H-2), 7.31(d, *J* = 8.6 Hz, 1 H, H-12), 7.48 (dd, *J* = 8.6, 2.2 Hz, 1 H, H-11), 7.77 (d, *J* = 2.2 Hz, 1 H, H-9); ^{13}C-NMR (150.7 MHz, $CDCl_3$): δ = 20.5, 20.6 (4 × CO*C*H$_3$), 61.3 (C-6), 63.3 (Ar*C*H$_2$OH), 66.7 (C-4), 67.8 (C-2), 70.5 (C-3), 71.3 (C-5), 100.8 (C-1), 119.9 (C-12), 123.2 (C-9), 131.7 (C-11), 137.2 (C-10), 141.2 (C-7), 148.4 (C-8), 169.5, 170.1, 170.2, 170.3 (4 × *C*OCH$_3$); $C_{21}H_{25}NO_{13}$ (499.42).

2,3,4,6-Tetra-*O*-acetyl-[2-nitro-4-(4-nitrophenoxycarbonyloxymethyl)phenyl]-β-D-galactopyranoside (16). To a solution of the benzyl alcohol **15** (400 mg, 0.80 mmol, 1.0 equiv.) and pyridine (126 mg, 129 μl, 1.59 mmol, 2.0 equiv.) in CH_2Cl_2 (20.0 ml) at 0 °C *p*-nitrophenylchloroformate (320 mg, 1.59 mmol, 2.0 equiv.) was added and the mixture was stirred for 2 h at 0 °C. Silica gel (500 mg) was added directly and the solvents were removed. From the residue the target molecule **16** (484 mg, 0.73 mmol, 91%) was obtained by column chromatography on silica (gradient: pentane-EtOAc = 2 : 1 → 1 : 2) as colourless foam. R_f 0.45 (EtOAc–pentane = 1 : 1) ; ^{1}H-NMR (300.1 MHz, $CDCl_3$): δ = 2.03, 2.08, 2.14, 2.20 (4 × s, zus. 12 H, 4 × $COCH_3$), 4.08–4.21 (m, 2 H, H-5, H-6_b), 4.27 (dd, *J* = 11.0, 6.8 Hz, 1 H, H-6_a), 5.13 (d, *J* = 7.3 Hz, 1 H, H-1), 5.13 (dd, *J* = 10.3, 2.4 Hz, 1 H, H-3), 5.30 (s, 2 H, H-10), 5.49 (dd, *J* = 3.4, 0.7 Hz, 1 H, H-4), 5.56 (dd, *J* = 10.5, 7.4 Hz, 1 H, H-2), 7.39 (d, *J* = 9.3 Hz, 2 H, H-16, H-20), 7.42 (d, *J* = 8.8 Hz, 1 H, H-11), 7.63 (dd, *J* = 8.7, 2.2 Hz, 1 H, H-12), 7.92 (d, *J* = 2.2 Hz, 1 H, H-9), 8.29 (d, *J* = 9.3 Hz, 2 H, H-17, H-19). ^{13}C-NMR (75.5 MHz, $CDCl_3$); δ = 20.48, 20.55, 20.58 (4 × CO*C*H$_3$), 61.23 (C-6), 66.58 (C-4), 67.66 (C-2), 68.76 (C-13), 70.36 (C-3), 71.42 (C-5), 100.5 (C-1), 119.6 (C-8), 121.6 (C^{-16}, C-20), 125.3 (C^{-17}, C-19), 125.4 (C-11), 130.1 (C-10), 133.8 (C-9), 141.1 (C-12), 145.4 (C-18), 149.6 (C-7), 152.2 (C-15), 155.2 (C-14), 169.3, 170.0, 170.1, 170.2 (4 × *C*OCH$_3$); *m*/*z* (ESI) 703.10223 (M^+ + K, $C_{28}H_{28}N_2O_{17}K$ requires 703.10196), 682.2 (17%), 687.1 (100), 703.1, 1351.3

Synthesis of the aminoalcohol spacer prodrug

***N*,*N*-Methyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-2-aminoethanol (19).** To a solution of benzylalcohol **15** (300 mg, 0.60 mmol, 1.0 equiv.) in CH_2Cl_2 pyridine (71.3 mg, 72.7 μL, 0.90 mmol, 1.5 equiv.) followed by one portion of *p*-nitrophenylchloroformate (60.5 mg, 0.30 mmol, 1.5 equiv.) was added at 0 °C and stirring continued for 30 min at

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0 °C and another 1.5 h at ambient temperature. After cooling to 0 °C, liquid *N*-methyl-2-aminoethanol (**17**) (63.7 mg, 0.34 mmol, 1.7 equiv.) and DMAP (41.5 mg, 0.34 mmol, 1.7 equiv.) were added and stirring at ambient temperature was continued for 2 h. The reaction was stopped through addition of silica gel (1.38 g) and removal of the solvents under reduced pressure. After column chromatography on silica (gradient: EtOAc–pentane = 1 : 1 → 2 : 1→ 4 : 1) the aminoalcohol **19** (258 mg, 0.43 mmol, 72%) was obtained as colourless syrup. R_f 0.20 (EtOAc–pentane = 4 : 1); $[\alpha]_D^{23}$ = +45.0 (*c* = 0.5, $CHCl_3$) ; λ_{max} (CH_3CN)/nm = 214.0, 255.0, 309.0 and 429.5 (lg ε 1.2243, 0.5020, 0.2498 and 0.1354); $\tilde{\nu}_{max}$/cm^{-1} = 2943, 1753, 1699, 1623, 1538, 1370, 1235, 1150, 1073, 914, 822, 768, 590; ^{1}H-NMR (599.7 MHz, $CDCl_3$): δ = 1.97, 2.03, 2.08, 2.14 (4 × s, 12 H, 4 × $COCH_3$), 2.78, 2.96 (s_{br}, 3 H, NCH_3), 3.41 (t, *J* = 5.2 Hz, 2 H, H_2-15), 3.65–3.79 (m, 2 H, H_2-16), 4.05 (t, *J* = 6.5 Hz, 1 H, H-5), 4.12 (dd, *J* = 11.4, 6.1 Hz, 1 H, H-6_a), 4.21 (dd, *J* = 11.4, 7.0 Hz, 1 H, H-6_b), 5.04 (d, *J* = 8.2 Hz, 1 H, H-1), 5.06 (dd, *J* = 10.6, 3.2 Hz, 1 H, H-3) 5.07 (s_{br}, 2 H, H-13), 5.42 (dd, *J* = 3.3, 0.7 Hz, 1 H, H-4), 5.49 (dd, *J* = 10.2, 8.2 Hz, 1 H, H-2), 7.30 (d, *J* = 8.4 Hz, 1 H, H-12), 7.48 (dd, *J* = 8.6, 2.2 Hz, 1 H, H-11), 7.77 (s_{br}, 1 H, H-9); ^{13}C-NMR (125.7 MHz, $CDCl_3$): δ = 20.48, 20.55, 20.56, 20.58 (4 × $COCH_3$), 34.47, 35.25, 35.54 (NCH_3), 50.78, 51.82 (C-15), 60.39, 60.95 (C-16), 61.25 (C-6), 65.19, 65.37 (C-13), 66.63 (C-4), 67.72 (C-2), 70.43 (C-3), 71.31 (C-5), 100.5 (C-1), 119.6 (C-12), 124.5 (C-9), 133.0, 133.1 (C^{-10}, C-11), 141.0 (C-8), 148.8 (C-7), 157.0, (C-14), 169.3, 170.0, 170.1, 170.3 (4 × $COCH_3$); *m*/*z* (ESI) 623.17010 (M^+ + Na, $C_{25}H_{32}N_2O_{15}Na$ requires 623.16949), 623.2 (100%), 1223.3 (68).

***N*,*N*-Methyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-2-aminoethanol-carbonyl-chloride (22).** To a solution of **19** (120 mg, 0.20 mmol, 1.0 equiv.) in CH_2Cl_2 (5.0 mL) phosgene (20% in toluene, 0.63 mL, 1.44 mmol, 7.2 equiv.) and Et_3N (33.3 μL, 0.24 mmol, 1.2 equiv.) were slowly added at 0 °C. After 1 h at 0 °C silica gel (350 mg) was added, all solvents were removed and the activated alcohol **22** was purified by column chromatography on silica (EtOAc–pentane = 2 : 1) to give a colourless sirup (112 mg, 170 μmol, 85%) which was used directly for the following reaction. R_f 0.49 (EtOAc–pentane = 2 : 1); ^{1}H-NMR (300.1 MHz, $CDCl_3$, strong internal dynamics): δ = 1.98, 2.04, 2.09, 2.16 (4 × s, 12 H, 4 × $COCH_3$), 2.95, 2.97, 3.00 (3 × s, 3 H, NCH_3), 3.40–3.79 (m, 4 H, H-15, H-16), 4.02–4.32 (m, 3 H, H-5, H-6_a, H-6_b), 5.02–5.12 (m, 4 H, H-1, H-3, H-13), 5.44 (d, *J* = 3.1 Hz, 1 H, H-4), 5.51 (dd, *J* = 10.6, 8.1 Hz, 1 H, H-2), 7.31, 7.33 (2 × d, *J* = 8.3 Hz, 1 H, H-12), 7.50 (d, *J* = 8.7 Hz, 1 H, H-11), 7.77 (s_{br}, 1 H, H-9); ^{13}C-NMR (125.7 MHz, $CDCl_3$): δ = 20.51, 20.58, 20.61 (4 × $COCH_3$), 31.08, 31.87, 35.28, 35.41, 35.57, 35.67, 35.73 (NCH_3), 41.28, 41.54, 46.81, 47.47, 47.53, 48.24, 50.36, 50.85, 51.28, 51.88 (C^{-15}, C-16), 61.30 (C-6), 65.37, 65.40 (C-13), 66.70 (C-4), 67.80 (C-2), 70.50 (C-3), 71.38 (C-5), 100.6 (C-1), 119.6, 119.7, 120.0 (C-12), 124.5, 124.6, 124.8, 125.2 (C-9), 132.8, 133.1 (C-11), 133.2, 133.5, 133.6 (C-10), 141.1, 141.2 (C-8), 148.9, 149.0, 149.1 (C-7), 154.8, 155.5, 155.9 (C-14), 169.4, 170.1, 170.2, 170.3 (4 × $COCH_3$), 175.3 (C-17); $C_{26}H_{31}ClN_2O_{16}$ (662.98).

***N*,*N*-Methyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-2-aminoethanol-carbonyl-4-nitrophenol (24).** To a solution of alcohol **19** (241 mg, 0.40 mmol, 1.0 equiv.) in CH_2Cl_2 (15.0 mL) pyridine (47.6 mg, 48.6 μL, 0.60 mmol, 1.5 equiv.) and 4-nitrophenylchloroformate (121 mg, 0.60 mmol, 1.5 equiv.) were added at 0 °C and the mixture was stirred for 30 min at 0 °C and for 30 min 25 °C. Silica gel (350 mg) was added, solvents were removed and the crude material was purified by column chromatography (EtOAc–pentane = 2 : 1) yielding **24** as colourless foam (282 mg, 368 μmol, 92%). R_f 0.63 (EtOAc–pentane = 4 : 1) ; $[\alpha]_D^{23}$ = +35.0 (*c* = 0.9, $CHCl_3$) ; λ_{max} (CH_3CN)/nm = 213.0, 264.0 and 431.0 (lg ε 1.4111, 1.0752 and 0.0800); $\tilde{\nu}_{max}$/cm^{-1} = 2964, 1754, 1704, 1619, 1595, 1536, 1492, 1370, 1351, 1219, 1165, 1073, 954, 897, 860, 769, 664, 590, 496; ^{1}H-NMR (599.7 MHz, $CDCl_3$): δ = 1.97, 2.02, 2.08, 2.15 (4 × s, 12 H, 4 × $COCH_3$), 3.00 (s, 3 H, NCH_3), 3.63 (t, *J* = 5.2 Hz, 2 H, H_2-15), 4.03 (q, *J* = 6.6 Hz, 1 H, H-5), 4.12 (m_c, 1 H, H-6_a), 4.20 (m_c, 1 H, H-6_b), 4.37 (dt, *J* = 20.2, 5.3 Hz, 2 H, H_2-16), 5.04 (m_c, 2 H, H-1, H-3), 5.09 (s, 1 H, H-13), 5.42 (s_{br}, 1 H, H-4), 5.49 (dd, *J* = 10.4, 7.9 Hz, 1 H, H-2), 7.29 (m_c, 2 H, H-19, H-23), 7.32 (d, *J* = 9.0 Hz, 1 H, H-12), 7.49 (dd, *J* = 8.8, 2.3 Hz, 1 H, H-11), 7.77 (dd, *J* = 7.6, 1.6 Hz, 1 H, H-9), 8.23 (ddd, *J* = 10.2, 5.3, 3.3 Hz, 2 H, H-20, H-22); ^{13}C-NMR (150.8 MHz, $CDCl_3$, internal dynamics): δ = 20.48, 20.55, 20.57 (4 × $COCH_3$), 35.26, 35.62 (NCH_3), 47.33, 47.95 (C-15), 61.20 (C-6), 65.37, 65.52 (C-13), 66.44 (C-16), 66.58 (C-4), 67.68 (C-2), 70.40 (C-3), 71.32 (C-5), 100.6 (C-1), 119.5, 119.6 (C-12), 121.6, 121.7 (C^{-19}, C-23), 124.4, 124.6 (C-9), 125.2 (C^{-20}, C-22), 132.6, 132.8, 133.0, 133.1 (C^{-10}, C-11), 141.2 (C-8), 145.4 (C-21), 148.9 (C-7), 152.3 (C-18), 155.1, 155.3, 155.5, 156.0 (C^{-14}, C-17), 169.3, 170.0, 170.1, 170.2 (4 × $COCH_3$); $C_{32}H_{35}N_3O_{19}$ (765.63).

(+)-*N*,*N*-Methyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-*O*-{{methyl-{(1*S*,10*R*)-1-(10-chloro-ethyl)-3-[(5-(2-(*N*,*N*-dimethylamino)-ethoxy)-indol-2-yl)-carbonyl]-1,2-dihydro-3*H*-benz[*e*]indol-5-yl]}}-2-aminoethanol-carbonate ((1*S*,10*R*)-26). To a mixture of the activated alcohol **24** (42.5 mg, 55.5 μmol, 1.0 equiv.) and *seco*-drug (+)-**3** (32.7 mg, 63.6 μmol, 1.15 equiv.) in DMF (6.0 mL) DMAP (16.7 mg, 138 μmol, 2.5 eq.) was added at 0 °C in one portion. After 1 h at 0 °C additional DMAP (16.7 mg, 138 μmol, 2.5 eq.) was added and stirring continued for 2 h at ambient temperature. The mixture was diluted with CH_2Cl_2 (4 mL) and sat. LiBr solution (4 mL). Phases were separated and the aqueous layer was re-extracted with CH_2Cl_2 (4 × 4 mL), the combined organic layers were dried (Na_2SO_4), the solvents removed and benzol (2 × 5 mL) distilled from the residue. After column chromatography on silica (CH_2Cl_2–MeOH = 6 : 1) the acetylated spacer prodrug **26** (35.7 mg, 32.3 μmol, 58%) was obtained as slightly yellow solid. R_f 0.33 (CH_2Cl_2–MeOH = 6 : 1); ^{1}H-NMR (599.8 MHz, DMSO-d_6, 60 °C): δ = 1.68 (d, *J* = 6.6 Hz, 3 H, H_3-11′), 1.95, 2.00, 2.14 (3 × s, 12 H, 4 × $COCH_3$), 2.30 (s, 6 H, $N(CH_3)_2$), 2.73 (t, *J* = 5.8 Hz, 2 H, H_2-2‴), 2.95 (s_{br}, 3 H, NCH_3), 3.67 (d, *J* = 4.7 Hz, 2 H, H_2-15), 4.09–4.17 (m, 4 H, H–1‴, H-6_a, H-6_b), 4.36 (td, *J* = 9.4, 2.2 Hz, 1 H, H-1′), 4.40 (m, 1 H, H-5), 4.45 (t, *J* = 5.2 Hz, H_2-16), 4.69 (dd, *J* = 11.0, 2.4 Hz, 1 H, H-2_a′), 4.80 (t, *J* = 10.2 Hz, 1 H, H-2_b′), 4.85 (dq, *J* = 6.6, 2.4 Hz, 1 H, H-10′), 5.14 (s_{br}, 2 H, H_2-13), 5.20–5.30 (m, 2 H, H-2, H-3), 5.36 (d, *J* = 0.9 Hz, 1 H, H-4), 5.49 (d, *J* = 5.0 Hz, 1 H, H-1), 6.95 (dd, *J* = 8.9, 2.4 Hz, 1 H, H-6″), 7.19 (d, *J* = 1.9 Hz, 1 H, H-3″), 7.20 (d, *J* = 2.2 Hz, 1 H, H-4″), 7.39 (d, *J* = 8.7 Hz, 1 H, H-12), 7.43 (d, *J* = 8.8 Hz, 1 H, H-7″), 7.51 (m_c, 1 H, H-7′), 7.62 (t, *J* = 7.5 Hz, 1 H, H-8′), 7.70 (dd, *J* = 8.7, 1.6 Hz, 1 H, H-11), 7.86, 7.88 (2 × s_{br}, 2 H, H-9, H-9′), 8.08 (d, *J* = 8.4 Hz, 1 H, H-6′), 8.34 (s, 1 H, H-4′), 11.47 (s,

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1 H, NH); ^{13}C-NMR (125.7 MHz, DMSO-d_6, 35 °C): δ = 20.15, 20.20, 20.25, 20.31 (4 × COCH_3), 23.32 (11′-CH_3), 34.16, 34.76 (NCH_3), 45.31 ($N(CH_3)_2$), 46.05 (C-1′), 46.83, 46.93, 47.32 (C-15), 51.85 (C-2′), 57.61 (C-2′′′), 61.11 (C-6), 61.20 (C-10′), 64.74, 64.82 (C-13), 66.01 (C-1′′′), 66.16, 66.42 (C-16), 67.00 (C-4), 67.62 (C-2), 69.86 (C-3), 70.71 (C-5), 98.56 (C-1), 103.3 (C-4′′), 105.7 (C-3′′), 110.1, 110.2 (C-4′), 113.2 (C-7′′), 116.1 (C-5a′, C-6′′), 117.7 (C-12), 122.8, 122.9, 123.1, 123.4, 123.5, 123.6 (C-6′, C-9, C-9′), 124.1 (C-9b′), 125.2 (C-8′), 127.5 (C-3a′′), 127.6 (C-7′), 129.5, 130.4, 131.8 (C-2′′, C-7a′′, C-9a′), 132.4 (C-10), 133.0, 133.2 (C-11), 140.1 (C-8), 141.3 (C-3a′), 146.3, 146.4 (C-17), 147.8 (C-7), 152.9 (C-5′, C-5′′, 2 Signale), 155.0, 155.1, 155.6 (C-14), 160.1 (NC=O), 168.7, 169.4, 169.7, 169.8 (4 × $COCH_3$); *m/z* (ESI) 1104.34806 (M^+ + H, $C_{53}H_{59}N_5O_{19}Cl$ requires 1104.34863), 1104.3 (100%).

Synthesis of a biscarbamate spacer prodrug

***N,N′*-Dimethyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-*N′*-(*tert*-butyloxycarbonyl)-ethylendiamine (20a).** In analogy to the synthesis of alcohol **19** benzylalcohol **15** (100 mg, 0.20 mmol, 1.0 equiv.) was treated with pyridine and *p*-NCF (1.5 equiv. each). After 1 h liquid amine **18** (63.7 mg, 0.34 mmol, 1.7 equiv.) and DMAP (41.5 mg, 0.34 mmol, 1.7 equiv.) were added and the solution was stirred for 4.5 h at 25 °C. Addition of silica gel (400 mg) followed by purification by column chromatography (EtOAc–pentane = 2 : 1) gave **20a** as colourless foam (125 mg, 0.18 mmol, 88%). R_f 0.50 (EtOAc–pentane = 4 : 1) ; $[\alpha]_D^{23}$ = +37.4° (*c* = 0.35, $CHCl_3$) ; λ_{max} (CH_3CN)/nm = 212.5, 254.5 and 309.5 (lg ε 1.2757, 0.5179 and 0.2521); $\tilde{\nu}_{max}$/cm^{-1} = 2978, 1754, 1699, 1623, 1538, 1484, 1402, 1368, 1233, 1163, 1127, 1073, 953, 823, 767, 590; ^{1}H-NMR (599.7 MHz, $CDCl_3$): δ = 1.39 (s, 9 H, $C(CH_3)_3$), 1.97, 2.03, 2.08, 2.15 (4 × s, together 12 H, 4 × $COCH_3$), 2.78, 2.84 (2 × s, 3 H, NCH_3Boc), 2.91 (s, 3 H, NCH_3), 3.19–3.53 (m, 4 H, H-15, H-16), 4.04 (t, *J* = 6.6 Hz, 1 H, H-5), 4.13 (dd, *J* = 11.5, 6.2 Hz, 1 H, H-6_a), 4.21 (dd, *J* = 11.2, 7.0 Hz, 1 H, H-6_b), 5.03 (d, *J* = 7.9 Hz, 1 H, H-1), 5.06 (dd, *J* = 10.5, 3.4 Hz, 1 H, H-3) 5.06 (s_{br}, 2 H, H-13), 5.43 (dd, *J* = 3.4, 1.0 Hz, 1 H, H-4), 5.50 (dd, *J* = 10.5, 7.9 Hz, 1 H, H-2), 7.30 (d, *J* = 8.6 Hz, 1 H, H-12), 7.48 (d, *J* = 8.8 Hz, 1 H, H-11), 7.75 (d, *J* = 1.9 Hz, 1 H, H-9); ^{13}C-NMR (125.7 MHz, $CDCl_3$): δ = 20.50, 20.58, 20.60 (4 × COCH_3), 28.31 ($C(CH_3)_3$), 34.47, 34.83 (2 × NCH_3, 14 signals in total), 35.27, 35.35 ($C(CH_3)_3$), 46.45, 46.70, 46.76, 46.95 (C-15, C-16, 15 signals in total), 61.24 (C-6), 65.16, 65.38 (C-13), 66.62 (C-4), 67.73 (C-2), 70.45 (C-3), 71.34 (C-5), 100.7 (C-1), 119.7 (C-12, 2 signals), 124.5 (C-9), 133.0, 133.2 (C-10, C-11), 141.1 (C-8), 148.8, 148.9 (C-7), 155.6, (C-14), 169.3, 170.0, 170.1, 170.2 (4 × $COCH_3$); *m/z* (ESI) 736.25347 (M^+ + H, $C_{31}H_{44}N_3O_{16}$ requires 736.25355), 736.3 (100%), 1449 (20).

***N,N′*-Dimethyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-ethylendiamine-hydrochloride (20b).** The amine **20a** (40 mg, 56 µmol, 1.0 equiv.) was cooled to 0 °C and a freshly prepared, pre-cooled solution of HCl (3 M in EtOAc, 5.0 mL) was added and the mixture was stirred for 30 min at ambient temperature. The solvent was removed under reduced pressure and benzol (2 × 5.0 mL) distilled from the residue. The crude amine salt **20b** (37.0 mg) was obtained analytically pure and used directly in the following reaction. R_f 0.00 (EtOAc–pentane = 4 : 1) ; ^{1}H-NMR (599.7 MHz, DMSO-d_6, 85 °C): δ = 1.94, 2.01, 2.02, 2.14 (4 × s, together 12 H, 4 × $COCH_3$), 2.55 (s_{br}, 3 H, $NCH_3H_2^+$), 2.92 (s_{br}, 3 H, NCH_3CO), 3.58 (t, *J* = 5.2 Hz, 2 H, H-15), 3.05 (t, *J* = 5.6 Hz, 2 H, H-16), 4.12 (dd, *J* = 11.5, 5.3 Hz, 1 H, H-6_a), 4.15 (dd, *J* = 11.4, 5.3 Hz, 1 H, H-6_b), 4.48 (t, *J* = 5.5 Hz, 1 H, H-5), 5.13 (s, 2 H, H-13), 5.24 (dd, *J* = 10.1, 7.6 Hz, 1 H, H-2), 5.28 (dd, *J* = 10.3, 3.2 Hz, 1 H, H-3), 5.37 (dd, *J* = 2.9 Hz, 1 H, H-4), 5.56 (d, *J* = 7.6 Hz, 1 H, H-1), 7.45 (d, *J* = 8.3 Hz, 1 H, H-12), 7.73 (d, *J* = 8.1 Hz, 1 H, H-11), 7.88 (s_{br}, 1 H, H-9), 9.11 (s_{br}, 2 H, $NCH_3H_2^+$); ^{13}C-NMR (125.7 MHz, DMSO-d_6, 35 °C): δ = 20.16, 20.23, 20.37 (4 × COCH_3), 32.43, (N$CH_3H_2^+$), 34.09, 34.55 (NCH_3CO), 44.42, 44.58, 44.76 (C-16), 45.68, 46.11 (C-15), 61.06 (C-6), 64.80 (C-13), 66.96 (C-4), 67.58 (C-2), 69.77 (C-3), 70.65 (C-5), 98.42 (C-1), 117.7 (C-12), 123.7 (C-9), 132.2 (C-10), 133.2, 133.4 (C-11), 140.0 (C-8), 147.7 (C-7), 154.8, 155.6 (C-14), 168.7, 169.3, 169.7, 169.8 (4 × $COCH_3$); *m/z* (ESI) 614.21908 (M^+ + H, $C_{26}H_{36}N_3O_{14}$ requires 614.21918), 614.2 (100%).

***N,N′*-Dimethyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-ethylendiamine carbamoylchloride (21).** The amine salt **20b** (54.7 mg, 76.6 µmol, 1.0 equiv.) was suspended in CH_2Cl_2 (2.10 mL), cooled to 0 °C and phosgene (20% in toluene, 0.24 mL, 0.55 mmol, 7.2 equiv.) followed by Et_3N (12.9 µL, 93.0 µmol, 1.2 equiv.) were added slowly. After 1 h at this temperature silica gel (200 mg) was added and the solvents were removed under reduced pressure. After conventional column chromatography on silica (EtOAc–pentane = 5 : 1) the activated amine **21** (50.0 mg, 74.0 µmol, 97%) was obtained as colourless foam. R_f 0.48 (EtOAc–pentane = 5 : 1); ^{1}H-NMR (599.7 MHz, $CDCl_3$): δ = 1.98, 2.04, 2.09, 2.16 (4 × s, together 12 H, 4 × $COCH_3$), 2.93, 2.96, 3.04, 3.05, 3.12 (5 × s_{br}, 6 H, 2 × NCH_3), 3.44–3.57 (m, 2 H, H-15), 3.58, 3.62 (2 × t, *J* = 5.9 Hz, 2 H, H-16), 4.05 (t, *J* = 6.2 Hz, 1 H, H-5), 4.14 (dd, *J* = 11.4, 6.2 Hz, 1 H, H-6_a), 4.21 (dd, *J* = 11.2, 7.0 Hz, 1 H, H-6_b), 5.01–5.12 (m, 4 H, H-1, H-3, H-13), 5.40 (dd, *J* = 3.0 Hz, 1 H, H-4), 5.51 (dd, *J* = 10.4, 8.0 Hz, 1 H, H-2), 7.32 (dd, *J* = 8.5, 2.1 Hz, 1 H, H-12), 7.49, 7.55 (2 × d, *J* = 8.4 Hz, 1 H, H-11), 7.77, 7.78 (2 × s_{br}, 1 H, H-9); ^{13}C-NMR (125.7 MHz, $CDCl_3$, 25 °C, strong internal dynamics): δ = 20.53, 20.61, 20.65 (4 × COCH_3), 34.58, 35.14, 35.17, 35.41, 36.97, 37.38, 38.74, 39.35 (2 × NCH_3), 45.88, 46.13, 46.65, 46.85, 48.27, 49.17, 50.19, 50.57 (C-15, C-16), 61.27 (C-6), 65.32, 65.45, 65.59, 65.75 (C-13), 66.62 (C-4), 67.70 (C-2), 70.46 (C-3), 71.34 (C-5), 100.6 (C-1), 119.7, 119.8 (C-12), 124.5, 124.6, 124.8, 125.0 (C-9), 132.4, 132.6, 132.7, 132.9, 133.1 (C-11), 133.6, 133.7 (C-10), 141.1 (C-8), 148.8, 148.9, 149.0, 149.1 (C-17), 149.8, 150.2 (C-7), 155.4, 155.5, 155.9, 156.1 (C-14), 169.4, 170.1, 170.3 (4 × $COCH_3$); *m/z* (ESI) 693.20190 (M^+ + NH_4, $C_{27}H_{38}ClN_4O_{15}$ requires 693.20167), 698.15708 (M^+ + Na, $C_{27}H_{34}ClN_3O_{15}Na$ requires 698.15750), 698.2 (100%), 714.1 (75), 1373.3 (40), 1389.3 (25).

***N,N′*-Dimethyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-ethylendiamine carbamoyl-4-nitrophenol (23).** The crude amine salt **20b** (27.2 mg, 41.8 µmol, 1.0 equiv.) in CH_2Cl_2 (5.0 mL) was treated with pyridine (9.90 mg, 10.1 µL, 126 µmol, 3.0 equiv.) and *p*-NCF (16.85 mg, 83.6 µmol, 2.0 equiv.) for 30 min at 0 °C. After 4 h additional pyridine (3.0 equiv.) and *p*-NCF (2.0 equiv.) were added and the reaction mixture was stirred for 2 h. Silica gel (300 mg) was added, the solvents were removed and product **23** (32.1 mg, 41.2 µmol, 98% starting from **20b**) was obtained as colourless sirup after

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column chromatography (EtOAc–pentane = 4 : 1). R_f 0.39 (EtOAc–pentane = 3 : 1); $[\alpha]_D^{23}$ = + 18.3° (*c* = 1.31, MeOH) ; λ_{max} (CH_3CN)/nm = 272.0 (lg ε 1.0096) ; $\tilde{\nu}_{max}$/cm^{-1} = 2961, 1754, 1704, 1616, 1595, 1537, 1404, 1348, 1220, 1162, 1073, 864, 821, 749, 685, 590; ^{1}H-NMR (599.7 MHz, $CDCl_3$, strong internal dynamics): δ = 1.99, 2.04, 2.10, 2.17 (4 × s, together 12 H, 4 × $COCH_3$), 2.95, 2.97, 3.03, 3.07, 3.12 (5 × s_{br}, 6 H, 2 × NCH_3), 3.43–3.64 (m, 4 H, H-15, H-16), 3.97–4.09 (m, 1 H, H-5), 4.13 (dd, *J* = 11.2, 6.3 Hz, 1 H, H-6_a), 4.18–4.23 (m, 1 H, H-6_b), 5.00–5.11 (m, 4 H, H-1, H-3, H-13), 5.44 (d, *J* = 2.4 Hz, 1 H, H-4), 5.51 (dd, *J* = 10.1, 8.7 Hz, 1 H, H-2), 7.12, 7.21 (2 × d, *J* = 8.9 Hz, 1 H, H-19, H-23), 7.26–7.31 (m, 1 H, H-12), 7.46 (t, *J* = 10.0 Hz, 1 H, H-11), 7.77 (m, 1 H, H-9), 8.15–8.24 (m, 2 H, H-20, H-22); ^{13}C-NMR (125.7 MHz, $CDCl_3$, 27 °C, very strong internal dynamics): δ = 20.53, 20.60, 20.62 (4 × $COCH_3$), 34.57, 34.91, 35.23, 35.35, 35.44, 35.48 (2 × NCH_3), 46.12, 46.50, 46.75, 46.91, 47.30, 47.39, 47.49 (C^{-15}, C-16), 61.25 (C-6), 65.26, 65.42, 65.52, 65.80 (C-13), 66.64 (C-4), 67.78 (C-2), 70.49 (C-3), 71.41 (C-5), 100.7 (C-1), 119.5, 119.6, 119.7, 119.8 (C-12), 122.0, 122.1, 122.4 (C^{-19}, C-23), 124.5, 124.6 (C-9), 125.0, 125.1 (C^{-20}, C-22), 132.3, 132.7, 132.9, 133.2, 133.3, 133.7 (C^{-10}, C-11), 141.1, 141.3 (C-8), 144.8, 145.1 (C-21), 148.9, 149.0, 149.1 (C-7), 153.1 153.3, 153.6 (C-18), 155.6, 155.7, 155.9, 156.0, 156.1, 156.2 (C^{-14}, C-17), 169.3, 170.1, 170.2, 170.3 (4 × $COCH_3$); $C_{33}H_{38}N_4O_{18}$ (778.67).

(+)-*N*,*N*′-Dimethyl-[4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-*O*-{{methyl-{(1*S*,10*R*)-1-(10-chloro-ethyl)-3-[(5-(2-(*N*,*N*-dimethylamino)-ethoxy)-indol-2-yl)-carbonyl]-1,2-dihydro-3*H*-benz[*e*]indol-5-yl]}}-ethylendiamine carbamate ((1*S*,10*R*)-25). To a cooled solution (0 °C) of the activated amine **21** (30.0 mg, 44.5 μmol, 1.0 equiv.) and *seco*-drug (+)-**3** (22.8 mg, 63.6 μmol, 1.0 equiv.) in DMF (6.0 mL) DMAP (53.7 mg, 443 μmol, 10 equiv.) was slowly added (10 min) followed by dropwise addition of Et_3N (9.25 μl, 66.8 μmol, 1.5 equiv.) and stirring was continued at 0 °C for 2 h. The mixture was diluted with CH_2Cl_2 (4.0 mL) and sat. LiBr solution (2.5 mL). Phases were separated and the aqueous layer was re-extracted with CH_2Cl_2 (4 × 5 mL), the combined organic layers were dried (Na_2SO_4), the solvents removed and benzol (2 × 5 mL) was destilled from the residue. After column chromatography on silica (CH_2Cl_2–MeOH = 6 : 1) the acetylated spacer prodrug **25** (18.9 mg, 16.9 μmol, 38%) was obtained as colourless solid. R_f 0.36 (CH_2Cl_2–MeOH = 5 : 1); $[\alpha]_D^{23}$ = +24.0° (*c* = 0.4, MeOH); λ_{max} (CH_3CN)/nm = 204.0, 250.0, 296.5 and 332.0 (lg ε 1.8025, 1.3737, 1.5361 and 1.4955); $\tilde{\nu}_{max}$/cm^{-1} = 2934, 1754, 1626, 1537, 1461, 1408, 1370, 1232, 1123, 1072, 758; ^{1}H-NMR (599.8 MHz, DMSO-d_6, 70 °C): δ = 1.68 (d, *J* = 6.6 Hz, 3 H, H_3-11′), 1.95, 1.99, 2.00, 2.14 (4 × s, together 12 H, 4 × $COCH_3$), 2.29 (s, 6 H, $N(CH_3)_2$), 2.72 (t, *J* = 5.8 Hz, 2 H, H_2-2‴), 2.90–3.81 (m, 10 H, 2 × NCH_3, H_2-15, H_2-16), 4.08–4.14 (m, 4 H, H_2-1‴, H_2-6), 4.33 (td, *J* = 9.5, 2.4 Hz, 1 H, H-1′), 4.39 (t, *J* = 6.3 Hz, 1 H, H-5), 4.68 (dd, *J* = 11.0, 1.7 Hz, 1 H, H-2_a′), 4.77 (t, *J* = 10.2 Hz, 1 H, H-2_b′), 4.84 (dq, *J* = 6.6, 2.4 Hz, 1 H, H-10′), 5.14 (s_{br}, 2 H, H_2-13), 5.21–5.27 (m, 2 H, H-2, H-3), 5.36 (d, *J* = 2.1 Hz, 1 H, H-4), 5.47 (d, *J* = 6.3 Hz, 1 H, H-1), 6.94 (dd, *J* = 8.9, 2.2 Hz, 1 H, H-6″), 7.16 (s_{br}, 1 H, H-3″), 7.20 (d, *J* = 2.0 Hz, 1 H, H-4″), 7.36 (s_{br}, 1 H, H-12), 7.43 (d, *J* = 8.9 Hz, 1 H, H-7″), 7.45 (m_c, 1 H, H-7′), 7.58 (t, *J* = 7.6 Hz, 1 H, H-8′), 7.66 (s_{br}, 1 H, H-11), 7.85 (s_{br}, 2 H, H-9, H-9′), 8.03 (d, *J* = 8.4 Hz, 1 H, H-6′), 8.20 (s, 1 H, H-4′), 11.40 (s, 1 H, NH); ^{13}C-NMR (125.7 MHz, DMSO-d_6, 35 °C): δ = 20.17, 20.20, 20.25, 20.30 (4 × $COCH_3$), 23.35 (11′-CH_3), 33.76, 34.23, 34.48, 34.62, 34.75, 35.67 (2 × NCH_3), 45.36 ($N(CH_3)_2$), 46.04 (C-1′), 45.94, 46.23, 46.33, 46.64, 46.77 (C^{-15}, C-16), 51.89 (C-2′), 57.66 (C-2‴), 61.08 (C-6), 61.25 (C-10′), 64.60, 64.71, 64.81, 64.87 (C-13), 66.09 (C-1‴), 66.99 (C-4), 67.63 (C-2), 69.86 (C-3), 70.71 (C-5), 98.58 (C-1), 103.3 (C-4″), 105.6 (C-3″), 110.4, 110.5, 110.7 (C-4′), 113.1 (C-7″), 116.0 (C-6″), 117.7 (C-5a′, C-12), 123.0 (C-6′, C-9b′), 123.4 (C-9′), 123.6, 123.7, 123.9, 124.0 (C-9), 124.7 (C-7′), 127.2 (C-8′), 127.5 (C-3a″), 129.4, 130.5, 131.7 (C-2″, C-7a″, C-9a′), 132.5, 132.6 (C-10), 133.1, 133.3 (C-11), 140.0, 140.1 (C-8), 141.3 (C-3a′), 147.2, 147.3, 147.8 (C-7), 152.9, 153.7 (C-5′, C-5″), 155.5 (C-14), 160.0 (NC=O), 162.2 (C-17), 168.7, 169.4, 169.7, 169.8 (4 × $COCH_3$); *m/z* (ESI) 1117.38056 (M^+ + H, $C_{54}H_{62}N_6O_{18}Cl$ requires 1117.38036), 1117.4 (100%).

(+)-*N*,*N*′-Dimethyl-[4-(β-D-galactopyranosyl]-3-nitrobenzyl-oxycarbonyl]-*O*-{{methyl-{(1*S*,10*R*)-1-(10-chloro-ethyl)-3-[(5-(2-(*N*,*N*-dimethylamino)-ethoxy)-indol-2-yl)carbonyl]-1,2-dihydro-3*H*-benz[*e*]indol-5-yl]}}-ethylendiamine carbamate ((1*S*,10*R*)-11). The acetylated prodrug **25** (14.6 mg, 13.1 μmol, 1.0 equiv.) was dissolved in MeOH (3.00 mL) and treated at 0 °C with NaOMe (0.065 M in MeOH, 100 μl, 6.50 μmol, 0.5 equiv.) for 30 min under stirring. Neutralisation was realised using a solution of HCl (0.10 M in MeOH, 65.0 μl, 0.5 equiv.) and the solvents removed. Conventional column chromatography on silica (CH_2Cl_2–MeOH = 3 : 1), removal of the solvents to 1 mL and filtration through a membrane filter following by solvent evaporation under reduced pressure gave the spacer prodrug (1*S*,10*R*)-**11** (9.80 mg, 10.3 μmol, 79%) as colourless, amorphous solid. R_f 0.12 (CH_2Cl_2–MeOH = 3 : 1); λ_{max} (CH_3CN)/nm = 253.0, 297.0 and 330.5 (lg ε 1.1978, 1.2604 and 1.2348), $\tilde{\nu}_{max}$/cm^{-1} = 3386, 2925, 1715, 1623, 1533, 1409, 1212, 1072, 759; ^{1}H-NMR (599.8 MHz, DMSO-d_6, 70 °C): δ = 1.67 (d, *J* = 6.6 Hz, 3 H, H_3-11′), 2.29 (s, 6 H, $N(CH_3)_2$), 2.72 (t, *J* = 5.8 Hz, 2 H, H_2-2‴), 2.88–3.39 (m, 6 H, 2 × NCH_3, underneath H_2O signal), 3.42 (dd, *J* = 9.4, 3.1 Hz, 1 H, H-3), 3.45–3.64 (m, 6 H, H-2, H-5, H_2-6, H-15, H-16), 3.75 (d, *J* = 2.9 Hz, 1 H, H-4), 4.11 (t, *J* = 5.8 Hz, 2 H, H_2-1″), 4.34 (m_c, 1 H, H-1′), 4.39–4.62 (m_{br}, 4 H, 4 × OH), 4.67 (dd, *J* = 11.2, 2.3 Hz, 1 H, H-2_a′), 4.77 (t, *J* = 10.4 Hz, 1 H, H-2_b′), 4.84 (m_c, 1 H, H-10′), 5.47 (d, *J* = 7.1 Hz, 1 H, H-1), 5.11 (s_{br}, 2 H, H_2-13), 6.94 (dd, *J* = 8.9, 2.4 Hz, 1 H, H-6″), 7.16 (s_{br}, 1 H, H-3″), 7.19 (d, *J* = 2.1 Hz, 1 H, H-4″), 7.34 (s_{br}, 1 H, H-12), 7.42 (d, *J* = 9.0 Hz, 1 H, H-7″), 7.46 (m_c, 1 H, H-7′), 7.59 (m_c, 2 H, H-11, H-8′), 7.79, 7.87 (2 × s_{br}, 2 H, H-9, H-9′), 8.03 (d, *J* = 8.4 Hz, 1 H, H-6′), 8.20 (s, 1 H, H-4′), 11.41 (s, 1 H, NH); ^{13}C-NMR (125.7 MHz, DMSO-d_6, 35 °C): δ = 23.38 (11′-CH_3), 31.19, 31.50, 34.67, 34.80, 34.84, 38.52, 38.61 (2 × NCH_3), 45.33 ($N(CH_3)_2$), 46.03 (C-1′), 40.41, 40.49, 46.40 (C^{-15}, C-16), 51.90 (C-2′), 57.62 (C-2‴), 60.11 (C-6), 61.29 (C-10′), 64.81, 64.83, 64.95 (C-13), 66.03 (C-1‴), 67.82 (C-4), 70.00 (C-5), 73.30 (C-3), 75.68 (C-2), 101.1 (C-1), 103.3 (C-4″), 105.6 (C-3″), 110.5, 110.7 (C-4′), 113.2 (C-7″), 116.0 (C-6″), 117.0 (C-5a′, C-12), 122.2, 123.1, 123.5, 123.6 (C-6′, C^{-9}, C-9′, C-9b′), 124.4 (C-7′), 127.3 (C-8′), 127.5 (C-3a″), 129.4, 130.6, 131.7 (C-2″, C-7a″, C-9a′), 131.7 (C-10), 133.3 (C-11), 139.8 (C-8), 141.3 (C-3a′), 147.2 (C-7), 150.6 (C-5′), 152.9 (C-5″), 155.5 (C-14), 160.0 (NC=O), 162.7 (C-17); *m/z* (ESI) 949.33796 (M^+ + H, $C_{46}H_{54}ClN_6O_{14}$ requires 949.33810), 949.3 (100%), 971.2 (41).

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In vitro cytotoxicity assay

Adherent cells of line A549 were sown in triplicate in 6 multiwell plates at concentrations of 10^2, 10^3, and 10^4 cells per cavity. Culture medium was removed using suction after 24 h and cells were washed in the incubation medium UltraCulture™ (UC, serum-free special medium, purchased from Lonza). Incubation with compound **11** was then performed in UltraCulture™ medium at 6–8 various concentrations for 24 h. All substances were used as freshly prepared solutions in DMSO (Merck, Darmstadt, Germany) diluted with incubation medium to a final concentration of DMSO of 1% in the wells. After 24 h of exposure the test substance was removed and the cells were washed with fresh medium. Cultivation was accomplished at 37 °C and 7.5% CO_2 in air for 9–10 days. The medium was removed and the clones were dried and stained with Löffler's methylene blue (Merck, Darmstadt, Germany). They were then counted macroscopically.

The IC_{50} values are based on the relative clone forming rate, which was determined according to the following formula: relative clone forming rate [%] = 100 × (number of clones counted after exposure)/(number of clones counted in the control). The obtained data points and graph can be found in the ESI.‡

Liberation of the drug from its glycosidic prodrug was achieved by addition of 4 U mL^{-1} β-galactosidase (E.C. 3.2.1.23) from *Escherichia coli* G 5635 (*Sigma*), to the cells during incubation with the substances.

HPLC-MS investigation of prodrug stability

An aliquot of a stock solution of prodrug **11** in DMSO (2 µL, 0.32 µmol) was dissolved in UltraCulture™ medium (*Cambrex*, pH 7.4, 198 µL) and incubated at 37 °C for 24 h. At $t = 0$ h and $t = 24$ h an aliquot of the reaction mixture was investigated by HPLC-MS. Column: Synergi Max-RP C12 (*phenomenex*, 150 × 2 mm, particle size: 4 µm), eluent A: water with 0.05% (v/v) formic acid (*Roth*), eluent B: methanol (VWR) with 0.05% (v/v) formic acid (*Roth*), flow: 0.3 mL min^{-1}, gradient: A/B = 70/30 (0 min) → 0/100 (15 min), DAD: 200–800 nm, MS: ESI^+, m/z 100–2000.

ESI-FTICR-MS investigation of DNA alkylation

The double-stranded oligonucleotide 5′-d(GCG ACT AAT TGA CCG)-3′ (*IBA*) was used as aqueous solution (0.1 mM) of the corresponding sodium-salt. Stock solutions of **3**, **2** and **11** were prepared in DMSO. 1 µL of stock solution was diluted with water, one aliquot of this solution mixed with 100 µL of oligonucleotide in water and the reaction mixture incubated at 25 °C for 24 h. At $t = 0$ h and $t = 24$ h an aliquot of the reaction mixture was diluted with an equivalent amount of methanol and introduced directly into the ion source by a syringe pump (74900 series, *Cole-Parmer*, Vernon Hills, USA) with a flow rate of 2 µL min^{-1}. High-resolution mass spectrometry was performed using a 7 T-FTICR-MS instrument (APEX IV, *Bruker Daltonics*) equipped with an APOLLO electrospray ion source. The ions generated in the negative ion mode were accumulated in the hexapole region for 0.8 s and transferred subsequently into the ICR cell. For gentle desolvatisation the drying gas temperature was set to 100 °C and the capillary exit voltage to −100 V. Enhanced fragmentation of alkylated oligonucleotides was achieved by capillary-skimmer dissociation (CSD) with a capillary exit voltage of −150 V. CID-MS/MS measurements were carried out by fragmentation of ions isolated in the ICR cell using Argon as collision gas.

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