DOI: 10.1002/cmdc.200800249

Synthesis and Properties of a Mitochondrial Peripheral Benzodiazepine Receptor Conjugate

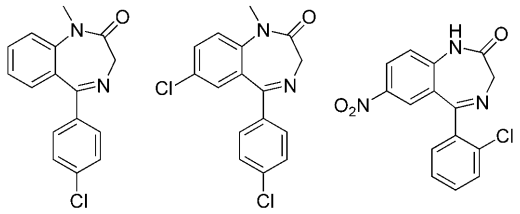
Abdessamad El Alaoui,[a, b] Frédéric Schmidt,*[a, b] Marianne Sarr,[c, d] Didier Decaudin,[c, e, f] Jean-Claude Florent,[a, b] and Ludger Johannes[c, d]

Peripheral benzodiazepine receptors are potential targets for cancer therapeutics through the use of specific ligands such as the pro-apoptotic benzodiazepine RO5-4864. However, the poor water solubility of this compound has been a limitation to its application in vivo. Herein we describe an efficient synthesis for the conjugation, via a cleavable linker arm, of RO5-4864 to a novel tumour-delivery tool, the B-subunit of Shiga toxin (STxB). The conjugate is water soluble and specifically targets cancer cells that overexpress the glycolipid Gb3, the cellular Shiga toxin receptor that is found on several human tumours. After internalisation via retrograde transport, the prodrug is cleaved inside cells to release the active principle. Delivery by STxB therefore increases the cytotoxic activity of RO5-4864 and its tumour specificity.

Introduction

Apoptosis is a programmed cell death mechanism that uses complex membrane and cytoplasmic regulation. In recent years, it has been widely accepted that apoptosis is under the control of mitochondria. The permeability transition pore (PTP), a multiprotein complex located at the contact site between the mitochondrial inner and outer membranes, plays a key role in this regulation.[1,2] The loss of mitochondrial membrane integrity leads to a drop of the transmembrane potential and remodelling of mitochondrial ultrastructure, which causes the release of toxic intermembrane proteins into the cytoplasm.[3] The mitochondrial peripheral benzodiazepine receptor (mPBR) is part of the PTP complex and participates in the regulation of apoptosis. Due to its localisation within a regulating structure of apoptosis, the mPBR can be considered a potential target for therapeutic intervention. The mPBR is overexpressed in various human cancers, including breast,[4,5] ovarian, and colon cancers.[6] A correlation between mPBR expression and colorectal cancer patient survival has also been reported.[7]

Natural mPBR ligands[8–11] as well as synthetic ligands, including benzodiazepines,[12–17] have been identified. For therapeutic applications, ligands should have high affinity for the peripheral mPBR and low affinity for GABA benzodiazepine receptors that are located in the central nervous system. One of the ligands that fulfill this requirement is RO5-4864, a 4′-chloro derivative of diazepam that binds specifically the mPBR with a K_i value of only 23 nM.[18]



RO5-4864 has highly interesting in vitro and in vivo properties.[19] Because of its low solubility in water, however, no satisfactory galenic forms of the molecule exist. Our aim was to obtain a soluble form with increased selectivity for tumour cells. Some soluble peptidic pro-benzodiazepine derivatives have already been published. in vitro, these compounds are stable, and their in vivo hydrolysis by peptidases leads to the release of a benzodiazepine precursor, which then spontaneously cyclises to yield the active drug (Scheme 1).[20–22] However, these compounds lack selectivity for cancer cells.

The nontoxic receptor-binding B-subunit of Shiga toxin (STxB) has been described as a tumour delivery tool[23–26] Shiga toxin is produced by intestinal pathogenic bacteria. STxB is noncovalently associated with the catalytic A-subunit that modifies ribosomal RNA in the cytosol of target cells, leading to protein biosynthesis inhibition. The STxB-based delivery

[a] *Dr. A. El Alaoui, Dr. F. Schmidt, Dr. J.-C. Florent*
Institut Curie, Centre de Recherche
Conception, Synthèse et Vectorisation de Biomolécules
26 rue d'Ulm, 75248 Paris Cedex 05 (France)

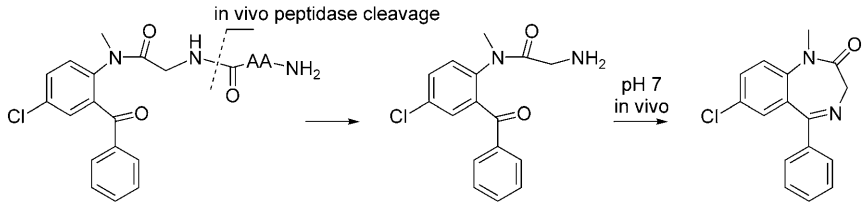
[b] *Dr. A. El Alaoui, Dr. F. Schmidt, Dr. J.-C. Florent*
CNRS, UMR 176
Fax: (+33) 1-56246664
E-mail: Frederic.Schmidt@curie.fr

[c] *M. Sarr, Dr. D. Decaudin, Dr. L. Johannes*
Institut Curie, Centre de Recherche
Laboratoire Trafic, Signalisation, et Ciblage Intracellulaires
26 rue d'Ulm, 75248 Paris Cedex 05, (France)

[d] *M. Sarr, Dr. L. Johannes*
CNRS, UMR 144

[e] *Dr. D. Decaudin*
Institut Curie, Hôpital
Department of Clinical Haematology/Laboratory of Preclinical Investigation
26 rue d'Ulm Paris 75248 (France)

[f] *Dr. D. Decaudin*
Institut Curie
Laboratory of Preclinical Investigation, Translational Research Department



Scheme 1. Release of benzodiazepine from its prodrug. AA = various amino acids.

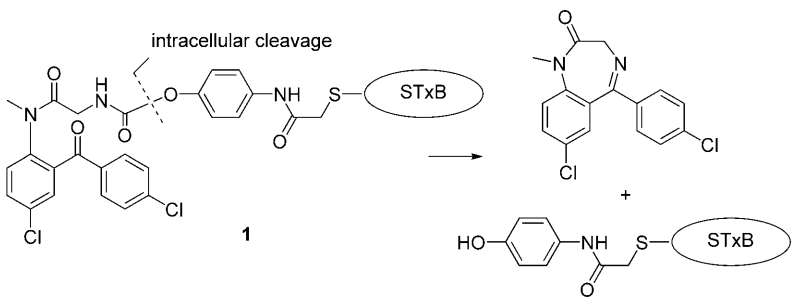
strategy exploits the extracellular stability of the protein, its capacity to cross tissue barriers, and the fact that its cellular receptor, the glycolipid globotriaosyl ceramide (Gb3), is highly expressed on a number of human tumours. After binding to Gb3, STxB is internalised into primary tumours cells and tumour cell lines, targeted to endosomes, the Golgi apparatus, and the endoplasmic reticulum, via the retrograde route. This specific trafficking allows avoiding degradation in lysosomes or recycling to the plasma membrane, two processes that often decrease the efficiency of therapeutic delivery.

We describe herein the development of a soluble derivative of RO5-4864 with specific tumour targeting properties brought about by conjugation with STxB. A linker arm was synthesised that is stable in serum, but undergoes an intracellular cleavage event liberating the RO5-4864 moiety.

Results and Discussion

Synthesis

The conjugate consists of a benzodiazepine precursor linked to STxB through a spacer. The benzodiazepine itself is difficult to functionalise. Our choice was to work on an open form of the central ring. A ketoamine cyclises spontaneously to afford the benzodiazepine structure and offers a free amine function for derivatisation. For the linkage of this compound with a spacer unit, a carbamate function seemed very attractive. This group, while being prone for enzymatic cleavage by esterases,[27] is more stable in the circulation than ester or carbonate moieties.[28–30] For the coupling to STxB, a variant was chosen that carries a C-terminal cysteine, termed STxB/Cys. The free thiol group of this residue allows site-directed chemical coupling to spacer arms. In consideration of these points, conjugate **1** appeared to be an appropriate choice (Scheme 2).



Scheme 2. Cleavage of prodrug **1**.

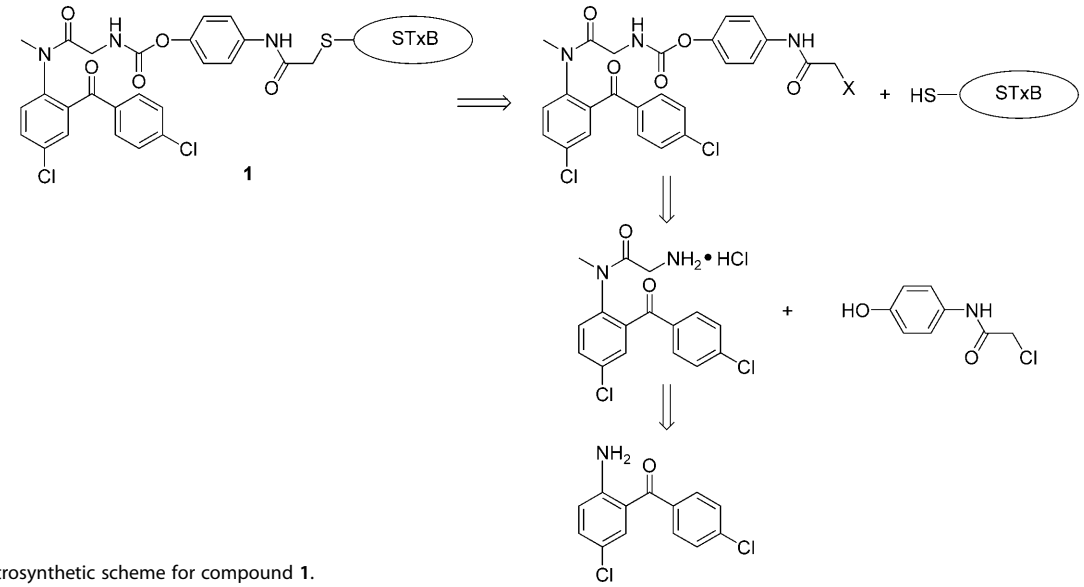
Among the retrosynthetic possibilities, introducing STxB/Cys at the end of the reaction scheme appeared advantageous, thereby avoiding synthesis steps in the presence of the protein. The spacer could be coupled to the benzodiazepine precursor that was used as its chlorohydrate in order to prevent premature cyclisation (Scheme 3).

First, the benzodiazepine was built starting from the aminobenzophenone **6** (Scheme 4). For this, *p*-chloroaniline **2** was protected as a Boc derivative. Ortho-deprotonation with *t*BuLi,[31] and condensation with *p*-chlorobenzaldehyde gave alcohol **4**. Oxidation with MnO_2 and deprotection of the Boc group afforded dichlorobenzophenone **6**.

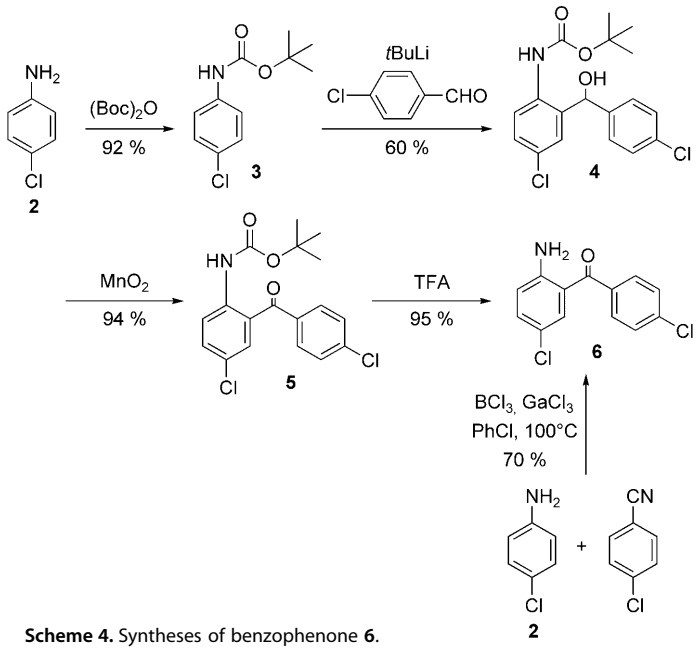
The amine of benzophenone **6** was methylated with methyl sulfate in the conditions of Mouzin et al.,[32] and then acylated with excess chloroacetyl chloride to afford chloroacetate **8** (Scheme 5). The chloro group was transformed into an amine by substitution by an azide, followed by hydrogen reduction of the azide with PtO_2 as catalyst. At this stage, it was important to remain in acidic medium in which the chlorohydrate **10** is stable, contrary to the free base that spontaneously cyclises to benzodiazepine. Finally, the spacer was introduced as a *p*-nitrophenyl chloroformate. The coupling worked well if an excess of chloroformate was used at 0 °C, otherwise cyclisation to benzodiazepine RO5-4864 occurred as the major product. Reduction of the nitro group to amine was tested in different conditions (catalytic hydrogenation, $SnCl_2$, SmI_2). The best conditions were reduction with iron albeit with a very modest yield of 25%. All other conditions gave by-product as cyclised compounds or loss of the chlorine atoms. At the end of the synthesis, reaction of the amine **12** with bromoacetic anhydride afforded compound **13** ready for coupling with the thiol group of STxB/Cys. The global yield of this 11-steps synthesis was 4.7%.

In the previous scheme, two problems became apparent: the construction of the substituted benzophenone **6** was rather tedious, and the reduction of the nitro group in one of the last steps had a yield of only 25%. Therefore, we set out to find a more efficient synthesis. For this, we adopted the Sugasawa reaction for the synthesis of such aminobenzophenones.[33–35] The *p*-chloroaniline **2** reacted with *p*-chlorobenzonitrile in the presence of boron trichloride and a Lewis acid such as aluminum trichloride or gallium trichloride (Scheme 4). The reaction was regioselective and efficient (70% yield for benzophenone **6**), and there was no need for protecting groups.

The transformation of **6** into chlorohydrate **10** was done as previously. To avoid the nitro reduction step, a carbamate bond was generated by using the already functionalised chloroacetyl aminophenol **14**. The chlorohydrate **10** was treated with phosgene, and without isolation of the formed isocyanate, the



Scheme 3. Retrosynthetic scheme for compound **1**.



Scheme 4. Syntheses of benzophenone **6**.

phenol **14** was introduced and gave chloroacetate **15** with a good yield (65% for the two steps) (Scheme 6). However, it turned out that chloroacetate **15** was not reactive enough for efficient coupling to STxB/Cys. This problem was solved by exchanging the chlorine atom by a bromine atom. With these improvements, the seven-step synthesis had a yield of 15%. STxB/Cys was coupled in Hepes buffer at pH 7.5 with an excess of bromide **13**.

Solubility improvement

RO5-4864 itself is insoluble in water, and can be taken up in DMSO or ethanol. After coupling to STxB/Cys, RO5-4864 could be introduced into aqueous solutions. Indeed, conjugate **1** had water solubility properties similar to those of the free STxB/Cys.

Stability of the carbamate linkage and drug release

For sensitivity reasons, model compound **16** was prepared to follow the stability and the cleavage of the carbamate by HPLC. This molecule was obtained by acetylation of compound **12** (R = *p*-NH-CO-C_6H_4-NH_2) with CH_3COCl, and possesses the same carbamate linkage as compound **1**. As shown in Table 1, the carbamate linkage was comparatively stable in neutral or acidic medium, but was rapidly cleaved in alkaline medium.

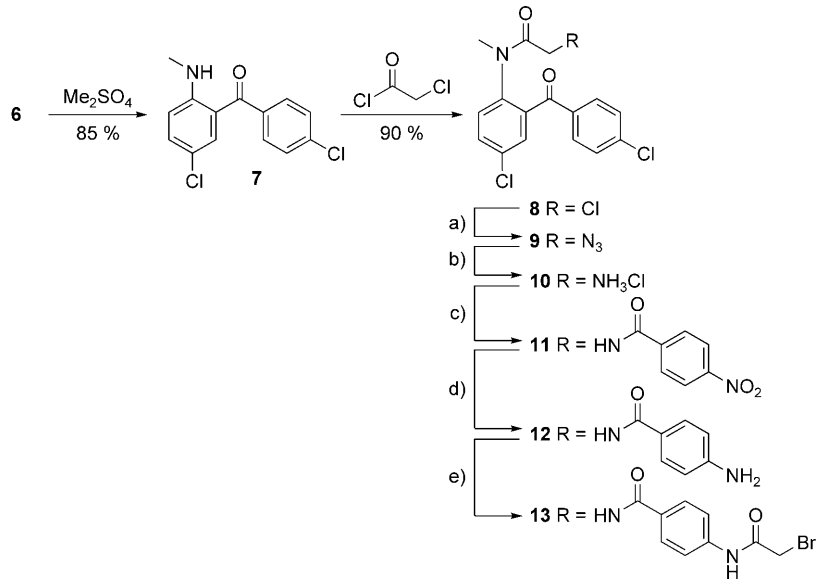
In biological medium, model compound **16** was slowly cleaved. After 6 h incubation at 37 °C in pure foetal calf serum, 15% of total RO5-4864 was released. This value is compatible with in vivo use. Indeed, previous experiments demonstrated tumour targeting by STxB as early as one hour after injection.[24]

To validate the mechanism of cleavage by esterases, model compound **16** was incubated with porcine liver esterase. Starting material disappeared and free RO5-4864 was formed, as shown by HPLC. Cleavage was 10% after 1.5 h, and 50% after 37 h. Such slow release is compatible with the prolonged localisation of STxB on tumour cells up to several days,[24,25] due to trafficking via the retrograde transport route.

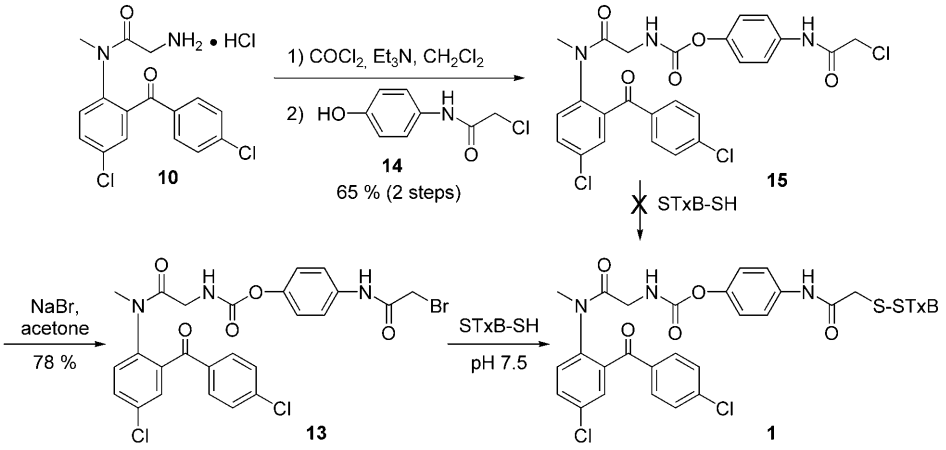
Cytotoxicity

Cytotoxicity measurements were performed on three cell lines (HT-29, A431, CHO) (Figure 1)). As a specificity control, we also used HT-29 and A431 cells on which Gb3 expression was inhibited using the glycosylceramide synthase inhibitor PPMP.[36] CHO cells do not express Gb3. IC_{50} values were determined for **1**, and compared with free RO5-4864.

For free RO5-4864, IC_{50} values were 40 μM (HT-29) or >100 μM (A431), independently of Gb3 expression (Figure 1). In contrast, conjugate **1** had an IC_{50} value of 0.2 μM (HT-29) or



Scheme 5. Synthesis of bromide **13**: a) NaN_3, DMF, 93%; b) H_2, PtO_2, HCl, quant.; c) *p*-$ClCOOC_6H_4NO_2$ (3 equiv) 76%; d) Fe/NH_4Cl, 25% e) $(BrCH_2CO)_2O$, 67%.



Scheme 6. Final steps of the synthesis.

Table 1. Stability of compound **16**

pH	Time for 10% decomposition [h]
9	1.5
7	18.5
5	63

0.4 μM (A431) on Gb3 expressing cells, and 10 μM (HT-29) or 2.8 μM (A431) on non-expressing cells. This difference revealed specificity for Gb3-expressing tumour cells, which was further demonstrated by the finding that non-Gb3-expressing CHO cells were not affected by STxB–RO5-4864, while their viability was efficiently reduced by free RO5-4864 (Figure 1). Free STxB was not cytotoxic. Importantly, we also found that **1** was more cytotoxic than free RO5-4864 (0.2 μM versus 40 μM). A likely explanation of this finding is the increased solubility of the conjugate, thereby increasing the bioavailability of the active principle.

Mitochondrial transmembrane potential

To evaluate the impact of STxB–RO5-4864 on the mitochondrial transmembrane potential ($\Delta\Psi_m$), HT29 cells were cultured for 24 and 48 h in the presence of 1 μM RO5-4864, STxB, or STxB–RO5-4864. Valinomycin (100 μM; Sigma) was used as positive control. After JC-1 staining, we observed a decrease of $\Delta\Psi_m$ in cells that were cultured with **1**, but not with RO5-4864 or STxB alone, demonstrating therefore a direct mitochondrial effect of the conjugate (Figure 2).

Conclusions

We describe a novel benzodiazepine formulation in which two critical requirements for the development of a tumour therapy tool are met: bioavailability of the benzodiazepine and specificity of tumour targeting. In our approach, we exploit the retrograde delivery capacity of STxB. We have previously shown that by reaching membranes of the biosynthetic/secretory pathway, the protein stably associates with primary tumour cells,[24] leading to the slow release of therapeutic compounds.[25] In combination with highly active RO5-4864 ($K_i = 23$ nM toward mPBR),[18] an interesting tool for the development of innovative cancer treatment strategies becomes available.

In the light of mPBR overexpression in a large variety of human cancers, it can be considered that this protein constitutes a tumour-specific intracellular component. Furthermore, mPBR binding by high-affinity ligands enhances apoptosis induction of numerous inducers and in various types of human tumours.[3] In combination with the retrograde delivery capacity of STxB, these parameters are expected to potentiate the efficiency of treatment modalities that build on Gb3-based tumour targeting. Thereby, mPBR ligation may synergise with Gb3-specific delivery approaches to bypass tumour cell resistance, ensuring more effective cancer cell eradication.

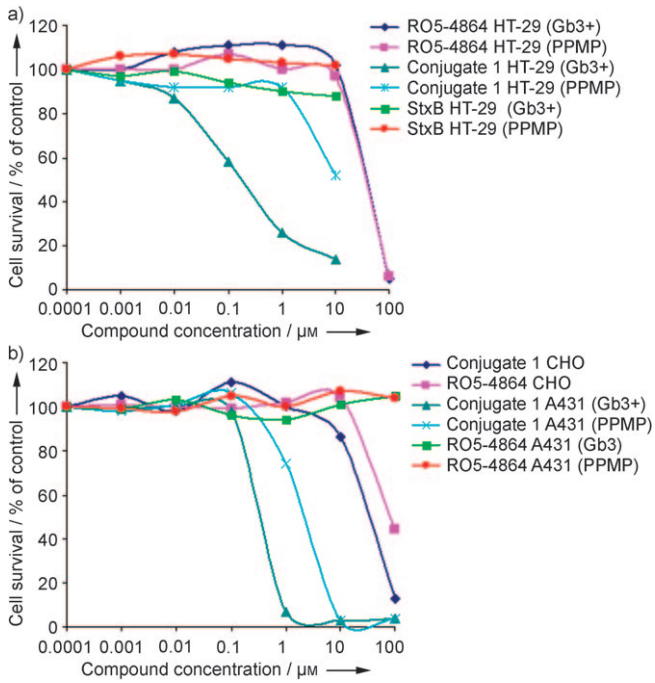


Figure 1. Cytotoxic activity of compounds toward HT-29, CHO, and A431 tumour cells.

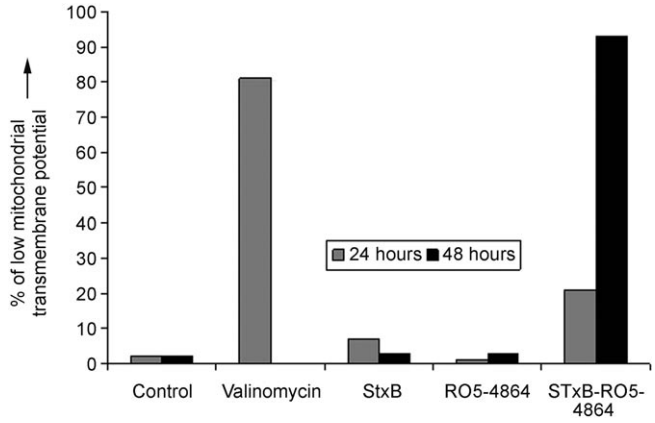


Figure 2. Determination of the mitochondrial transmembrane potential.

Experimental Section

General procedures: All reaction conditions dealing with air- and moisture-sensitive compounds were carried out in a dry reaction vessel under argon atmosphere. Melting points (mp) were measured with an electrothermal digital melting point apparatus and are uncorrected. NMR spectra were recorded at 300 (^{1}H) or 75 MHz (^{13}C). Chemical shifts δ are reported in ppm, and J values in Hz. Mass spectra were recorded using chemical ionisation (CI-MS; NH_3, positive ion mode) or FAB (positive ion mode, either MB ("magic bullet"), or NBA (3-nitrobenzyl alcohol) as the matrix). Electrospray ionisation mass spectra (ESI-MS) were acquired with a quadripole instrument with a mass of charge (m/z) range of 2000. Chromatography was conducted over silica gel (230–400 mesh).

4-chloro-*N*-*tert*-butoxycarbonylaniline (3): To a solution of 4-chloroaniline (10 g, 0.078 mol) in anhydrous THF (65 mL), $(Boc)_2O$ (19 g, 0.085 mol, 1.1 equiv) and Et_3N (12.1 mL, 0.085 mol, 1.1 equiv) were added. The mixture was stirred at room temperature for 48 h. After solvent evaporation, the residue was taken up in EtOAc (300 mL), washed with a saturated ammonium chloride solution (2×100 mL), then with H_2O (2×100 mL), and dried over $MgSO_4$. After removal of EtOAc, a pure white solid (16.3 g, 92%) was recovered. R_f: 0.41 (cyclohexane/EtOAc 7:3); mp: 100 °C; ^{1}H NMR ($CDCl_3$): δ = 7.31–7.21 (m, 4H, $H_{aromatics}$), 6.54 (bs, 1H, NH), 1.51 ppm (s, 9H, $(CH_3)_3$); ^{13}C NMR ($CDCl_3$): δ = 152.6 (CO), 136.9, 128.9, 128.8, 127.8, 119.7, 80.4 ($C(CH_3)_3$), 28.3 and 28.2 ppm ($(CH_3)_3$); MS (CI/NH_3): m/z: 245 $[M+NH_4]^+$; Anal. calcd for $C_{11}H_{14}NO_2Cl$: C 58.03, H 6.20, N 6.15, found: C 58.21, H 6.26, N 6.26.

4′,5-dichloro-2-(*N*-*tert*-butoxycarbonylamino)benzhydrol (4): To a solution of compound **3** (5 g, 0.022 mol) in freshly distilled THF (65 mL) at −78 °C, a solution of *tert*-butyllithium (1.7 M, 35 mL, 0.059 mol, 2.7 equiv) were added dropwise. The first equivalent gave a colourless solution, then a yellow solution after 1.5 equiv. After 15 min at −78 °C, the solution was left to heat till −20 °C (orange colour). To this solution 4-chlorobenzaldehyde (3.86 g, 0.0275 mol, 1.25 equiv) in THF (19 mL) was added. The mixture was stirred at −20 °C for 2.5 h. Et_2O (100 mL) and H_2O (100 mL) were then added at room temperature. The organic phase was washed with brine, dried over $MgSO_4$, and evaporated. After purification over silica gel column (eluent: CH_2Cl_2), compound **4** (4.83 g, 60%) was obtained as a yellow solid. R_f: 0.51 (CH_2Cl_2); mp: 123–124 °C; ^{1}H NMR ($CDCl_3$): δ = 7.69 (d, 1H, J = 8.6 Hz, H3), 7.47 (bs, 1H, NH), 7.33–7.21 (m, 5H, $H_{aromatics}$), 7.00 (d, 1H, J = 2.3 Hz, H6), 5.75 (s, 1H, CHO), 3.45 (s, 1H, OH), 1.41 ppm (s, 9H, $(CH_3)_3$); ^{13}C NMR ($CDCl_3$): δ = 153.3 (CO), 139.3, 135.4, 133.9 133.6, 128.7, 128.6, 128.3, 127.5, 124.1, 80.8 ($C(CH_3)_3$), 73.8 (CHOH), 28.2 ppm ($(CH_3)_3$); MS (CI/NH_3): m/z: 367$[M]^+$; Anal. calcd for $C_{18}H_{19}NO_3Cl_2$: C 58.71, H 5.20, N 3.80, found: C 58.43, H 5.14, N 3.68.

4′,5-dichloro-2-(*N*-*tert*-butoxycarbonylamino)benzophenone (5): To a solution of compound **4** (3 g, 8 mmol) in freshly distilled CH_2Cl_2 (50 mL), MnO_2 (0.065 mmol, 8 equiv) was added. The mixture was stirred at room temperature for 24 h. After filtration over 545 Celite and evaporation, the residue was purified over silica gel (eluent: CH_2Cl_2/hexane 7:3). Compound **5** (2.76 g, 94%) was obtained and recrystallised in MeOH to obtain white crystals. R_f: 0.62 (CH_2Cl_2); mp: 96 °C; ^{1}H NMR ($CDCl_3$): δ = 9.76 (s, 1H, NH), 8.40 (d, 1H, J = 9.0 Hz, H3), 7.66 (d, 2H, J = 8.3 Hz, H2′), 7.49 (d, 3H, J = 8.3 Hz, H4, H3′, H5′), 7.42 (d, 1H, J = 2.0 Hz, H6), 1.50 ppm (s, 9H, $(CH_3)_3$); ^{13}C NMR (CDCl3): δ = 196.6 (CO ketone), 152.7 (CO carbamate), 139.8 and 139.3, 136.3, 134.0, 132.1, 131.2, 128.8, 125.9, 123.6, 121.6, 81.1 ($C(CH_3)_3$), 28.2 ppm ($C(CH_3)_3$); MS (FAB+): m/z 366 $[M+H]^+$; Anal. calcd for $C_{18}H_{17}NO_3Cl_2$: C 59.03, H 4.68, N 3.82, found: C 58.86, H 4.53, N 3.78.

4′,5-dichloro-2-aminobenzophenone (6): *1) From compound **5**.* To a solution of compound **5** (2.5 g, 6.8 mmol) in anhydrous CH_2Cl_2 (40 mL), TFA (8 mL) was added at 0 °C. The cooling bath was removed and the mixture was stirred during 1 h. Excess TFA was removed in vacuum, the residue taken in CH_2Cl_2 (35 mL) and washed with a saturated $NaHCO_3$ solution till pH 7–8. After evaporation and purification (eluent: CH_2Cl_2), compound **6** (1.73 g, 95%) was obtained as yellow crystals. *2) Sugasawa reaction*: To a solution of BCl_3 (28.3 mL, 0.028 mmol, 1.3 equiv) in anhydrous chlorobenzene (20 mL), was added dropwise at 0 °C, a solution of 4-chloroaniline (5.56 g, 0.043 mmol, 2 equiv) in chlorobenzene (50 mL). The mixture was stirred for 5 min (formation of a white precipitate, that corresponds to the amine–BCl_2 complex). Then, *para*-chlorobenzonitrile (3 g, 0.022 mmol) and $GaCl_3$ (5 g, 0.028 mmol, 1.3 equiv) were added. The precipitate dissolved by addition of $GaCl_3$ (orange

solution). The reaction was then heated at reflux during 24 h. After cooling at 0 °C, a 2 N HCl (85 mL) solution was added and the mixture heated at 80 °C during 45 min. The compound was extracted with CH_2Cl_2, dried over $MgSO_4$ and evaporated. Purification on silica gel (eluent: CH_2Cl_2) afforded yellow crystals (4.05 g, 70%). R_f: 0.56 (CH_2Cl_2); mp: 117 °C (lit: 118–119[36]); ^{1}H NMR ($CDCl_3$): $\delta = 7.57$ (dd, 2H, $J = 6.7$ Hz and $J' = 1.9$ Hz, H2′, H6′), 7.45 (dd, 2H, $J = 6.7$ Hz and $J' = 1.9$ Hz, H3′, H5′), 7.36 (d, 1H, $J = 2.4$ Hz, H6), 7.24 (dd, 1H, $J = 8.7$ Hz and $J' = 2.4$ Hz, H4), 6.71 (d, 1H, $J = 8.7$ Hz, H3), 6.05 ppm (s, 2H, NH_2); ^{13}C NMR ($CDCl_3$): $\delta = 196.5$ (CO ketone), 149.4, 137.8, 137.5, 134.4, 132.8, 130.5, 128.6, 119.9, 118.6, 118.3 ppm; MS (CI/NH_3): *m/z* 266 $[M+H]^+$ and 283 $[M+NH_4]^+$; Anal. calcd for $C_{13}H_9NOCl_2$: C 58.67, H 3.41, N 5.26, found: C 58.48, H 3.29, N 5.15.

4′,5-dichloro-2-methylaminobenzophenone (7): To a solution of amine **6** (2.77 g, 0.010 mol) and tetrabutylammonium bromide (33.5 mg, 0.01 equiv) in THF (50 mL), NaOH pellets (1.66 g, 0.041 mol, 4 equiv) were added. The mixture was stirred for 10 min at room temperature, then dimethyl sulfate (2.96 mL, 0.031 mol, 3 equiv) was added. After 8 h at 60 °C and evaporation of THF, the residue was taken in EtOAc (80 mL), washed with H_2O to neutralisation, dried over $MgSO_4$ and evaporated to dryness. Purification on silica gel (eluent: CH_2Cl_2/hexane 4:6) afforded compound **7** (2.2 g, 85%) as a yellow solid and starting material 320 mg. R_f: 0.52 (hexane/CH_2Cl_2 1:1); mp: 122 °C (lit: 123 °C[31]); ^{1}H NMR ($CDCl_3$): $\delta =$ 8.41 (s, 1H, NH), 7.53 (dd, 2H, $J = 6.7$ Hz and $J' = 2.0$ Hz, H2′, H6′), 7.44 (dd, 2H, $J = 6.7$ Hz and $J' = 2.0$ Hz, H3′, H5′), 7.37–7.33 (m, 2H, H4, H6), 6.72 (d, 1H, $J = 8.8$ Hz, H3), 2.96 and 2.94 ppm (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$): $\delta = 196.7$ (CO ketone), 151.1, 138.0, 137.4, 135.0, 133.6, 130.4, 128.5, 118.3, 117.5, 112.8, 29.6 ppm (NCH_3) MS (CI/NH_3): *m/z* 280 $[M+H]^+$; Anal. calcd for $C_{14}H_{11}NOCl_2$: C 60.02, H 3.96, N 5.00, found: C 59.88, H 3.98, N 4.91.

4′,5-dichloro-*N*-methyl-2-chloroacetamidobenzophenone (8): To a solution of amine **7** (300 mg, 1.07 mmol) in EtOAc/H_2O (1:1, 25 mL), were added K_2CO_3 (162 mg, 1.17 mmol, 1.1 equiv) and chloroacetyl chloride (341 μL, 4.28 mmol, 4 equiv). The mixture was stirred during 40 min at room temperature. The two phases were separated. The organic phase was evaporated and taken in CH_2Cl_2 (20 mL), washed four times with H_2O, dried over $MgSO_4$, and evaporated to dryness. The residue was purified on silica gel (eluent: CH_2Cl_2/acetone 9:1) and afforded compound **8** (343 mg, 90%) that crystallise by cooling (white solid). R_f: 0.23 (CH_2Cl_2); mp: 160 °C (lit: 161–162 °C[28]); ^{1}H NMR ($CDCl_3$): $\delta = 7.71$ (d, 2H, $J =$ 8.3 Hz, H2′, H6′′); 7.61 and 7.58 (d, 1H, $J = 2.1$ Hz, H6); 7.44–7.35 (m, 4H, H3, H4, H3′, H5′); 3.98–3.81 (m, 2H, CH_2Cl); 3.41 and 3.04 ppm (rotamers) (2 s, 3H, NCH_3); ^{13}C NMR (CDCl3): $\delta = 193.0$ and 192.1 (CO ketone), 166.7 and 166.5 ($COCH_2$), 141.1, 140.1, 139.9, 139.4, 138.1, 137.9, 134.7, 134.3, 133.9, 132.9, 132.4, 131.9, 131.5, 131.3, 131.2, 129.5, 129.3, 129.2, 128.9, 128.8, 41.6 and 41.1 (CH_2Cl), 39.5 and 38.0 ppm (NCH_3 rotamers) MS (ESI+): *m/z* 378 $[M+Na]^+$; Anal. calcd for $C_{16}H_{12}NO_2Cl_3$: C 53.89, H 3.39, N 3.93, found: C 53.68, H 3.31, N 3.88.

4′,5-dichloro-*N*-methyl-2-azidoacetamidobenzophenone (9): To a solution of chloride **8** (340 mg, 0.955 mmol) in DMF (10 mL), NaN_3 (500 mg, 7.64 mmol, 8 equiv) was added at once. The mixture was stirred at room temperature during 15 h. The reaction was quenched with H_2O (10 mL), then extracted three times with Et_2O. The organic phases were washed with H_2O (2×20 mL), dried over $MgSO_4$ and evaporated. The compound was purified by chromatography over silica gel (eluent: CH_2Cl_2 then CH_2Cl_2/acetone 95:5). A white solid (323 mg, 93%) was obtained. R_f: 0.23 (CH_2Cl_2); mp: 117–119 °C; IR ($CHCl_3$): $\tilde{\nu} = 2109$ (N_3); 1673 cm^{-1} (CO); ^{1}H NMR ($CDCl_3$): $\delta = 7.73$ (d, 2H, $J = 8.4$ Hz, H2′, H6′); 7.60 (d, 1H, $J = 2.3$ Hz, H6), 7.52–7.36 (m, 3H, H4, H3′, H5′), 7.31 (d, 1H, $J = 8.4$ Hz, H3); 3.83 (d, 1H, $J = 15.5$ Hz, AB, CH_2N_3), 3.53 (d, 1H, $J = 15.5$ Hz, AB, CH_2N_3), 3.27 and 3.02 (rotamers) (2 s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$): $\delta = 192.1$ (CO ketone), 166.7 ($COCH_2$), 141.1, 140.0, 138.9, 138.1, 137.9, 134.7, 134.3, 133.9, 132.9, 132.4, 131.9, 131.5, 131.3, 130.9, 129.6, 129.4, 129.3, 129.0, 128.8, 50.7 (CH_2N_3), 38.4 and 37.5 (NCH_3 rotamers); MS (CI/NH_3) *m/z* 363 $[M+H]^+$; Anal. calcd for $C_{16}H_{12}N_4O_2Cl_2$: C 52.91, H 3.33, N 15.43, found: C 52.73, H 3.39, N 15.36.

4′,5-dichloro-*N*-methyl-2-aminoacetamidobenzophenone chlorohydrate (10): Azide **9** (316 mg, 0.87 mmol) was dissolved in absolute EtOH (27 mL). PtO_2 (69 mg) and concentrated HCl (318 μL, 10.44 mmol, 12 equiv) were added. The mixture was hydrogenated for 4 h. After filtration over 545 Celite, washing with absolute EtOH, and evaporation to dryness, chlorohydrate **10** (325 mg, quantitative) was obtained as a white solid. Due to stability problems, the compound could not be purified and was used crude in the next step. R_f: 0.37 (CH_2Cl_2/acetone 95:5); mp: 170 °C (decomposition); ^{1}H NMR (D_2O): $\delta = 7.60$–7.50 (m, 3H, H6, H2′, H6′), 7.37–7.15 (m, 4H, H3, H4, H3′, H5′), 3.69 (m, 1H, AB, CH_2NH_2); 3.53 (m.1H, AB, CH_2NH_2), 3.02 and 2.84 (rotamers) (2 s, 3H, NCH_3); MS (FAB+): *m/z* 337 $[M+H-HCl]^+$.

4′,5-dichloro-*N*-methyl-2-*para*-nitrophenoxycarbonylaminoacetamidobenzophenone (11): To a solution of chlorohydrate **10** (110 mg, 0.29 mmol) and *para*-nitrophenylchloroformate (137 mg, 0.67 mmol, 2.3 equiv) in freshly distilled THF, three drops of Et_3N were added at 0 °C. After removal of the ice bath, the mixture was stirred at room temperature for 2 h. After evaporation, the residue was purified on silica gel (eluent: hexane/acetone 7:3). A white slightly pasty solid (114 mg, 78%) was isolated. R_f: 0.60 (CH_2Cl_2/acetone 95:5); mp: 97–100 °C; ^{1}H NMR ($CDCl_3$): $\delta = 8.24$ (d, 2H, $J =$ 9.2 Hz, H3′′, H5′′), 7.76 (d, 2H, $J = 8.5$ Hz, H2′, H6′), 7.63 and 7.60 (d, 1H, $J = 2.3$ Hz, H6), 7.48 (dd, 3H, $J = 8.5$ Hz and $J' = 2.3$ Hz, H4, H3′, H5′), 7.32 (m, 3H, H3, H2′′, H6′′), 6.02 and 5.90 (bs, 1H, NH), 4.06 and 3.84 (d, 2H, $J = 2.7$ Hz, CH_2), 3.35 and 3.11 ppm (rotamers) (2 s, 3H, NCH_3), ^{13}C NMR ($CDCl_3$): $\delta = 193.1$ and 192.0 (CO ketone), 168.2 and 168.0 ($CONCH_3$), 155.8, 155.7, 152.9 and 152.8 (OCONH), 144.7, 141.1, 138.6, 137.9, 134.9, 134.4, 133.9, 132.7, 132.2, 131.5, 131.4, 131.1, 130.0, 129.6, 129.4, 128.8, 125.0, 121.9, 43.4 and 43.0 (CH_2); 38.1 and 37.5 ppm (NCH_3 rotamers). MS (FAB+): *m/z* 502 $[M+H]^+$; Anal. calcd for $C_{23}H_{17}N_3O_6Cl_2$: C 55.00, H 3.41, N 8.37, found: C 55.24, H 3.48, N 8.32.

4′,5-dichloro-*N*-methyl-2-*para*-aminophenoxycarbonylaminoacetamidobenzophenone (12): To a solution of compound **11** (60 mg, 0.118 mmol) in THF (3 mL) were added iron (80 mg, excess) and three drops of a NH_4Cl (1N) solution. The mixture was stirred for 90 min at room temperature. After filtration over 545 Celite and evaporation, the residue was purified on silica gel (eluent: hexane/acetone 7:3). Compound **12** (14 mg, 25%) was obtained as yellow solid. R_f: 0.50 (CH_2Cl_2/MeOH 95:5); mp: 54–56 °C ^{1}H NMR ($CDCl_3$): $\delta = 7.75$ (d, 2H, $J = 8.4$ Hz, H9), 7.60 and 7.50 (d, 1H, $J = 2.3$ Hz, H5), 7.49–7.32 (m, 4H, H10, H3 and H2), 6.89 (d, 2H, $J = 8.4$ Hz, H13), 6.72 (d, 2H, $J = 8.4$ Hz, H14), 5.88 and 5.79 (bs, 1H, NH), 3.83 (m, 2H, CH_2), 3.31 and 3.07 ppm (rotamers) (2 s, 3H, NCH_3); MS (FAB+): *m/z* 472 $[M+H]^+$; Anal. calcd for $C_{23}H_{19}N_3O_4Cl_2$: C 58.49, H 4.05, N 8.90, found: C 58.30, H 3.93, N 8.76.

4-chloroacetamidophenol (14): To a solution of potassium carbonate (4.0 g, 0.0302 mmol, 1.1 equiv) in acetone/H_2O (3:1, 40 mL) *para*-aminophenol (3.0 g, 0.027 mmol, 1 equiv) was added. The mixture was cooled to −10 °C, then chloroacetyl chloride (2.19 mL, 0.027 mmol, 1 equiv) was added dropwise during 30 min. The

whole mixture was stirred for 75 min at −10 °C. After evaporation of acetone, the mixture was taken in EtOH (30 mL), dried over $MgSO_4$ and evaporated to dryness. The residue was purified on silica gel (eluent: CH_2Cl_2/MeOH 95:5). A brown solid (3.05 g, 60%) was obtained. R_f: 0.56 (CH_2Cl_2/MeOH 9:1); mp: 146 °C; ^{1}H NMR ($CDCl_3$): δ = 7.32 and 7.31 (d, 2H, J = 6.8 Hz, H3, H5), 6.76 and 6.75 (d, 2H, J = 6.8 Hz, H2, H6), 4.08 (s, 2H, CH_2Cl); ^{13}C NMR ($CDCl_3$): δ = 165.7 ($COCH_2$), 154.4, 129.5, 122.1, 114.4, 42.5 (CH_2Cl); MS (CI/NH_3): m/z 186 $[M+H]^+$; Anal. calcd for $C_8H_8NO_2Cl$: C 51.77, H 4.34, N 7.55, found: C 51.93, H 4.29, N 7.62.

4′,5-dichloro-*N*-methyl-2-chloroacetamido-*para*-aminophenoxycarbonylaminoacetamidobenzophenone (15): To a suspension of chlorohydrate **10** (60 mg, 0.16 mmol) in freshly distilled CH_2Cl_2 (6 mL), a phosgene solution (165 μL, 1.28 mmol, 8 equiv) and three drops of Et_3N were added at 0 °C. After 45 min stirring at 0 °C, phenol **14** (32.5 mg, 0.17 mmol, 1.1 equiv) was added. The cooling bath was removed and the stirring was continued for 3 h 30. The mixture was the diluted with CH_2Cl_2 (10 mL), washed four times with H_2O, dried over $MgSO_4$ and evaporated. Silica gel purification (eluent: CH_2Cl_2 then CH_2Cl_2/acetone 8:2) afforded a white solid (57 mg, 65%). R_f: 0.44 (CH_2Cl_2/acetone 8:2); mp: 123–125 °C; ^{1}H NMR ($CDCl_3$): δ = 8.29 (bs, 1H, NH), 7.74 (d, 2H, J = 6.3 Hz, H2′, H6′), 7.59–7.22 (m, 7H, H3, H4, H6, H3′, H5′, H3″, H5″), 7.06 (d, 2H, J = 6.9 Hz, H2″, H6″), 5.95 and 5.84 (bs, 1H, NH), 4.13 (s, 2H, CH_2Cl), 4.01–3.68 (m, 2H, CH_2), 3.31 and 3.06 ppm (2 s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$): δ = 193.1 and 192.0 (CO ketone), 168.4 and 168.3 ($CONCH_3$), 163.8 ($COCH_2$), 154.3 and 154.2 (OCONH), 147.8, 147.7, 141.0, 140.0, 139.9, 138.8, 137.9, 134.8, 134.0, 132.9, 132.7, 132.1, 131.5, 131.1, 131.4, 129.9, 129.5, 129.3, 128.8, 122.1, 121.0, 43.4 (CH_2Cl), 43.0 and 42.8 (CH_2), 38.0 and 37.5 ppm (NCH_3); MS (MALDI): m/z 570 $[M+Na]^+$ and 586 $[M+K]^+$; Anal. calcd for $C_{25}H_{20}N_3O_5Cl_3$: C 54.71, H 3.67, N 7.66, found: C 54.55, H 3.58, N 7.59.

4′,5-dichloro-*N*-methyl-2-bromoacetamido-*para*-aminophenoxycarbonylaminoacetamidobenzophenone (13): *1) 1st synthesis*. To a solution of amine **12** (6 mg, 0.013 mmol) in anhydrous THF (1.5 mL), bromoacetyl anhydride (5 mg, 0.019 mmol, 1.5 equiv) and Et_3N (2 μL, 0.010 mmol, 0.8 equiv) were added. The mixture was stirred for 30 min. The reaction was quenched with H_2O (1 mL), then extracted with CH_2Cl_2 (2×3 mL), dried over $MgSO_4$ and evaporated. The crude compound was purified on silica gel (eluent: CH_2Cl_2/acetone 9:1) to give compound **13** (5.2 mg, 67%). *2) 2nd synthesis*. To a solution of chloride **15** (39 mg, 0.071 mmol) in freshly distilled acetone, NaBr (260 mg, dried over P_2O_5), was added. The mixture was heated for 48 h at reflux. After filtration, washing with acetone and evaporation, the product was purified on silica gel (eluent: CH_2Cl_2/acetone 9:1). The bromide **13** (33 mg, 78%) was isolated as a white solid. R_f: 0.44 (CH_2Cl_2/acetone 8:2); mp: 120–122 °C; ^{1}H NMR ($CDCl_3$): δ = 8.20 (bs, 1H, NH), 7.75 (d, 2H, J = 8.4 Hz, H2′, H6′), 7.60 and 7.58 (d, 1H, J = 2.1 Hz, H6), 7.49–7.41 (m, 5H, H3, H3′, H5′, H3″, H5″), 7.34 (dd, 1H, J = 8.4 Hz and J' = 2.1 Hz, H4), 7.07 (d, 2H, J = 8.7 Hz, H2″, H6″), 5.92 and 5.82 (bs, 1H, NH), 4.02 and 3.82 (m, 2H, CH_2), 3.97 (s, 2H, CH_2Br), 3.33 and 3.07 ppm (2 s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$): δ = 193.1 and 192.0 (CO ketone), 168.4 and 168.3 ($CONCH_3$), 163.6 ($COCH_2$), 154.6 and 154.5 (OCONH), 147.6, 147.5, 141.1, 140.1, 138.7, 137.8, 137.7, 134.8, 134.4, 134.3, 134.0, 132.9, 132.7, 131.5, 131.1, 131.9, 129.9, 129.5, 129.3, 128.8, 122.0, 121.0, 43.5 and 43.0 (CH_2), 38.1 and 37.6 (NCH_3), 29.4 and 29.2 ppm (CH_2Br); MS (FAB+): m/z 592 $[M+H]^+$; Anal. calcd for $C_{25}H_{20}N_3O_5BrCl_2$: C 50.61, H 3.40, N 7.08, found: C 50.39, H 3.29, N 6.97.

4′,5-dichloro-*N*-methyl-2-acetamido-*para*-aminophenoxycarbonylaminoacetamidobenzophenone (16): To a solution of amine **12** (6 mg, 0.013 mmol) in anhydrous CH_2Cl_2 (1 mL), acetyl chloride (5 μL, 0.019 mmol, 1.5 equiv) and Et_3N (2 μL, 0.011 mmol, 0.9 equiv) were added. The mixture was stirred for 30 min. The reaction was quenched with H_2O (1 mL), extracted with CH_2Cl_2, dried over $MgSO_4$ and evaporated. The compound was purified on silica gel (eluent: CH_2Cl_2/acetone 7:3). Compound **16** (5 mg, 75%) were obtained as a white slightly yellowish solid. R_f: 0.21 (CH_2Cl_2/acetone 8:2); mp: 125 °C; ^{1}H NMR ($CDCl_3$): δ = 7.75 (d, 2H, J = 8.1 Hz, H2′, H6′), 7.59 (d, 1H, J = 8.4 Hz, H2), 7.49–7.40 (m, 5H, H6, H3′, H5′, H3″, H5″), 7.36 (dd, 1H, J = 8.4 Hz and J' = 2.0 Hz, H4), 7.03 and 6.97 (d, 2H, J = 8.7 Hz, H2″, H6″), 5.88 and 5.78 (bs, 1H, NH), 4.01 and 3.97 (m, 2H, CH_2), 3.32 and 3.07 (2 s, 3H, NCH_3), 2.62 and 2.13 ppm (s, 3H, $COCH_3$); ^{13}C NMR ($CDCl_3$): δ = 193.1 and 192.1 (CO ketone), 168.4 and 168.3 ($CONCH_3$), 163.1 ($COCH_3$), 154.3 and 154.2 (OCONH), 147.7 and 147.6 (C12), 141.1, 140.1, 138.7, 137.8, 137.7, 134.8, 134.1, 132.8, 132.7, 131.5, 131.1, 131.4, 129.9, 129.5, 129.3, 128.8, 122.0, 121.0, 38.1 and 37.5 (NCH_3), 18.1 ppm (CH_3); MS (FAB+): m/z 514 $[M+H]^+$; Anal. calcd for $C_{25}H_{21}N_3O_5Cl_2$: C 58.38, H 4.12, N 8.17, found: C 58.28, H 4.18, N 8.21.

Chemical coupling with STxB: STxB/Cys (2.5 mg mL^{-1}) in 20 mM HEPES buffer (pH 7.5, 150 mM NaCl) was incubated volume to volume with a ninefold molar excess of compound **13**. The final DMSO concentration was 10%. The reaction was carried out overnight at room temperature in the dark with end-over-end mixing. PD-10 gel filtration columns, equilibrated with PBS, were used to separate conjugates from excess of compounds.

Mass spectrometry of conjugate 1: After dialysis of the conjugates against H_2O, mass spectroscopy was performed on a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, USA) operated in the delayed extraction and linear mode. A solution of sinapinnic acid in acetonitrile/TFA (30%/0.1%) was used as the matrix. Samples were prepared by mixing with the matrix at a ratio of 1:1. The mixture was spotted onto a MALDI-TOF plate and allowed to dry: m/z: calculated 8304, found 8308 (Figure 3).

HPLC conditions for conjugate 1: Analysis was carried out on a reversed-phase column (Waters X Bridge BEH300 C_{18}, 3.6 μm, 150× 4.6 mm) using a gradient (1 mL min^{-1}) of H_2O + 0.05% TFA/acetonitrile + 0.05% TFA (100:0 to 60:40 in 30 min) with UV detection at 215 nm. t_R: 22.38; purity: 87.95% (Figure 4).

HPLC conditions for cleavage of model compound 16: Analysis was carried out on a reversed-phase column (X-Terra MS C_{18} Waters, 5 μm, 150×4.6 mm) using isocratic conditions (1 mL min^{-1}) of 50% phosphate buffer (0.02 M, pH 3) and 50% acetonitrile with UV detection at 226 nm (extracted from PDA 3D spectra).

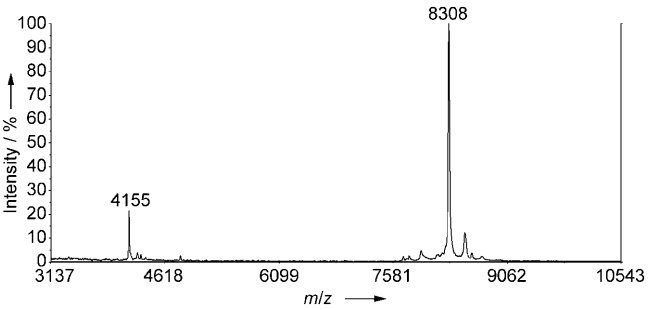


Figure 3. Mass spectrum (MALDI-TOF) of conjugate **1**.

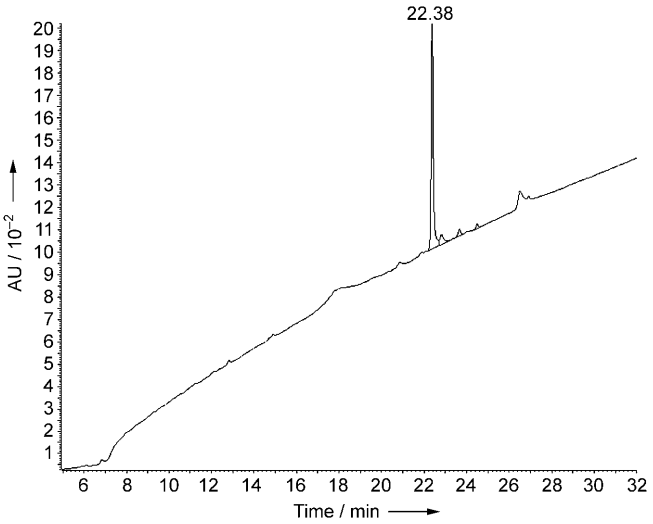


Figure 4. Purity of conjugate **1** (HPLC).

Stability of compound 16: A solution of 190 μM of compound **16** in phosphate buffer (0.02 M, various pH) or in pure foetal calf serum (FCS) was incubated at 37 °C. Aliquots (50 μL) were taken at various times and analyzed by HPLC after dilution with the eluent (150 μL).

Enzymatic cleavage of compound 16 by PLE: A solution of 190 μM of compound **16** in phosphate buffer (0.02 M, pH 7.2) was incubated at 37 °C in the presence of 12.5 U mL^{-1} porcine liver esterase (PLE, Sigma). Aliquots (50 μL) were taken at various times and analyzed by HPLC after dilution with the eluent (150 μL).

In vitro cytotoxicity: HT-29 (human colorectal adenocarcinoma) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*v/v*) FCS, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, and kept under 5% CO_2 at 37 °C. When indicated, 5 μM PPMP was added for six days to decrease Gb3 expression to 2% of control levels. 96-well plates were seeded with 3000 control or PPMP-treated HT-29 cells per well in 100 μL medium. Twenty-four hours later, serial dilutions of conjugate **1** (solution in PBS) or RO5-4864 (DMSO stock solution, then dilution in PBS) were added. The cells were exposed to the conjugates for five days at 37 °C. Numbers of viable cells were determined with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent, and IC_{50} values were calculated as the concentration of compounds inducing a 50% inhibition of cell proliferation. Measurements were made in triplicate, taking into account the errors on the measures, it was possible to give a maximum and a minimum value for each IC_{50} (Table 2).

Mitochondrial transmembrane potential: The drop of the cellular $\Delta\Psi_m$ value was evaluated after JC-1 staining, as described.[38]

Gb3 extraction procedures and overlay: Lipid extraction was done according to the method of Bligh and Dyer.[39] The indicated numbers of cells in 1 mL aqueous buffer were injected into 3.75 mL chloroform/MeOH (1:2). After mixing, 1.25 mL chloroform and 1.25 mL H_2O were added. Phases were separated after mixing, and the hydro-alcoholic phase was washed once with 1.5 mL chloroform. The combined chloroform phases were dried under nitrogen, and lipids were saponified at 56 °C for 1 h in 1 mL MeOH/KOH. The saponification reaction was once again extracted as described above, and the chloroform phase was washed once with MeOH/H_2O (1:1). The isolated neutral glycolipids were spotted on high-performance thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) and separated with chloroform/MeOH/H_2O (65:25:4). Dried plates were soaked in 0.1% poly(isobutylmethacrylate) in hexane, floated for 1 h in blocking solution, followed by incubation with STxB (20 nM), primary polyclonal anti-STxB, and secondary horseradish peroxidase, or alkaline phosphatase-coupled anti-rabbit antibodies. Reactive bands were revealed with the use of enhanced chemiluminescence or chemifluorescence (Amersham Pharmacia Biotech, Little Chalfont, UK) and PhosphorImager.

Table 2. Inhibition measurements.

Experiment	IC_{50} [μM]	Max. IC_{50} [μM]	Min. IC_{50} [μM]
Conjugate **1** HT-29 (Gb3+)	0.2	0.38	0.08
Conjugate **1** HT-29 (PPMP)	10	12	8
RO5-4864 HT-29	40	44	32
Conjugate **1** A431 (Gb3+)	0.4	0.47	0.32
Conjugate **1** A431 (PPMP)	2.8	3.6	1.8

Acknowledgements

*We acknowledge the help from Wolfgang Faigle, Jean-Marc Clavel, and Damarys Loew (mass spectrometry laboratory of the Institut Curie) as well as the help of Sylvie Dubruille for HPLC measurements. For the characterisation of conjugate **1** (HPLC and MALDI), Hervé Drobecq and Oleg Melnyk from the Institut de Biologie de Lille are acknowledged. Our work was supported by funding from the Institut Curie (PIC Vectorisation) and Cancer-opôle Ile-de-France.*

Keywords: antitumor agents · drug delivery · prodrugs · benzodiazepine · shiga toxin

[1] S. A. Susin, N. Zamzami, M. Castedo, E. Daugas, H. G. Wang, S. Geley, F. Fassy, J. C. Reed, G. Kroemer, *J. Exp. Med.* **1997**, *186*, 25.
[2] R. R. Anholt, P. L. Pedersen, E. B. De Souza, S. H. Snyder, *J. Biol. Chem.* **1986**, *261*, 576.
[3] D. Decaudin, *Anticancer Drugs* **2004**, *15*, 737.
[4] A. Beinlich, R. Strohmeier, M. Kaufmann, H. Kuhl, *Biochem. Pharmacol.* **2000**, *60*, 397–402.
[5] W. Li, M. Hardwick, D. Rosenthal, M. Culty, V. Papadopoulos, *Biochemical Pharmacology* **2007**, *73*, 491–503.
[6] Y. Katz, G. Ben-Baruch, Y. Klog, J. Menczert, M. Gavish, *Clin. Sci.* **1990**, *78*, 155–158.
[7] K. Maaser, P. Grabowski, A. P. Sutter, M. Höpfner, H. D. Foss, H. Stein, G. Berger, M. Gavish, M. Zeitz, H. Scherübl, *Clin. Cancer Res.* **2002**, *8*, 3205–3209.
[8] M. Corda, M. Ferrari, A. Guidotti, D. Konkel, *Neurosci. Lett.* **1984**, *47*, 310–324.
[9] C. Mantione, M. Goldman, B. Martin, G. Bolger, H. Lueddens, S. Paul, *Biochem. Pharmacol.* **1988**, *37*, 339–347.
[10] A. Verma, J. Nye, S. Snyder, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 2256–2260.
[11] G. Wendler, P. Lindemann, J. Lacapere, *Biochem. Biophys. Res. Commun.* **2003**, *311*, 847–852.
[12] G. Le Fur, F. Guilloux, P. Rufat, J. Benavides, A. Uzan, C. Renault, M. Dubroeucq, C. Gueremy, *Life Sci.* **1983**, *32*, 1849–1856.
[13] E. Romeo, J. Auta, A. Kozikowski, D. Ma, V. Papadopoulos, G. Puia, E. Costa, *J. Pharmacol. Exp. Ther.* **1992**, *262*, 971–978.
[14] A. Kozikowski, D. Ma, J. Brewer, S. Sun, E. Costa, E. Romeo, A. Guidotti, *J. Med. Chem.* **1993**, *36*, 2908–2920.

[15] S. Langer, S. Arbilla, S. Tan, K. Lloyd, P. George, J. Allen, A. Wick, *Pharmacopsychiatry* **1990**, *23*, 103–107.
[16] T. Okubo, R. Yoshikawa, S. Chaki, S. Okuyama, A. Nakazato, *Bioorg. Med. Chem.* **2004**, *12*, 423–438.
[17] G. Primofiore, F. Da Settimo, S. Taliani, F. Simorini, M. Patrizi, E. Novellino, G. Greco, E. Abignente, B. Costa, B. Chelli, C. Martini, *Med. Chem.* **2004**, *47*, 1852–1855.
[18] F. Bono, I. Lamarche, V. Prabonnnaud, G. Le Fur, J. M. Herbert, *Biochem. Biophys. Res. Commun.* **1999**, *265*, 457–461.
[19] D. Decaudin, M. Castedo, F. Nemati, A. Beurdely-Thomas, G. De Pinieux, A. Caron, P. Pouillard, J. Wijdenes, D. Rouillard, G. Kroemer, M.-F. Poupon, *Cancer Res.* **2002**, *62*, 1388–1393.
[20] K. Hirai, T. Ishiba, H. Sugimoto, K. Sasakura, T. Fujishita, T. Toyoda, Y. Tsukinoki, H. Joyama, H. Hatakeyama, K. Hirose, *J. Med. Chem.* **1980**, *23*, 764–773.
[21] K. Hirai K, T. Ishiba, H. Sugimoto, T. Fujishita, Y. Tsukinoki, K. Hirose, *J. Med. Chem.* **1981**, *24*, 20–27.
[22] H. Bundgaard, *Adv. Drug Delivery Rev.* **1989**, *3*, 39–65.
[23] L. Johannes, D. Decaudin, *Gene Ther.* **2005**, *18*, 1360–1368.
[24] K.-P. Janssen, D. Vignjevic, R. Boisgard, T. Falguières, G. Bousquet, D. Decaudin, F. Dolle, D. Louvard, B. Tavitian, S. Robine, L. Johannes, *Cancer Res.* **2006**, *14*, 7230–7236.
[25] A. El Alaoui, F. Schmidt, M. Amessou, M. Sarr, D. Decaudin, J.-C. Florent, L. Johannes, *Angew. Chem.* **2007**, *119*, 6589–6592; *Angew. Chem. Int. Ed.* **2007**, *46*, 6469–6472.
[26] T. Falguières, M. Maak, C. von Weyhern, M. Sarr, X. Sastre, M. F. Poupon, S. Robine, L. Johannes, K. P. Janssen, *Mol. Cancer Ther.* **2008**, *7*, 2498–2508.
[27] C. L. Morton, M. Wierdl, L. Oliver, M. K. Ma, M. K. Danks, C. F. Stewart, J. L. Eiseman, P. M. Potter, *Cancer Res.* **2000**, *60*, 4206–4210.
[28] T. L. Huang, A. Szekacs, T. Uematsu, E. Kuwano, A. Parkinson, B. D. Hammock, *Pharm. Res.* **1993**, *10*, 639–648.
[29] R. B. Greenwald, A. Pendri, C. D. Conover, H. Zhao, Y. H. Choe, A. Martinez, K. Shum, S. Guan, *J. Med. Chem.* **1999**, *42*, 3657–3667.
[30] R. B. Greenwald, Y. H. Choe, C. D. Conover, K. Shum, D. Wu, M. Royzen, *J. Med. Chem.* **2000**, *43*, 475–487.
[31] J. Muchowski, M. Venuti, *J. Org. Chem.* **1980**, *45*, 4789–4801.
[32] G. Mouzin, H. Cousse, J.-M. Autin, *Synthesis* **1981**, 448–449.
[33] T. Sugasawa, T. Toyada, M. Adachi, K. Sasakura, *J. Am. Chem. Soc.* **1978**, *100*, 4842–4852.
[34] D. Walsh, *Synthesis* **1980**, 677–688.
[35] I. Houpis, A. Molina, A. Douglas, L. Xavier, J. Lynch, R. Volante, P. Reider, *Tetrahedron Lett.* **1994**, *35*, 6811–6814.
[36] A. Abe, J. Inokuchi, M. Jimbo, H. Shimeno, A. Nagamatsu, J. A. Shayman, G. S. Shukla, N. S. Radin, *J. Biochem.* **1992**, *111*, 191.
[37] Z. Vejdelek, Z. Polivka, M. Protiva, *Collect. Czech. Chem. Commun.* **1985**, *50*, 1064–1069.
[38] S. Smiley, M. Reers, C. Mottola-Hartshorn, M. Lin, A. Chen, T. Smith, G. Steele, L. Chen, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 3671–3675.
[39] E. Bligh, W. Dyer, *Can. J. Biochem. Physiol.* **1959**, 37, 911–917.

Received: July 28, 2008
Published online on October 9, 2008

