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Tuning a modular system – synthesis and characterisation of a boron-rich s-triazine-based carboxylic acid and amine bearing a galactopyranosyl moiety

The cover shows that smugglers can succeed in smuggling carborane clusters undetected into cancer cells using a false “galactopyranose” flag. The boat is the hydrophilic transport vehicle or bioconjugate.

Artwork: Dr. Christoph Selg

See Evamarie Hey-Hawkins et al., Dalton Trans., 2020, 49, 57.
Tuning a modular system – synthesis and characterisation of a boron-rich s-triazine-based carboxylic acid and amine bearing a galactopyranosyl moiety†

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Introduction

Since the first report by Locher in 1936, boron neutron capture therapy (BNCT) has been developed as a very promising approach for cancer treatment.1 It combines two non-toxic components to produce cytotoxic species, which are able to destroy malignant tissue. Boron-10 containing drugs bearing a tumour-selective moiety to address the infected site are highly advantageous.2–8 Ideally, these bioconjugates accumulate selectively in the malignant tissues in a required amount of 10–30 µg g–1 tumour tissue and can then be irradiated with thermal or epithermal neutrons.2,9 Depending on the biomolecule, the BNCT agent is either just accumulated in the tumour tissue or internalised into the cancer cells, which of course increases the efficacy of this treatment.10,11,12–20 The particles which are generated upon neutron capture are lithium and helium nuclei (α particles).2,21,22 These particles with a high linear energy transfer (LET) have a mean free path of about 5 to 10 µm, and thus, a limited radius of destructive action.2,5,6,21,22 The combination of suitable boron-rich molecules with tumour-selective biomolecules opens up a very selective tumour therapy which only affects malignant tissue and spares normal tissue.10,12,14–17,23 However, there are still some major challenges, including the selectivity of the chosen biomolecules for a specific type of tumour, the required high concentration of boron-10 in the cancer cell, the water solubility of the final bioconjugate, and the neutron beam quality,24 which are also the focus of current research.13,17–19,25,26

Recently, we reported the synthesis of s-triazine-based boron-rich carboxylic acids.27 Preliminary studies showed that the incorporation of more than one s-triazine-based bis(carbonyl) derivative into the breast tumour-selective peptide [F7,P34]-neuropeptide Y leads to a decrease or even total loss of the hY1 receptor activation potency. It was assumed that this was caused by the strong hydrophobic character of the carborane clusters attached to the peptide producing a hydrophobic collapse of the bioconjugate.28 Here, we describe the incorporation of an α-ω-galactopyranosyl-substituted glycine derivative to reduce the hydrophobicity and improve the water solubility, which are important features for successful BNCT.5–7,29 We also demonstrate the expansion of potential receptor targets for tumour addressing by utilising the recently developed gastrin-releasing peptide receptor (GRPR, BB2) selective peptide agonist [d-Phe6, β-Ala11, Ala13, Nle14]Bn(6–14)
The GRPR is commonly used as a drug shuttle system because of its frequent overexpression in breast and prostate cancer and its ability to repetitively internalise together with the peptide drug conjugate leading to an intracellular drug accumulation. Furthermore, inclusion of either a carboxylic acid or an amine moiety as functional group further extends the scope for potential coupling partners (Fig. 1).

The only comparable compound using an s-triazine scaffold in combination with carboranes, monosaccharides and carboxylic acids was reported by Panza and co-workers. In this case, ortho-carborane is used, which is a potential problem as this isomer is prone to undergo deboronation reactions if good nucleophiles associated with basic or even neutral conditions are present. This may cause problems with bioavailability or side effects when employed in therapy. Using meta-carborane assures the integrity of the carborane cluster.

Results and discussion

Based on the synthetic procedures described in our previous work, [4,6-bis(1,7-dicarba-closo-dodecaboran-9-ylthio)-1,3,5-triazin-2-yl]glycine (3) was prepared from glycine and 2-chloro-4,6-bis(1,7-dicarba-closo-dodecaboran-9-ylthio)-1,3,5-triazine (2), which was synthesised from 9-mercapto-(1,7-dicarba-closo-dodecaborane)(12) (1) and cyanuric chloride. The following step involved the base-assisted introduction of the monosaccharide moiety as 1,2,3,4-di-O-isopropylidene-6-deoxy-α-D-galactopyranosyl-6-triflate (4), prepared from 1,2,3,4-di-O-isopropylidene-α-D-galactopyranose (Scheme 1, step c). A protection strategy is required also to retain the monosaccharide as the α anomer. Although, acetyl protecting groups are well known and widely used, we favour isopropylidene (acetoni) protecting groups, because the corresponding α-D-galactopyranose is commercially available, whereas the respective 1,2,3,4-tetraacetyl derivative has to be synthesised. Furthermore, there is no interference of the acetoni groups with the cluster signals in 1H NMR spectroscopy. In addition, acetonides are only cleavable under strongly acidic (and aqueous) conditions, whereas acetyl protecting groups are also cleaved by strong acids and bases and nucleophiles like enolates, organolithium compounds and Grignard reagents.

Unexpectedly, the reaction of 3 with 4 did not give N-[4,6-bis(1,7-dicarba-closo-dodecaboran-9-ylthio)-1,3,5-triazin-2-yl]-
N\{(1',2':3',4'-di-O-isopropylidene-6'-deoxy-α-D-galactopyranos-6'-yl)glycine (5); instead the carboxylic acid ester \{(1',2':3',4'-di-O-isopropylidene-6'-deoxy-α-D-galactopyranos-6'-yl)\} [4,6-bis(1,7-dicarba-closo-dodecaboran-9-ythio)-1,3,5-triazin-2-yl]glycinate (6) was obtained with N,N-diisopropylethylamine (DIPEA) or potassium carbonate as base (Scheme 1, step d). A similar reaction was observed by Hughes et al. when reacting α-trifluoromethyl benzyl triflates with octanoic acid.40

These observations led to a different synthetic approach (Scheme 2). Starting from cyanuric chloride and tert-butyl glycinate hydrochloride the dichloro species tert-butyl(4,6-dichloro-1,3,5-triazin-2-yl)glycinate (7) was prepared to introduce a protected carboxylic acid group and thus avoid the side reaction leading to 6 (Scheme 1, step d). Subsequently, the carborane clusters were introduced under mild basic conditions (b in Scheme 2). However, only a mixture of the mono- (8) and disubstituted derivative (9) could be obtained, which could not be separated by column chromatography (details are given in the ESI†). Attempts to introduce a galactopyranosyl moiety via the reaction of the mixture of 8 and 9 with the triflate 4 failed irrespective of the used base (DIPEA or K2CO3). This indicates that the secondary amine group in 8 and 9 is a weak nucleophile and not able to undergo a substitution reaction. A possible reason for this may be the resonance stabilisation of the adjacent s-triazine ring which might even be enhanced by the electron-withdrawing effect of the chloro and carborane substituents.

Since the carboxylate could act as a nucleophile towards triflates (Scheme 1, step d) and the directly attached secondary amine at the s-triazine backbone is a weak nucleophile and undergoes no substitution reaction (Scheme 2), these observations led to a third approach in the synthesis of compound 5 (Scheme 3).

Starting from 1,2:3,4-di-O-isopropylidene-6-deoxy-α-D-galactopyranosyl-6-triflate (4) and tert-butyl glycinate hydrochloride the monosubstituted s-triazine derivative tert-butyl-N-(4,6-dichloro-1,3,5-triazin-2-yl)-N\{(1',2':3',4'-di-O-isopropylidene-6'-deoxy-α-D-galactopyranos-6'-yl)\} glycinate (11) was prepared in a one-pot reaction via tert-butyl-N\{(1',2':3',4'-di-O-isopropylidene-6'-deoxy-α-D-galactopyranos-6'-yl)\} glycinate (10) as intermediate (a and b in Scheme 3). Subsequently, the introduction of the two 9-mercaptopo-meta-carborane clusters (1) was achieved with K2CO3 as base resulting in almost quantitative formation of tert-butyl-N\{4,6-bis(1,7-dicarba-closo-dodecaboran-9-ythio)-
1,3,5-triazin-2-yl]-N-(1',2',3',4'-di-O-propylidene-6'-deoxy-α-D-galactopyranos-6'-yl)glycinate (12) from 11.

Successful reproduction of the mentioned synthetic approach (Scheme 3, steps a to c) was already reported using a three carborene-bearing mercapto derivative as a nucleophile.26

Attempts to prepare 12 or 5 directly in one-pot syntheses, using DIPEA as a base, failed and only produced the mono-substituted derivative tert-butyl-N-[4-chloro-6-(1,7-dicarba-closododecaboran-9-ythio)-1,3,5-triazin-2-yl]-N-(1',2',3',4'-di-O-isopropylidene-6'-deoxy-α-D-galactopyranos-6'-yl)glycinate (SP1) in 15% yield or the side product N-ethyl-N-isopropyl-4,6-bis-(1,7-dicarba-closododecaboran-9-ythio)-1,3,5-triazine-2-amine (SP2) in 10% yield (details are given in the ESI†). Selective cleavage of the ester group of 12 (under maintenance of the isopropylidene protecting groups of the galactopyranosyl moiety) with trifluoroacetic acid in anhydrous toluene or dichloromethane gave the final product 5.38,41

The synthetic approach described in Scheme 3 was adapted for ethylene diamine (Scheme 4) to introduce an additional functional group for incorporation of various other biomolecules. In this case, tert-butyl-N-(2-aminoethyl)carbamate and triflate 4 were converted to tert-butyl-N-[2-[[4,6-dichloro-1,3,5-triazin-2-yl]-2-(1',2',3',4'-di-O-isopropylidene-6'-deoxy-α-D-galactopyranos-6'-yl)-amino]ethyl]carbamate (14), with compound 13 as intermediate, in 68% yield over two steps. Introduction of 9-mercapto-dicarba-closododecaborane (1), in the same manner as described for compound 12, gave tert-butyl-N-[2-[4,6-bis(1,7-dicarba-closododecaboran-9-ythio)-1,3,5-triazin-2-yl]-2-(1',2',3',4'-di-O-isopropylidene-6'-deoxy-α-D-galactopyranos-6'-yl)-amino]ethyl]carbamate (15) in almost quantitative yield. The observed high yields in the reaction of 1 with 11 or 14 show the pronounced nucleophilicity of the 9-mercapto-dicarba-closododecaborane (1) under mild basic conditions. Cleavage of the tert-butoxycarbonyl protecting group in 15 was achieved as described for 5 (Scheme 3). The primary amine 16, N5-[4,6-bis(1,7-dicarba-closododecaboran-9-ythio)-1,3,5-triazin-2-yl]-N3-(1',2',3',4'-di-O-isopropylidene-6'-deoxy-α-D-galactopyranos-6'-yl)ethane-1,2-diamine, was obtained in 91% yield.

All isolated compounds were fully characterised by NMR and IR spectroscopy, mass spectrometry and melting point determination. Additionally, compounds 6, 11 and 12 were characterised by single crystal X-ray diffraction.

The final product 5 was characterised by high-resolution mass spectrometry and NMR spectroscopy. A very broad signal in the 1H NMR spectrum at 6.34 ppm was assigned to a protonated tertiary amine indicating the presence of a zwitterion (NH3+/CO2−). In comparison, typical carboxylic acid protons have chemical shifts around 9 to 13 ppm.39

The characterisation of 6 was very challenging, as the obtained spectroscopic data were not unambiguous for the proposed structure. However, colourless crystals suitable for X-ray diffraction were obtained from an acetone solution confirming the formation of 1H)N6-[4,6-bis(1,7-dicarba-closododecaboran-9-ythio)-1,3,5-triazin-2-yl]glycinate (6). Two independent molecules of 6 are linked by hydrogen bonds between the hydrogen atom of the secondary amine group and one nitrogen atom of the s-triazine ring (Fig. 2). The observation of hydrogen bonds was already described for compounds of this substance class.27

Compound 11 crystallised from chloroform solution with two molecules in the asymmetric unit (Fig. 3).
Fig. 2 Molecular structure of 6. Hydrogen atoms are omitted for clarity, except the hydrogen atoms attached to the secondary amine groups drawn with a fixed atom radius of 13.5 pm. Thermal ellipsoids are drawn at the 50% probability level. Selected bond lengths, distances (pm) and bond angles (°): N4–H1N4 86(4), N4–N7 213(4), N4–N7 299.4(4), N8–H1N8 85(4), H1N8–N3 217(4), N8–N3 302.5(4), N4–C5 133.7(4), S1–B10 186.9(4), S1–C3 174.8(4), S2–B20 186.4(4), S2–C4 175.7(3), N8–C26 133.5(4), S3–B30 186.7(4), S4–B40 187.0(4), S4–C25 175.8(4); N4–H1N4–N7 173(4), N8–H1N8–N3 176(3), N1H4–N4–C5 121(3), C5–N4–C6 120.5(3), C6–N4–H1N4 114(3), C3–S1–B10 107.2(2), C4–S2–B20 106.9(2), H1N8–N8–C26 116(3), C6–N8–C27 122.4(3), C27–N8–H1N8 122(3), C24–S3–B30 108.3(2), C25–S4–B40 106.4(2).

Fig. 3 Molecular structure of 11. Hydrogen atoms are omitted for clarity. Thermal ellipsoids are drawn at the 50% probability level. Only one of the two symmetry-independent molecules is shown. Selected bond length (pm) and bond angles (°): N4–C3 133.4(3) [133.7(3), N4–C4 145.5(3) [145.1(3)], N4–C10 146.3(3) [146.4(3)], C1–C11 172.7(2) [172.3(2), C2–C12 172.9(2) [172.4(2)], C3–N4–C4 116.2(2) [116.2(2), C4–N4–C10 120.8(2) [119.1(1)], C10–N4–C3 120.9(2) [122.2(2)] (values for the second molecule are given in brackets [ ]).

In the case of compound 12, mass spectrometry was a very informative characterisation method, as the characteristic isotopic pattern for molecules with 20 boron atoms was observed.42,43 Single crystals of 12 could be obtained from n-hexane/ethyl acetate solution. The molecular structure is depicted in Fig. 4.

The carbamate 14 and the following products 15 and 16 were characterised by NMR and mass spectrometry confirming the successful synthesis. For compound 15, the mass spectrum was very characteristic, because the isotopic pattern clearly showed the replacement of two chloro substituents by two carborane clusters.42,43

In summary, the two target molecules 5 and 16 were obtained in good to excellent yield. Due to their different functional groups [R–COOH in 5 and R–NH2 in 16], coupling with a large variety of different biomolecules as tumour-selective carriers can be envisaged for application in BNCT.4,8,12,15,17,20

Biological studies

The galactopyranosyl derivative 5 was incorporated into the recently developed GRPR selective peptide [D-Phe6, β-Ala11, Ala13, Nle14]Bn(6–14) (sBB2L)10 to investigate, whether the ratio of one galactopyranosyl unit per two carborane clusters is sufficient to counterbalance the hydrophobicity of the carborane clusters yielding biologically active bioconjugates. The necessity for increased hydrophilicity was concluded from previous studies concerning the introduction of meta-carboranes as single clusters17 or s-triazine-based derivatives bearing no monosaccharide group.25 Three different peptide conjugates were synthesised (Fig. 5A) by a combination of automated and manual solid phase peptide synthesis (SPPS).30,44
azine derivative 5 as well as the previously published derivative without any galactopyranosyl moiety (compound #4 in Kellert et al., ref. 27) were coupled manually in three-fold molar excess to an N-terminally introduced three ethylene glycol-unit spacer (EG3). The reaction was carried out overnight at room temperature in dimethylformamide (DMF) containing three equivalents 1-hydroxybenzotriazole (HOBt) and N,N-diiiso-propylcarbodiimide (DIC), yielding conjugates 17 and 18. To enhance the boron loading per molecule a branching step was introduced using (2S)-2,3-diaminopropanoic acid (Dap) allowing the incorporation of two carborane building blocks 5 as deprotected moieties 5* (Fig. 5A). This strategy facilitated the generation of conjugate 19 bearing 40 boron atoms.

Analyses of all three conjugates were performed with analytical reversed-phase high performance liquid chromatography (RP-HPLC) and electrospray ionisation mass spectrometry (ESI-MS) as well as with MALDI-TOF-MS (Fig. 5B and C). The exemplary RP-HPLC chromatogram of 18 displayed the main peak at 17.2 min retention time and a small shoulder on the right side. Since this shoulder had the same mass as the desired product and conjugate 17 did not exhibit any shoulders, the deoxygalactosyl moiety is suggested to cause this shoulder by mutarotation (Fig. 5B).

The three bioconjugates were tested in receptor activation and internalisation studies. For this purpose, the human GRPR was selected due to its remarkable overexpression on various tumour tissues like small cell lung, breast and prostate cancer. The neuromedin B receptor (NMBR) and the bombesin receptor subtype 3 (BRS-3), which are also part of the bombesin receptor family are only occasionally expressed in these tumours.

The use of the recently published GRPR selective ligand (sBB2L) allows the specific targeting of cancer cells while the accumulation in healthy tissues is kept to a minimum. Thus, the risk of side effects is reduced. Compared to the unmodified ligand sBB2L, which displayed an EC50 value of 0.12 nM at the GRPR, conjugate 17 exhibited a ca. 20-fold reduced potency (EC50 2.2 nM; Fig. 6A). This can be explained by the insufficient solubility of derivate #4 which was recently demonstrated by the incorporation into the even longer and more hydrophilic peptide [F7,P34]-NPY. However, conjugate 18 bearing an additional deoxygalactopyranosyl moiety regained the activity at the GRPR and, with an EC50 value of 0.17 nM, demonstrated wild type like potency. In addition, fluorescence microscopy studies revealed that 17 and 18 induce internalisation of the GRPR at a peptide concentration of 100 nM. After one hour of stimulation, the membrane bound receptor was completely translocated into intracellular vesicles, as was observed for the unmodified sBB2L (Fig. 6B).

Due to the improved receptor activation of conjugate 18, Dap was introduced to allow the incorporation of more than one carborane-based building block per peptide molecule. The resulting conjugate 19, bearing two deprotected molecules 5*...
(40 boron atoms), displayed a strongly reduced potency at the GRPR and showed nearly no internalisation after 1 h of stimulation with 100 nM peptide. This indicates that building block 5, featuring a ratio of one deoxygalactopyranosyl unit per two carborane clusters, is not optimal for the generation of highly carborane-loaded peptide conjugates. Nevertheless, the incorporation of the deoxygalactopyranosyl unit improved the hydrophilicity, which allowed the synthesis of a double modified conjugate (19). This was previously not possible with building block #4 using standard peptide purification methods (data not shown). These observations demonstrate the necessity of hydrophilicity providing moieties to generate highly boron-loaded bioconjugates for tumour delivery. Therefore, conjugate 18 can be considered as a promising selective boron delivery agent in BNCT.

Conclusions

By modifying s-triazine derivatives with a galactopyranosyl moiety the modular system based on readily available building blocks like 9-mercapto-1,7-dicarba-closo-dodecaborane (1) and cyanuric acid 5 and amine 16, are highly suitable for the synthesis of bioconjugates, shown here exemplarily for 5 being introduced to the GRPR selective peptide [D-Phe6, 16

Experimental section

General experimental part

All reactions were carried out under nitrogen atmosphere using Schlenk techniques, if not reported otherwise. Anhydrous dichloromethane, toluene and tetrahydrofuran were obtained with an MBRAUN solvent purification system MB SPS-800 and stored over molecular sieves (3 Å) under nitrogen atmosphere. Acetonitrile and 2,4,6-collidine were dried over CaH₂, distilled and stored over molecular sieves (3 Å) under nitrogen atmosphere. 9-Mercapto-1,7-dicarba-closo-dodecaborane(12) (1), 2-chloro-4,6-bis(1,7-dicarba-closo-dodecaboran-9-ylthio)-1,3,5-triazine (2), [4,6-bis(1,7-dicarba-closo-dodecaboran-9-ylthio)-1,3,5-triazin-2-yl]glycine (3) and 1,2,3,4-di-O-isopropylidene-6-deoxy-α-d-galactopyranosyl-6-triflate (4) were prepared and characterised according to the literature procedure. All other solvents and chemicals were purchased and used as received. Thin-layer chromatography (TLC) with silica gel 60 F254 on glass available from Merck KGaA was used for monitoring the reactions. Carborane-containing spots were visualised with a 5-10% solution of PdCl₂ in methanol.

For chromatography, silica gel (60 Å) with a particle diameter in the range of 0.035 to 0.070 mm, the Biotage® Isolera 1 or the Biotage® Isolera 4 automatic purification system with SNAP (particle diameter: 0.040 to 0.065 mm) and SNAP Ultra (spherical particle, diameter: 0.025 mm) cartridges were used. The triazine and carborane species were detected by an integrated UV/Vis detector (Isolera 1, Biotage) or evaporative light scattering detector (ELSD) A-120 (Isolera 4, Biotage). For chromatography, solvents were distilled before use. NMR measurements were carried out on a Bruker AVANCE III HD spectrometer with an Ascend™ 400 magnet at room temperature. Tetramethylsilane was used as internal standard for 1H and 13C[1H] NMR spectra, and 11B and 11B[1H] NMR spectra were referenced to the Ξ scale. NMR spectra were recorded at the following frequencies: 1H: 400.16 MHz, 13C: 100.63 MHz, 11B: 128.38 MHz; chemical shifts are reported in ppm. Assignment of the 1H and 13C signals was based on 2D NMR experiments (data not shown). These observations demonstrate the advantageous influence of galactopyranosyl moieties for the development of highly carborane-loaded biomolecules.

X-ray diffraction experiments

Measurements were performed with a Gemini diffractometer (Rigaku Oxford Diffraction) with Mo Kα radiation (λ = 71.073 pm), o-scan rotation. Data reduction was performed with Crysalis Pro including the program SCALE3 ABSPACK for empirical absorption correction. The structures were solved by direct methods (SHELXT-2014) and the refinement of all non-hydrogen atoms was performed with SHELXL-2018. Hydrogen atoms were mostly located on difference Fourier maps calculated at the final stage of the structure refinement, and only for disordered regions or poor diffacting samples they were calculated on idealised positions using the riding model. Structure figures were generated with Diamond. CCDC 1958031 (6), 1958032 (8), 1958033 (9), 1958034 (11), 1958035 (12), 1958036 (SP1), 1958037 (SP2) and 1958038 (SP3) contain supplementary crystallographic data for this paper.
(′1,2′,3′,4′-Di-O-isopropylidene-6′-deoxy-α-β-galactopyranos-6′-yl)[4,6-bis(1′,7′-dicarba-closo-dodecaboran-9-ythio)-1,3,5-triazin-2′-yl]glycinate (6). A 100 mL Schlenk flask was charged with 90 mg (0.18 mmol, 1.00 eq.) [4,6-bis(1′,7′-dicarba-closo-dodecaboran-9-ythio)-1,3,5-triazin-2′-yl]glycine (3) and 0.13 g (0.94 mmol, 5.22 eq.) K₂CO₃, evacuated and purged with nitrogen. 30 mL tetrahydrofuran were added, the mixture was heated to 40 °C and stirred for 3 h. Subsequently, 0.15 g (0.38 mmol, 2.11 eq.) 1,2,3,4-di-O-isopropylidene-6′-deoxy-α-β-galactopyranosyl-6-triflate (4), dissolved in 20 mL tetrahydrofuran, were added to the mixture. The reaction mixture was stirred for 2 d at ambient temperature. The reaction was stopped by adding 30 mL H₂O and the aqueous layer was extracted three times with 25 mL Et₂O. The combined organic layers were dried over MgSO₄, filtered and the solvent was removed under reduced pressure. After column chromatography (n-hexane/ethyl acetate, 3:1 to 100% ethyl acetate, v/v) 84 mg (0.11 mmol, 65%, Rf = 0.49, n-hexane/ethyl acetate, 1:1, v/v) of compound 6 was obtained as a pure white solid. Colourless crystals of 6 suitable for X-ray structure determination were obtained from acetonitrile at room temperature. Crystallographic data are given in Table S1, and the molecular structure is depicted in Fig. 2. 7a: 167–168 °C (ethyl acetate).

IR (KBr): v = 3436 (m), 3056 (w), 2988 (w), 2936 (w), 2608 (s), 1749 (m), 1563 (s), 1497 (s), 1455 (w), 1412 (m), 1383 (m), 1298 (m), 1252 (m), 1211 (m), 1174 (m), 1116 (w), 1070 (s), 1001 (m), 955 (w), 921 (w), 892 (w), 864 (m), 850 (m), 804 (w), 759 (w), 730 (w), 677 (w), 627 (w), 512 (w), 432 (w) cm⁻¹. ¹H NMR (acetone-d₆): δ = 1.316 (s, 3H, C₁₅ or 15 H₃), 1.322 (s, 3H, C₁₅ or 15 H₃), 1.38 (s, 3H, C₁₅ or 15 H₃), 1.47 (s, 3H, C₁₅ or 15 H₃), 1.50 to 3.50 (br, 18H, 2 × Br(CH₂)₃), 3.69 (br, s, 2H, 2 × C₁ or 1 H), 3.74 (br, 2H, 2 × C₁ or 1 H), 4.06 (m, 1H, C₃H), 4.19 (m, 2H, C₄H₂), 4.29 (m, 1H, CH₃), 4.33 (m, 2H, C₄H₂), 4.37 (m, 1H, C₄H₂), 4.64 (dd, J₁H₁H = 2.5 Hz, 1H, C₁H), 5.47 (d, J₁H₃H = 5.0 Hz, 1H, C₃H), 7.05 ppm (t, J₁H₆H = 6.6 Hz, 1H, N₆H).

¹³C[¹H] NMR (acetone-d₆): δ = 24.7, 25.2, 26.3 and 26.4 (s, CH₃, C₁₅ or 15 H₃, C₁₅ or 15 H₃, C₁₅ or 15 H₃), 42.9 (s, CH₂, C₄H₂), 55.6 and 55.9 (br, s, CH₂, 2 × C₁ and 1 H), 64.7 (s, CH₂, C₄H₂), 66.6 (s, CH, C₃H), 71.3 (s, CH₁, C₁₅ or 15 H₃), 71.5 (s, CH₁, C₁₅ or 15 H₃), 71.8 (s, CH₁, C₃H), 97.1 (s, CH, C₁₅ or 15 H₃), 109.2 (s, C₄, C₁₅ or 15 H₃), 109.9 (s, C₆, C₁₅ or 15 H₃), 164.7 (s, C₆, C₁₅ or 15 H₃), 170.7 (s, C₇, 2 × C₁), 179.8 ppm (s, C₇, C₆, C₁₅ or 15 O₆). ¹⁵B[¹H] NMR (acetone-d₆): δ = −18.5 (s, 2B), −17.0 (s, 2B), −14.1 (s, 4B), −12.8 (s, 4B), −10.4 (s, 2B), −5.9 (s, 4B), −3.4 ppm (s, 2B, 2 × BS). ¹¹B NMR (acetone-d₆): δ = −20.4 to −9.2 (br, 14B), −5.9 (d, J₁H₁ ≈ 149 Hz, 4B), −3.4 ppm (s, 2B, 2 × BS). HRMS (ESI+): C₂₁H₄₄B₂O₂N₄S₂, m/z calcid: 768.45326 ([M + Na]⁺); found: 768.45314 (100%); m/z calcid: 746.47131 ([M novelist]); found: 746.47144 (17%).

tert-Butyl-(4,6-dichloro-1,3,5-triazin-2-yl)-N′-[1′,2′,3′,4′-di-O-isopropylidene-6′-deoxy-α-β-galactopyranos-6′-yl]glycinate (11). A 100 mL Schlenk flask was charged with 1.55 g (3.95 mmol, 1.00 eq.) 1,2,3,4-di-O-isopropylidene-6′-deoxy-α-β-galactopyranosyl-6-triflate (4) and 0.80 g (4.77 mmol, 1.21 eq.) tert-butyl glycinate hydrochloride, evacuated, purged with nitrogen and 40 mL MeCN were added. Subsequently, 2.00 mL (1.52 g, 11.8 mmol, 2.47 eq.) DIPEA were slowly added and the mixture was stirred for 2 d at 45 °C. A solution of 1.83 g (9.92 mmol, 2.51 eq.) cyanuric chloride and 0.87 mL (0.66 g, 15.12 mmol, 3.0 eq.) DIPEA in 10 mL MeCN was slowly added at 0 °C and the reaction mixture was stirred for 2 d at 35 °C. The reaction was stopped by adding 50 mL of a saturated NaCl solution; the resulting layers were separated. The aqueous layer was extracted three times with 50 mL ethyl acetate. The combined organic layers were dried over MgSO₄, filtered and the solvent was removed under reduced pressure. After column chromatography (ethyl acetate/n-hexane, 1:5, v/v) 1.59 g (3.05 mmol, 77%, Rf = 0.37) of the title compound was obtained as a slightly yellow solid. Colourless crystals of 11 suitable for X-ray structure determination were obtained from CHCl₃ at room temperature. Crystallographic data are given in Table S2, and the molecular structure is depicted in Fig. 3. Tm**: 122–124 °C (ethyl acetate). IR (KBr): v = 3441 (s), 2984 (m), 2937 (w), 1741 (s), 1568 (s), 1489 (s), 1458 (w), 1417 (w), 1372 (m), 1328 (m), 1289 (w), 1228 (s), 1172 (s), 1114 (m), 1071 (s), 1003 (m), 980 (w), 883 (w), 848 (m), 800 (m), 773 (w), 746 (w), 651 (w), 599 (w), 551 (w), 512 (w) cm⁻¹. ¹H NMR (CDCl₃): δ = 1.31 (s, 3H, C₁₅ or 15 H₃), 1.34 (s, 3H, C₁₅ or 15 H₃), 1.34 (s, 3H, C₁₅ or 15 H₃), 1.44 (s, 3H, C₁₅ or 15 H₃), 1.47 (s, 9H, C(CH₃)₃), 1.50 (s, 3H, C₁₅ or 15 H₃), 3.68 (dd, J₁H₁H = 14.3 Hz, J₁H₁H = 8.1 Hz, 1H, C₁H), 3.88 (dd, J₁H₁H = 14.3 Hz, J₁H₁H = 8.1 Hz, 1H, C₁H), 3.93 (dd, J₁H₁H = 14.3 Hz, J₁H₁H = 8.1 Hz, 1H, C₁H), 3.98 ppm (dd, J₁H₁H = 14.3 Hz, J₁H₁H = 8.1 Hz, 1H, C₁H), 3.98 ppm (dd, J₁H₁H = 14.3 Hz, J₁H₁H = 8.1 Hz, 1H, C₁H), 3.98 ppm (dd, J₁H₁H = 14.3 Hz, J₁H₁H = 8.1 Hz, 1H, C₁H)}. View Article Online
acetate, v/v) 3.01 g (3.75 mmol, 98%, determination were obtained from ethyl acetate layered with less solid. Colourless crystals of 757 (w), 731 (m) cm⁻¹, 1316 (m), 1301 (m), 1261 (s), 1250 (s), 1222 (s), 1182 (s), 1153 (m), 1126 (m), 1095 (s), 1055 (s), 973 (s), 932 (s), 831 (s), 775 (m), 757 (m), 731 (m), 697 (s), 666 (s), 644 (s), 559 (s), 512 (s), 493 (s), 462 (dd, J_HH = 14.4 Hz, J_HH = 4.6 Hz, 1H, C^{13}H), 4.37 (dd, J_HH = 17.8 Hz, 1H, C^{13}H), 4.58 (dd, J_HH = 8.0 Hz, 1H, C^{13}H), 5.39 ppm (d, J_HH = 5.0 Hz, 1H, C^{13}H). 1^1C{1H} NMR (CDCl₃): δ = 24.4, 25.1, 26.0 and 26.1 (s, CH₃, C^{16}H₃, C^{16}H₃, C^{17}H and C^{17}H), 28.1 (s, CH₃, C(C^{13}H)₃), 47.9 (s, CH₂, C^{14}H₃), 50.2 (s, CH₂, C^{15}H₂), 53.8 (br, s, CH, 4xCH₂), 66.4 (s, CH, C^{15}H), 70.5 (s, CH, C^{12}H), 70.8 (s, CH, C^{10}H), 71.5 (s, CH, C^{11}H), 81.4 (s, C(q, C^{16})), 96.3 (s, CH, C^{13}H), 108.8 (s, C(q, C^{16})), 109.2 (s, C(q, C^{14})), 113.2 (s, C(q, C^{14})), 116.9 (s, C(q, C^{16})), 119.3 ppm (s, C(q, C^{16})), 117B{1H} NMR (CDCl₃): δ = −18.7 (s, 2B), −17.4 (s, 2B), −14.0 (s, 4B), −12.8 (s, 4B), −10.0 (s, 2B), −5.6 (s, 4B), −3.1 ppm (s, 2B, 2 x BS). 11B NMR (CDCl₃): δ = −20.5 to −15.8 (br², 4B), −15.4 to −11.4 (br², 4B), −10.0 (d, J_HH = 150 Hz, 2B), −5.6 (d, J_HH = 158 Hz, 4B), −3.1 ppm (s, 2B, 2 x BS). HRMS (ESI+): C_{25}H_{32}B_{12}Cl_{2}N_{2}O_{25}, m/z calc: 802.53420 ([M + H]+); found: 802.53393 (100%).

N-[4,6-bis(1,7-dicarba-closo-dodecaboran-9-ythio)−1,3,5-triazin-2-yl]-N′-[1,2,3,4′,di-O-isopropylidene-6-deoxy-α-D-galactopyranos-6-y]glycinate (12). A 250 mL two-neck round-bottom flask, equipped with a condenser, was charged with 2.15 g (12.2 mmol, 3.18 eq.) mercapto-1,7-dicarba-closo-dodecaborane(12) (1) and 2.65 g (19.2 mmol, 4.99 eq.) K₂CO₃, evacuated and purged with nitrogen. The starting materials were suspended in 100 mL MeCN. A separate Schlenk flask was charged with 2.00 g (3.84 mmol, 1.00 eq.) tert-butyl-N-[4,6-dichloro-1,3,5-triazin-2-yl]-N′-[1,2,3,4′,di-O-isopropylidene-6-deoxy-α-D-galactopyranos-6-y]glycinate (11), evacuated, purged with nitrogen and then 40 mL MeCN were added. The solution containing the glycinate was added dropwise to the reaction mixture and the mixture was then stirred under reflux for 2 d. The reaction was stopped by adding 70 mL of a saturated aqueous NaCl solution. A saturated NH₄Cl solution was added until a pH value of about 7 to 8 was achieved. The resulting layers were separated and the aqueous layer was extracted three times with 40 mL ethyl acetate. The combined organic layers were washed with 20 mL saturated NaCl solution. The organic layer was then dried over MgSO₄, filtered and the solvent was removed under reduced pressure. After column chromatography (ethyl acetate/n-hexane, 1:3) the crude product was obtained as a colourless solid. Colourless crystals of 12 suitable for X-ray structure determination were obtained from ethyl acetate layered with n-hexane at room temperature. Crystallographic data are given in Table S2, and the molecular structure is depicted in Fig. 5. T_{rmt}: 240–242 °C (ethyl acetate, decomposition), IR (KBr): ν = 3253 (w), 3134 (w), 3064 (m), 3038 (m), 2983 (m), 2925 (m), 2602 (s), 2562 (m), 1967 (w), 1741 (s), 1614 (m), 1534 (s), 1510 (s), 1486 (s), 1421 (m), 1401 (w), 1383 (m), 1370 (s), 1331 (m), 1316 (m), 1301 (m), 1261 (s), 1250 (s), 1222 (s), 1182 (s), 1153 (s), 1102 (s), 1070 (s), 1044 (m), 1007 (s), 976 (m), 952 (s), 919 (w), 901 (m), 879 (w), 864 (m), 848 (s), 803 (m), 772 (w), 737 (w), 731 (m) cm⁻¹. 1H NMR (CDCl₃): δ = 1.29 (s, 3H, C^{16} or 16H), 1.34 (s, 3H, C^{15} or 15H), 1.44 (s, 12H, C^{14} or 14H and C(C^{13}H)₃), 1.46 (s, 3H, C^{17} or 17H), 1.66 to 3.56 (br², 18H, 2 x B₂H₁₀), 2.96 (br, s, 4H, 4xCH₂), 3.65 (dd, J_HH = 14.4 Hz, J_HH = 8.2 Hz, 1H, C^{13}H), 3.99 (dd, J_HH = 14.4 Hz, J_HH = 4.6 Hz, 1H, C^{13}H), 4.19 (m, 1H, C^{15}H), 4.26 (m, 2H, C^{11}H and C^{12}H), 4.37 (d, J_HH = 17.8 Hz, 1H, C^{13}H), 4.58 (dd, J_HH = 8.0 Hz, 1H, C^{13}H), 4.68 (d, J_HH = 17.8 Hz, 1H, C^{13}H), 5.49 ppm (d, J_HH = 5.0 Hz, 1H, C^{13}H). 1^1C{1H} NMR (CDCl₃): δ = 24.4, 25.1, 26.0 and 26.1 (s, CH₃, C^{16}H₃, C^{16}H₃, C^{17}H and C^{17}H), 28.1 (s, CH₃, C(C^{13}H)₃), 47.9 (s, CH₂, C^{14}H₃), 50.2 (s, CH₂, C^{15}H₂), 53.8 (br, s, CH, 4xCH₂), 66.4 (s, CH, C^{15}H), 70.5 (s, CH, C^{12}H), 70.8 (s, CH, C^{10}H), 71.5 (s, CH, C^{11}H), 81.4 (s, C(q, C^{16})), 96.3 (s, CH, C^{13}H), 108.8 (s, C(q, C^{16})), 109.2 (s, C(q, C^{14})), 113.2 (s, C(q, C^{14})), 116.9 (s, C(q, C^{16})), 119.3 ppm (s, C(q, C^{16})), 117B{1H} NMR (CDCl₃): δ = −18.7 (s, 2B), −17.4 (s, 2B), −14.0 (s, 4B), −12.8 (s, 4B), −10.0 (s, 2B), −5.6 (s, 4B), −3.1 ppm (s, 2B, 2 x BS). HRMS (ESI+): C_{25}H_{32}B_{12}Cl_{2}N_{2}O_{25}, m/z calc: 802.53420 ([M + H]+); found: 802.53393 (100%).
2 × C^1H and 2 × C^3H), 3.54 (dd, δ^HH = 14.4 Hz, δ^HH = 8.4 Hz, 1H, C^3HH), 4.10 (m, 1H, C^3HH), 4.19 (m, 1H, C^1H), 4.27 (m, 2H, C^1H and C^3H), 4.58 (m, 1H, C^1H), 4.64 (br, m, 2H, C^2H), 5.48 (d, δ^HH = 5.0 Hz, 1H, C^1H), 6.34 ppm (vbr s, 1H, C^3OH or NR,H). \textsuperscript{13}C\text{[\text{H}]} NMR (CDCl\textsubscript{3}): δ = 24.3, 25.1, 26.0 and 26.1 (s, CH\textsubscript{3}), 54.9 (s, CH\textsubscript{2}), 56.7 (s, CH\textsubscript{2}), 71.3 (s, CH\textsubscript{2}), 71.6 (s, CH\textsubscript{2}), 72.0 (s, CH\textsubscript{2}), 72.8 (s, CH\textsubscript{2}), 78.8 (s, CH\textsubscript{2}), 78.9 (s, CH\textsubscript{2}), 78.9 (s, CH\textsubscript{2}), 83.0 (s, CH\textsubscript{2}), 84.1 (s, CH\textsubscript{2}), 88.1 (s, CH\textsubscript{2}), 97.3 (s, CH\textsubscript{2}), 109.3 (s, CH\textsubscript{2}), 109.3 (s, CH\textsubscript{2}), 116.3 (s, CH\textsubscript{2}), 127.5 (s, CH\textsubscript{2}), 127.5 (s, CH\textsubscript{2}), 131.5 (s, CH\textsubscript{2}), 131.5 (s, CH\textsubscript{2}), 133.5 (s, CH\textsubscript{2}), 133.5 (s, CH\textsubscript{2}), 137.0 (s, CH\textsubscript{2}), 144.0 (s, CH\textsubscript{2}), 144.0 (s, CH\textsubscript{2}), 156.7 (s, CH\textsubscript{2}), 156.7 (s, CH\textsubscript{2}), 163.3 (s, CH\textsubscript{2}), 163.3 (s, CH\textsubscript{2}), 168.1 ppm (s, CH\textsubscript{2}).

The reaction mixture was stirred at 0 °C and the reaction mixture was stirred for 2 d at 35 °C. A solution of 1.56 g (1 eq.) 1,2:3,4-di-O-isopropylidenen-6-deoxy-α-D-galactopyranosyloxy)ethylene-1,2-diamine (5a) and 0.99% NaOH was added to the mixture. The reaction mixture was stirred at 0 °C and the reaction mixture was stirred for 2 d at 35 °C. The reaction was stopped by adding 25 mL of saturated aqueous NaCl solution. The resulting layers were separated and the aqueous layer was extracted with 90 °C and the reaction mixture was stirred for 2 d at 35 °C. The reaction was stopped by adding 30 mL of a saturated NaCl solution; the resulting layers were separated. The aqueous layer was extracted four times with 20 mL ethyl acetate. The combined organic layers were dried over MgSO\textsubscript{4}, filtered and the solvent was removed under reduced pressure. After column chromatography (ethyl acetate/n-hexane, 1:3, v/v) 1.90 g (3.46 mmol, 68%, R\textsubscript{f} = 0.31) of compound 14 was obtained as a colourless solid.

\textit{tert-Butyl-N-[2-[[4,6-dichloro-1,3,5-triazin-2-yl]-N-2-(1',2',3',4'-di-O-isopropylidenen-6-deoxy-α-D-galactopyranosyloxy)-ethanoy]amino]ethyl]carbamate} (14). A 100 mL Schlenk flask was evacuated and purged with nitrogen and diluted with 1.99 g (5.07 mmol, 1.00 eq.) 2,2':3,3'-tert-butyl-2-[[4,6-dichloro-1,3,5-triazin-2-yl]-N-2-(1',2',3',4'-di-O-isopropylidenen-6-deoxy-α-D-galactopyranosyloxy)-ethanoy]amino]ethyl]carbamate. Subsequently, 1.05 mL (0.80 g, 6.17 mmol, 1.22 eq.) DPEA was slowly added at room temperature to the mixture and the reaction mixture was stirred for 2 d at 40 °C. A solution of 1.56 g (8.46 mmol, 1.67 eq.) cyanoacrylate chloride and 1.05 mL (0.80 g, 6.17 mmol, 1.22 eq.) DPEA was slowly added at room temperature to the mixture and the reaction mixture was stirred for 2 d at 35 °C. The reaction was stopped by adding 30 mL of a saturated NaCl solution; the resulting layers were separated. The aqueous layer was extracted four times with 20 mL ethyl acetate. The combined organic layers were dried over MgSO\textsubscript{4}, filtered and the solvent was removed under reduced pressure. After column chromatography (ethyl acetate/n-hexane, 1:2, v/v) 1.50 g (1.80 mmol, 99%, R\textsubscript{f} = 0.21) of compound 15 was obtained as a colourless solid. IR (KBr): 3435 (m), 3048 (m), 2981 (m), 2935 (m), 2608 (s), 1715 (s), 1625 (w), 1543 (s), 1511 (s), 1478 (s), 1434 (m), 1383 (m), 1369 (m), 1315 (s), 1243 (s), 1214 (s), 1171 (s), 1104 (w), 995 (m), 954 (m), 918 (w), 903 (w), 864 (m), 847 (m), 802 (w), 759 (w), 731 (w), 678 (w), 627 (w), 585 (w), 512 (w), 450 (w), 340 (m), 292 (m), 222 (m), 182 (m), 140 (m), 138 (m), 130 (m), 124 (m), 118 (m), 108 (m), 97 (m), 88 (m), 87 (m), 78 (m), 70 (m), 63 (m), 54 (m), 52 (m), 40 (m), 29 (m), 27 (m), 25 (m), 23 (m), 21 (m), 19 (m), 17 (m), 15 (m), 13 (m), 11 (m), 9 (m), 7 (m), 6 (m), 4 (m), 3 (m), 2 (m), 1 (m).
di-O-isopropylidene-6′-deoxy-a-D-galactopyranos-6′-yl)-amino]-ethyl]carbamate (15), evacuated and purged with nitrogen, and 10 mL CH₂Cl₂ were added. Subsequently, 1.65 mL (2.44 g, 21.4 mmol, 50.4 eq.) trifluoroacetic acid was added and the mixture was stirred for 4 h at room temperature. Then the solvent was removed under reduced pressure. The raw product was purified by column chromatography (ethyl acetate/n-hexane, 2:1 (v/v) to ethyl acetate, 100%) and 283 mg (388 µmol, 91%, R₁ = 0.05 (100% ethyl acetate)) of compound 16 were obtained as an off-white solid. Tₘ: 147–150 °C (ethyl acetate, decomposition). IR (KBr): ν: 3442 (s), 3053 (m), 2989 (m), 2935 (m), 2609 (s), 2095 (w), 1680 (s), 1637 (m), 1531 (s), 1508 (s), 1483 (s), 1431 (m), 1343 (m), 1338 (w), 1338 (w), 1316 (m), 1281 (w), 1250 (m), 1207 (s), 1175 (s), 1139 (m), 1109 (w), 1069 (s), 998 (m), 954 (m), 902 (w), 864 (m), 849 (m), 802 (m), 760 (w), 724 (m), 700 (w), 626 (w), 588 (w), 513 (w) cm⁻¹. ¹H NMR (acetone-d₆): δ = 1.28 [s, 3 H, C₁5 or 15 H₃], 1.35 [s, 3 H, C₁6 or 16 H₃], 3.40 (bra, 18 H, 2 × B₁₀H₉), 3.56 (dd, 3 H, J₁BH = 2.5 Hz, 1 H, C₁₀H), 5.51 ppm (d, J₁BH = 5.0 Hz, 1 H, C₁H), 10.2 Hz, 1 H, C₇H), 3.80 and 3.82 (s, CH, 2 × C₁H and 2 × C₁H), 3.95 to 4.25 (br, m, 6 H, 2 × C₄HH, 2 × C₅HH, C₇H) and C₂H₅), 4.62 (dd, J₁HH = 7.9 Hz, 3 H, J₂HH = 5.5 Hz, 1 H, C₁H), 4.62 (dd, J₁HH = 7.9 Hz, 3 H, J₂HH = 2.5 Hz, 1 H, C₁H), 110.0 (s, C₂H, C₂H), 45.8 (s, C₂H, C₂H), 49.9 (s, C₂H, C₂H), 56.0 and 56.1 (s, C₁H, 2 × C₁H and 2 × C₁H, 67.2 (s, C₁H, C₁H), 71.3 (s, C₁H, C₁H), 71.8 (s, C₁H), C₁HH), 72.4 (s, C₁H, C₁HH), 97.3 (s, C₁H, C₁HH), 109.3 (s, C₁H), C₁HH), 116.0 (s, C₁H, C₁HH), 163.9 (s, C₁H, C₁HH), 179.0 and 179.5 ppm (s, C₁H, 2 × C₁H₃), 13C{¹H} NMR (acetone-d₆): δ = 24.7, 25.2, 26.3 and 26.4 (s, CH₃), 21.9 (s, CH₃), 145.8 (s, CH₂, C₂H₂), 49.9 (s, CH₂, C₂H₂), 56.0 and 56.1 (s, CH₂, 2 × C₂H and 2 × C₂H), 67.2 (s, C₁H), C₁HH), 71.3 (s, C₁H, C₁HH), 71.8 (s, C₁H, C₁HH), 72.4 (s, C₁H, C₁HH), 97.3 (s, C₁H, C₁HH), 109.3 (s, C₁H, C₁HH), 116.0 (s, C₁H, C₁HH), 163.9 (s, C₁H, C₁HH), 179.0 and 179.5 ppm (s, C₁H, 2 × C₁H₃), 13C{¹H} NMR (acetone-d₆): δ = −19.6 (s, 2B), −17.9 (s, 2B), −14.9 (s, 4B), −13.7 (s, 4B), −11.3 (s, 2B), −6.9 (s, 4B), −4.4 ppm (s, 2B, 2 × BS), 11B NMR (acetone-d₆): δ = −21.3 to −12.3 (br², 12B), −11.3 (d, J₁BH = 149 Hz, 2B), −6.9 (d, J₁BH = 165 Hz, 4B), −4.4 ppm (s, 2B, 2 × BS), HRMS (ESI⁺): C₁₂H₂₃N₂O₃S₂ m/z calc.: 774.5604 [M + C₂H₂N⁺]; found: 774.5620 (100%); m/z calc.: 731.5067 [M + H⁺]; found: 731.5082 (54%).

Peptide synthesis

All bioconjugates were synthesised on a NovaSyn® TGR R resin from Merck (Darmstadt, Germany) with automated peptide synthesiser (SYRO I, MultiSynTech) on solid support in a 15 µmol scale. All reactions were carried out in DMF and all amino acids (AA) were Nα-Fmoc-protected and standard side chain protecting groups were used. Each AA and the reagents oxyma and DIC were added in 8-fold molar excess (equiv.) and each coupling step was performed twice with a reaction time of 40 min. Fmoc protecting group cleavage was achieved by using 40% piperidine for 3 min followed by incubation with 20% piperidine for 10 min. The cycle of coupling and Fmoc cleavage was repeated until the desired peptide length was achieved. After the peptide backbone was finished on solid support, a three-unit ethylene glycol spacer (EG₃) was coupled manually to the N-terminus with 2 equiv. of Fmoc-NH-PEG₃-COOH and 1.9 equiv. of HATU (O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethylenuroniumhexafluorophosphate). DIPEA was added in 2-fold molar excess and the reaction was performed for approximately 18 h. Subsequently, Fmoc was cleaved using 30% piperidine for 10 min twice. The carboline building blocks #4 or 5 were coupled manually with 3 equiv. HOBT and DIC overnight to yield conjugates 17 and 18. A branching moiety was introduced to obtain conjugate 19. Fmoc-L-Dap-(Fmoc)-OH was coupled manually with 3 equiv. HOBT and DIC overnight. Manual Fmoc-cleavage was performed as described above, and building block 5 was coupled as described above using 3 equiv. of 5 per free amino group. After completed synthesis the conjugates were cleaved from the resin and the side chain protecting groups were removed by incubation with TFA/triisopropylsilane (TIS)/H₂O (90:5:5) for 2.5 h. Cleaved conjugates were precipitated in ice-cold Et₂O/n-hexane (1:4) and subsequently lyophilised. Purification was performed with preparative RP-HPLC by applying a linear binary gradient system of eluent A (0.1% TFA in water) and eluent B (0.08% TFA in MeCN). UV detection was measured at λ = 220 nm. To confirm correct identity and purity of the synthesised conjugates, analytical RP-HPLC, MALDI-ToF-MS (Ultraxflex III, Bruker) and ESI-MS (HCT, Bruker) were performed.

Stable transfection

The plasmid (pCMV6-GRPR-tGFP) was amplified using E. coli DH5α and purified (PureYield™, Promega, Madison, WI, USA). Correct constructs could be confirmed by sequencing. HEK293 cells were stably transfected with the plasmids using Lipofectamine™ 2000 and selected with G-418.

Cell culture

The generation and cultivation of stably transfected HEK293-GRPR-tGFP cells was described before.30 Briefly, cells were cultivated in T75 cell culture flasks and were grown in DMEM/HAM’S F12 (1:1, v/v) containing 15% FBS (v/v) and 1.0 mg mL⁻¹ G-418 under standard conditions in an incubator (37 °C, 5% CO₂, 95% humidity). After cells reached full confluence, they were split in desired ratios from 1:2 to 1:12 into new cell culture flasks, filled with fresh medium for further cultivation or seeded into cell culture vessels for assays.

Ca²⁺ mobilisation assay

HEK293-GRPR-tGFP cells were seeded into black poly-(L-lysine) coated 96-well plates (μCLEAR®, CELLSTAR®, Greiner Bio One) at a density of 180 000 cells per well and were incubated under standard conditions overnight. At the following day, the medium was aspirated, and the cells were incubated for 40 min under standard conditions with Fluo-2-AM solution (2.3 µM Fluo-2-AM (abcam, Cambridge, UK), 0.06% (v/v) Pluronic® F-127 (Sigma-Aldrich, St Louis, MO, USA) in assay buffer). The fluorescence dye solution was replaced by assay buffer (20 mM HEPES, 2.5 mM Probenecid (Sigma-Aldrich, St Louis, MO, USA) in HBSS, pH 7.5) and basal Ca²⁺ signal was measured for 20 s with a Flexstation 3 (Molecular Devices, Sunnyvale, CA, USA) before ligand was added and Ca²⁺ response was determined for another 40 s (Ex = 485 nm, Em = 525 nm). The x-over basal values were calculated, and data
were normalised with GraphPad Prism 5.0 to the universal ligand \( \text{o-Phe}^6, \beta\text{Ala}^{11}, \text{Phe}^{13}, \text{Nle}^{14} \text{Bn}(6-14) \).

Live cell microscopy

Receptor internalisation was investigated by using stably transfected HEK293 cells, which were seeded into 8-well µ-slides (ibiTreat, ibidi, Martinsried, Germany) and incubated for two days at standard conditions. At the assay day, cells were starved for 30 min with 200 µL OptiMEM® under standard conditions and OptiMEM® was subsequently replaced by 200 µL OptiMEM® containing \( 10^{-7} \) M peptide. Stimulation was carried out for 1 h and nuclei visualisation was achieved by addition of 1 µL Hoechst 33342 (Sigma-Aldrich, 0.5 mg mL\(^{-1}\)) 30 min before image recording. Subsequently, cells were washed twice with OptiMEM® to remove non-internalised conjugates. Images were taken directly after washing while cells were maintained in OptiMEM®, using an Axio Observer-Z1 microscope equipped with an ApoTome Imaging System and a Heating Insert P Lab-Tek S1 unit (Zeiss, Oberkochen, Germany). Image processing was performed with AxioVision 3.1.

Conflicts of interest

There are no conflicts to declare.

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