closo-Borane Conjugated Regulatory Peptides Retain High Biological Affinity: Synthesis of *closo*-Borane Conjugated Tyr^3-Octreotate Derivatives for BNCT

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Despite the improvements in cancer therapy during the past years, high-grade gliomas and many other types of cancer are still extremely resistant to current forms of therapy. Boron neutron capture therapy (BNCT) provides a promising way to destroy cancer cells without damaging healthy tissue. However, BNCT in practice is still limited due to the lack of boron-containing compounds that selectively deliver boron to cancer cells. Since many neuroendocrine tumors show an overexpression of the somatostatin receptor, it was our aim to synthesize compounds that contain a large number of boron atoms and still show high affinity toward this transmembrane receptor. The synthetic peptide Tyr^3-octreotate (TATE) was chosen as a high-affinity and internalizing tumor targeting vector (TTV). Novel boron cluster compounds, containing 10 or 20 boron atoms, were coupled to the N-terminus of TATE. The obtained affinity data demonstrate that the use of a spacer between TATE and the *closo*-borane moiety is the option to avoid a loss of biological affinity of *closo*-borane conjugated TATE. For the first time, it was shown that *closo*-borane conjugated regulatory peptides retain high biological affinity and selectivity toward their transmembrane tumor receptors. The results obtained and the improvement of spacer and boron building block chemistry may stimulate new directions for BNCT.

INTRODUCTION

In 1932, Chadwick (*1*) discovered the neutron (Nobel Prize in Physics in 1935); four years later, Locher (*2*) proposed boron neutron capture therapy (BNCT) as a potential strategy to treat cancer. It is based on the high cross section of ^{10}B (3838 barn) with thermal neutrons and the resulting nuclear processes. This reaction generates two charged particles, an α-particle and a ^{7}Li nucleus, having a range of about 10 μm, which is approximately one cell diameter. If ^{10}B could be accumulated selectively in tumor tissue, which subsequently could be irradiated with thermal neutrons, the nuclear reaction products would specifically damage tumor DNA due to their high linear energy transfer and would finally destroy tumor tissue selectively (cf. Figure 1).

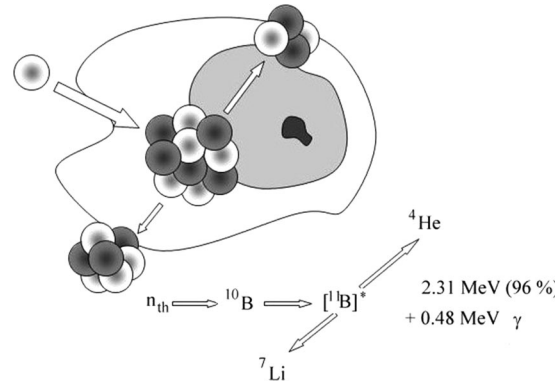

Figure 1. Simplified scheme of the BNCT reaction inside a cell.

However, there are adequate nontoxic and high-affinity boron-containing tumor targeting vectors (TTV) required, which deliver the necessary amount of boron to tumor cells (*3*). BPA (*p*-borophenylalanine) and BSH (sodium borocaptate), the only BNCT agents approved clinically, have been shown in many cases to exhibit insufficient selectivity and efficiency for BNCT.

In order to design better boron-containing candidates for BNCT, different targeting approaches have been described utilizing various properties of proliferating tumor cells, such as boronated nucleosides and nucleotides (*4, 5*), sugars (*6*), amino acids (*7*−*10*), and cytostatic target components (*11*).

Many neuroendocrine tumors overexpress somatostatin receptors (sst) that represent the molecular basis for the use of radiolabeled somatostatin analogues as tools for diagnostic and therapeutic applications in oncology (*12*). Due to the short plasma stability of the natural peptides somatostatin 14 (SRIF-14) and somatostatin 28 (SRIF-28) of approximately three minutes (*13*), they cannot be used directly as targeting vectors for these transmembrane receptors. The syntheses of octreotides—synthetic octapeptides containing the pharmacophoric part of somatostatin and threoninol at the C-terminus—provided derivatives with extended half-lives while even further increasing the affinity toward the sst. More recently, octreotate derivatives bearing a threonine instead of a threoninol moiety at the C-terminus have been investigated, such as D-Phe^1-c[Cys^2-Tyr^3-D-Trp^4-Lys^5-Thr^6-Cys^7]-Thr^8-OH (TATE). Compared to octreotide analogues, they show higher binding affinity and internalization rate (*14*−*16*).

Consequently, it was the concept of the present work to synthesize TATE based compounds that carry one, two, or more *closo*-borane units, i.e., a large number of boron atoms. Octreotate was selected as TTV instead of octreotide because its high internalization rate would allow an increased "shuttle"-type delivery of the boronated TTV. In this case, the cellular uptake is not necessarily limited by the initial number of

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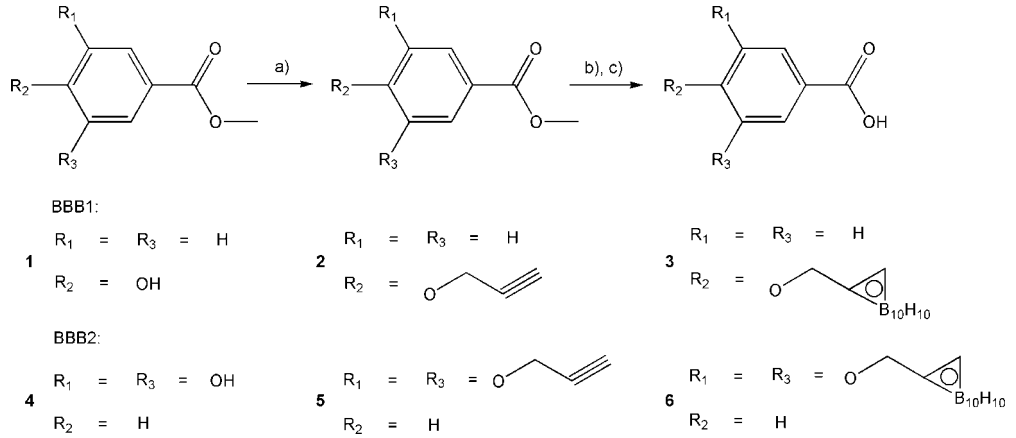
Scheme 1. Synthesis of Mono- and Di-*closo*-Borane Building Blocks BBB1 and BBB2[a]



[a] (a) 3-bromopropyne/K_2CO_3, acetone; (b) 1. decaborane, MeCN, 2. methyl prop-2-ynyloxybenzoate, toluene; (c) LiOH, THF/H_2O.

somatostatin receptors expressed at the tumor cell membrane. Syntheses of other octreotate derivatives, coupled with borane clusters, have recently been published; however, affinity studies were not performed (*17*). Prior results on our new compounds with one and two *closo*-boranes directly attached to the N-terminus of the TTV, i.e., TATE-5,6-dicarba-*closo*-dodecaboranyl hexynoic acid (*18*) and TATE-4-amino-bis-(propane-2,3-dicarba-*closo*-dodecaboranyl)-butynoic acid, showed 16 nM and >1000 nM binding affinity to sst_2, respectively. These initial results indicated for the first time that *closo*-borane conjugation to TATE may not annihilate the biological affinity of the peptide, if appropriate coupling chemistry between the borane and the peptide compartments is applied. In contrast, an inadequate design of conjugation may yield new *closo*-borane containing peptides, which are, however, not useful because of a dramatic loss of affinity. This led to the intention of inventing a conjugation chemistry that offers the possibility to obtain a variety of peptide conjugates within a modular system, useful both for octreotides or for any other peptidic TTV. We report the syntheses and characterization of a series of novel *closo*-borane conjugated octreotate derivatives as potential compounds for BNCT. We demonstrate that the introduction of spacers between *closo*-borane cluster(s) and peptide increases receptor binding affinities and leads to nanomolar IC_{50} values for the most relevant subtype sst_2.

EXPERIMENTAL PROCEDURES

Materials and Instrumentation. Wang (*p*-benzyloxybenzyl alcohol) resin, Fmoc, and other amino acids were supplied by Novabiochem (Darmstadt, Germany). All other starting materials and (dehydrated) solvents were purchased from Acros Organics (Geel, Belgium), VWR (Darmstadt, Germany), Fluka, and Sigma-Aldrich (Seelze, Germany) and were used without further purification. Decaborane was purchased from Katchem (Prague, Czech Republic). The silica gel SiO_2 (0.060−0.200 mm) used for column chromatography was purchased from Acros Organics. Thin-layer chromatography was performed using silica gel 60 F_{254} aluminum backed TLC plates, purchased from VWR.

The NMR spectra were taken on a 300 MHz FT-NMR spectrometer, AC 300 (Bruker Analytik GmbH), operating at 300 MHz for ^1H and 75.5 MHz for ^{13}C. ^{11}B-spectra were taken on a 400 MHz FT-NMR spectrometer, DIX 400 (Bruker Analytik GmbH), operating at 128.4 MHz.

The electron spray ionization mass spectra (ESI-MS) were recorded using a QTOF Ultima 3 system (Micromass/Waters). FD mass spectra were recorded using a MAT90 spectrometer (Finnigan). The matrix-assisted laser desorption-ionization time-of-flight mass spectra (MALDI-TOF-MS) were measured on an Axima-CFR (Shimadzu) spectrometer.

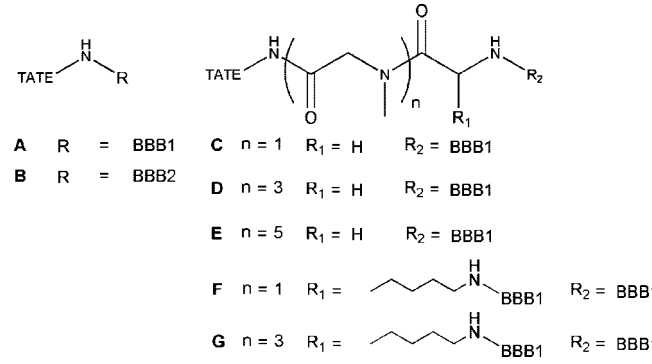


Figure 2. Overview on synthesized compounds.

Purification of the peptides was performed on a semipreparative Dionex HPLC system (P680 HPLC pump) equipped with a UV detector (UVD 170U) using a LiChroCART250 10 HPLC column LiChrosorbRP-select B (10 μ, 250 × 10 mm). The peptides were eluted applying various gradients of 0.1% TFA in 95% H_2O and 5% ACN (solvent A) and 0.1% TFA in 10% H_2O and 90% ACN (solvent B) at different flow rates. All solvent gradient programs are provided in Supporting Information. Effluent was monitored by detection of its optical absorption at 254 and 280 nm.

The synthetic chemistry was divided into three parts: the syntheses of boronated building blocks (BBB) which carry the necessary boron amount, the synthesis of the TTV using solid-phase chemistry including the conjugation with spacer units, and the coupling of various BBBs to the synthesized peptides.

***closo*-Borane Building Block Chemistry.** A hydroxybenzoate backbone was used as a linker molecule, which was conjugated with a triple bond. The introduction of a *closo*-borane cluster was achieved by coupling of decaborane to the triple bond (*19*). Due to promising experiences on the reaction of decaborane-acetonitrile adducts and triple bonds, this synthetic method was chosen for all BBBs. Two compounds, namely, *p*-(*O*-methylencarboranyl)-benzoic acid (BBB1) and 3,5-bis-(*O*-methylencarboranyl)-benzoic acid (BBB2), were synthesized starting from methyl 4-hydroxybenzoate or methyl 3,5-dihydroxybenzoate (cf. Scheme 1). The first step was the introduction of the essential triple bond via nucleophilic substitution with 3-bromopropyne, followed by the coupling with *closo*-borane cluster(s) in up to 70% yield (*20*). The use of equimolar amounts of LiOH in THF/H_2O gave the desired pure boronated benzoic

Table 1. Binding Affinities of the *closo*-Borane Conjugated Octreotates to the hsst Subtypes Expressed As IC_{50} in nM (Mean ± SEM; $n \geq 2$) with SRIF-28 as Reference

compound	boron atoms	$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$
SRIF-28	0	2.3 ± 0.2	2.6 ± 0.3	3.9 ± 0.3	4.0 ± 1.1	3.0 ± 0.3
A	10	>1000	19 ± 1.8	>1000	172 ± 3	975 ± 182
B	20	>1000	>1000	>1000	>1000	>1000
C	10	>1000	2.6 ± 0.2	>1000	82 ± 24	148 ± 30
D	10	>1000	2.6 ± 0.7	62 ± 9.3	33 ± 11	12 ± 2.4
E	10	>1000	1.8 ± 0.3	22 ± 1.2	8.6 ± 2	7.6 ± 1.2
F	20	>1000	415 ± 53	>1000	>1000	>1000
G	20	>1000	20 ± 5.8	89 ± 13	258 ± 33	146 ± 22

acids in yields of up to 92%. The overall yield of this three-step synthesis was up to 43%.

*Methyl-4-prop-2-ynyloxy-benzoate (***1***).* To a solution of methyl 4-hydroxybenzoate (9.12 g, 60 mmol) in acetone (100 mL), K_2CO_3 (11.58 g, 84 mmol) was added and the solution stirred for 30 min at room temperature under argon atmosphere. Propargylbromide (80% in toluene, 12.9 mL, 120 mmol) was added and the reaction mixture kept at 80 °C for 3 h. After cooling to room temperature, most of the solvent was removed in vacuo, and the residue was taken up in water (100 mL) and extracted with ethyl acetate (3 × 70 mL). The combined organic phases were successively washed with 10% NaOH, H_2O, and brine (3 × 75 mL) and dried over Na_2SO_4. The solvent was removed in vacuo and the pure product was obtained as pale yellow crystals (9.70 g, 85%). R_f = 0.75 (petrol ether/ethyl acetate, 2/1). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ: 7.99 (dd, ^3J(H,H) = 8.7 Hz, ^5J(H,H) = 1.2 Hz, 2 H, ArH), 6.98 (dd, ^3J(H,H) = 8.7 Hz, ^5J(H,H) = 1.2 Hz, 2 H, ArH), 4.73 (d, ^4J(H,H) = 2.4 Hz, 2 H, CH_2), 3.87 (s, 3 H, CH_3), 2.53 (tr, ^4J = 2,4 Hz, 1 H, C≡CH). ^{13}C NMR (75.5 MHz, $CDCl_3$, 298 K) δ: 165.90, 161.11, 131.25, 122.64, 114.95, 78.83, 78.75, 55.80, 51.97. MS (FD) *m/z*: calcd for $C_{11}H_{10}O_3$, 190.2; found, 190.3 $(M + H)^+$. Anal. ($C_{11}H_{10}O_3$) C, H.

*Methyl-4-(O-methylencarboranyl)-benzoate (***2***).* Decaborane (2.57 g, 23 mmol) was dissolved in dry acetonitrile (40 mL) and refluxed for 30 min. A solution of compound **1** (3.12 g, 16.5 mmol) in toluene (33 mL) was added to the yellow solution and the mixture was heated to reflux for a further 20 h. Methanol (5 mL) was added carefully, and the resulting mixture was refluxed for 30 min and afterward cooled to room temperature. The solvent was removed in vacuo, the crude product was purified by column chromatography (petrol ether/ethyl acetate, 4/1), and the pure product was obtained as a colorless solid (3.11 g, 61%). R_f = 0.33 (petrol ether/ethyl acetate, 4/1). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ: 7.99 (dd, ^3J(H,H) = 8.7 Hz, ^5J(H,H) = 1.2 Hz, 2 H, ArH), 6.85 (dd, ^3J(H,H) = 8.7 Hz, ^5J(H,H) = 1.2 Hz, 2 H, ArH), 4.45 (s, 2 H, CH_2), 4.05 (s, 1 H, CH), 3.88 (s, 3 H, CH_3), 1.25−3.1 (bm, 10 H, $B_{10}H_{10}$). ^{13}C NMR (75.5 MHz, $CDCl_3$, 298 K) δ: 165.78, 160.87, 131.35, 123.25, 114.98, 73.29, 68.59, 62.00, 52.01. ^{11}B-NMR (128.4 MHz, $CDCl_3$, 298 K) δ: −2.67 (bs, 1B), −4.47 (bs, 1B), −9.03 (bs, 2B), −11.59 (bs), −13.01 (bs, 6B). MS (FD) *m/z*: calcd for $C_{11}H_{20}B_{10}O_3$, 308.4; found, 308.6 $(M + H)^+$. Anal. ($C_{11}H_{20}B_{10}O_3$) C, H.

*4-(O-Methylencarboranyl)-benzoic Acid (***3** = *BBB1).* LiOH (60 mg, 2.5 mmol) was added in 4 portions to a solution of compound **2** (300 mg, 0.97 mmol) in THF/H_2O (36 mL, 2/1). The mixture was stirred for 22 h at room temperature. H_2O (25 mL) was added and most of the THF was removed in vacuo. The aqueous phase was washed with Et_2O (2 × 30 mL), adjusted to pH 2 with 1 M HCl, and extracted with ethyl acetate (3 × 30 mL), and the combined organic phases were dried over Na_2SO_4. Removal of the solvent in vacuo yielded the crude product which was purified by column chromatography (petrol ether/ethyl acetate, 2/1 + 3% formic acid). The pure product was obtained as a colorless solid (0.243 g, 82%). R_f = 0.55 (petrol ether/ethyl acetate, 2/1 + 3% formic acid). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K) δ: 12.75 (s, 1 H, COOH), 7.89 (d, ^3J(H,H) = 9.0 Hz, 2 H, ArH), 7.07 (d, ^3J(H,H) = 9.0 Hz, 2 H, ArH), 5.34 (s, 1 H, CH), 4.69 (s, 2 H, CH_2), 1.25−3.1 (bm, 10 H, $B_{10}H_{10}$). ^{13}C NMR (75.5 MHz, DMSO-d_6, 298 K) δ: 166.89, 160.62, 131.47, 124.42, 114.79, 73.35, 68.60, 61.98. ^{11}B-NMR (128.4 MHz, DMSO-d_6, 298 K) δ: −2.60 (bs, 1B), −4.50 (bs, 1B), −9.12 (bs, 2B), −11.62 (bs), −13.12 (bs, 6B). MS (FD) *m/z*: calcd for $C_{10}H_{18}B_{10}O_3$, 294.4; found, 294.3 $(M + H)^+$. Anal. ($C_{10}H_{18}B_{10}O_3$) C, H.

*Methyl-3,5-bis(prop-2-ynyloxy)-benzoate (***4***).* To a solution of methyl 3,5-dihydroxybenzoate (1.68 g, 10 mmol) in acetone (15 mL), K_2CO_3 (3.86 g, 28 mmol) was added and the solution was stirred for 30 min at room temperature under argon atmosphere. Propargylbromide (80% in toluene, 4.3 mL, 40 mmol) was added and the reaction mixture kept at 80 °C for 3 h. After cooling to room temperature, most of the solvent was removed in vacuo. The residue was taken up in water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic phases were successively washed with 10% NaOH, H_2O, and brine (3 × 50 mL) and dried over Na_2SO_4. The solvent was removed in vacuo and the pure product was obtained as pale yellow crystals (2.28 g, 93%). R_f = 0.51 (petrol ether/ethyl acetate, 4/1). ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ: 7.26 (d, ^4J(H,H) = 2.7 Hz, 2 H, ArH), 6.78 (tr, ^4J(H,H) = 2.7 Hz, ArH), 4.68 (d, ^4J(H,H) = 2.1 Hz, 4 H, CH_2), 3.88 (s, 3 H, CH_3), 2.53 (tr, ^4J(H,H) = 2,1 Hz, 2 H, C≡CH). ^{13}C NMR (75.5 MHz, $CDCl_3$, 298 K) δ: 165.92, 158.82, 158.39, 131.68, 109.97, 107.12, 106.24, 73.45, 68.67, 61.95, 52.33. MS (FD) *m/z*: calcd for $C_{14}H_{12}O_4$, 244.2; found, 244.2 $(M + H)^+$. Anal. ($C_{14}H_{12}O_4$) C, H.

*Methyl-3,5-bis-(O-methylencarboranyl)-benzoate (***5***).* Decaborane (1.57 g, 14 mmol) was dissolved in dry acetonitrile (20 mL) and refluxed for 30 min. A solution of compound **4** (1.22 g, 5 mmol) in toluene (10 mL) was added to the yellow solution and the mixture was heated to reflux for a further 20 h. Methanol (2.5 mL) was added carefully and the resulting mixture was refluxed for 30 min and afterward cooled to room temperature. The solvent was removed in vacuo. The crude product was purified by column chromatography (petrol ether/ethyl acetate, 4/1) and the pure product was obtained as a colorless solid (0.957 g, 40%). R_f = 0.49 (petrol ether/ethyl acetate, 4/1). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K) δ: 7.17 (d, ^4J(H,H) = 1.8 Hz, 2 H, ArH), 6.96 (d, ^4J(H,H) = 1.8 Hz, 1 H, ArH), 5.35 (s, 2 H, CH), 4.67 (s, 4 H, CH_2), 3.84 (s, 3 H, CH_3), 0.8−3.05 (bm, 20 H, $B_{10}H_{10}$). ^{13}C NMR (75.5 MHz, DMSO-d_6, 298 K) δ: 165.49, 158.31, 131.92, 109.21, 106.86, 73.29, 68.84, 61.86, 52.53. ^{11}B-NMR (128.4 MHz, $CDCl_3$, 298 K) δ: −3.47 (bs, 3 B), −9.64 (bs, 6 B), −11.31 to −15.30 (bm, 11 B). MS (FD) *m/z*: calcd for $C_{14}H_{32}B_{20}O_4$, 480.6; found, 480.7 $(M + H)^+$. Anal. ($C_{14}H_{32}B_{20}O_4$) C, H: calcd, 34.99, 6.71; found, 35.66, 6.64.

*3,5-Bis-(O-methylencarboranyl)-benzoic Acid (***6** = *BBB2).* LiOH (38.3 mg, 1.6 mmol) was added in 4 portions to a solution of compound **5** (300 mg, 0.62 mmol) in THF/H_2O (36 mL, 2/1). The mixture was stirred for 22 h at room temperature and the

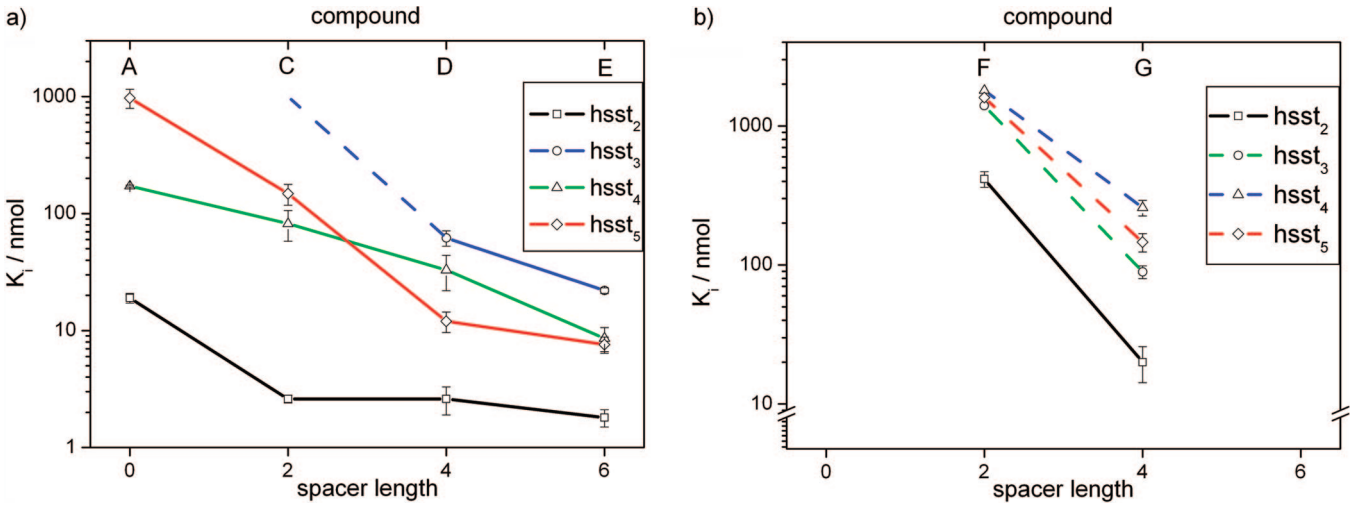


Figure 3. Structure−affinity correlation of (a) mono- and (b) di-*closo*-borane conjugated TATE-derivatives to $hsst_2$-$hsst_5$ subtypes.

solvent was removed in vacuo. Column chromatography (petrol ether/ethyl acetate, 2/1 + 3% formic acid) yielded the pure product as a colorless solid (0.215 g, 74%). $R_f = 0.43$ (petrol ether/ethyl acetate, 2/1 + 3% formic acid). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K) δ: 7.15 (d, ^4J(H,H) = 2.1 Hz, 2 H, ArH), 6.91 (d, ^4J(H,H) = 2.1 Hz, 1 H, ArH), 5.34 (s, 2 H, CH), 4.66 (s, 4 H, CH_2), 0.8−3.05 (bm, 20 H, $B_{10}H_{10}$). ^{13}C NMR (75.5 MHz, DMSO-d_6, 298 K) δ: 166.56, 158.24, 133.22, 109.30, 106.53, 73.30, 68.81, 61.89. ^{11}B-NMR (128.4 MHz, DMSO-d_6, 298 K) δ: −3.47 (bs, 3 B), −4.47 (bs, 1B), −9.64 (bs, 6B), −11.60 (bs), −15.37 (bm, 11 B). MS (FD) *m/z*: calcd for $C_{13}H_{30}O_4$, 466.6; found, 466.5 $(M + H)^+$. Anal. ($C_{13}H_{30}O_4$) C, H: calcd, 33.46, 6.48; found, 35.25, 6.48.

Peptide Synthesis. The octapeptide TATE was synthesized manually via solid-phase reaction on a Wang resin (loading capacity 0.59 mmol/g) using standard Fmoc-strategy, HOBt/HBTU as coupling reagents, and DMF as solvent. The following N-protected and side chain protected amino acids were used: Fmoc-Thr(tBu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Lys(Boc)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-D-Phe-OH. Cyclization was achieved with Tl(III)$(CF_3COO)_3$ in DMF.

Different spacer units were coupled successively to the N-terminus of TATE on the solid phase using Fmoc-Sar-OH, Fmoc-Gly-OH, and Fmoc-Lys(Fmoc)-OH.

The following general procedures have been applied to all synthesized compounds unless otherwise noted. All methods are given for batches of 0.1 mmol. After swelling of the resin (*A1*), the Fmoc-group was removed by method *A2*. The amino acids were attached subsequently using coupling procedure *A3*. Cyclization was performed after the addition and Fmoc-deprotection of D-Phe^1 using procedure *A4*. Additional amino acids for the spacer were coupled successively to TATE on the solid phase.

Swelling of the Wang Resin (A1). The N-α-Fmoc-O-tBu-threonine Wang resin (168 mg, 0.1 mmol) was treated with DCM (2 mL) in a 5 mL syringe equipped with a frit and soaked for 3 h using a shaker and washed with DMF (6 × 1 mL).

Deprotecting Procedure (A2). The attached Fmoc-protected amino acids were treated three times successively: 1, 3, and 5 min with piperidine (20% in DMF, 1 mL). After each treatment, the resin was washed three times with DMF (1 mL).

Coupling Procedure (A3). The amino acid (4 equiv) and HOBt-hydrate (4 equiv) were dissolved in DMF (1 mL). HBTU (4 equiv) was added and the resulting solution was kept for 5 min in an ultrasonic bath. Finally, DIPEA (8 equiv) was added to the clear solution. The reaction mixture was vortexed, centrifuged, incubated for 1 min, added to the resin, and shaken for 30 min. After complete reaction, the reaction mixture was removed and the resin was washed with DMF (6 × 1 mL).

Cyclization (A4). Thallium(III)-trifluoroacetate (400 μL, 0.4 M in DMF) was added to the resin and the mixture kept for 1 h on the shaker and washed afterward successively with DMF, CCl_4, DCM, and MeOH.

Cleavage from the Resin (A5). The resin was suspended with the cleavage solution (100 μL/mg resin; 95% TFA, 2.5% H_2O, 2.5% TIS (triisopropylsilane)) and kept for 1.5 h at room temperature. Afterward the mixture was filtered over a glass frit and the remaining solid was washed with TFA (4 mL). The filtrate was evaporated in vacuo to give the crude product.

Coupling. The peptide conjugate was coupled with the boronated linker molecule (4 equiv) using HOBt/HBTU (4 equiv) in DMF as coupling reagents on the solid phase. Reaction time was extended to 4 h. After coupling, the final compounds were cleaved from the resin using TFA/H_2O/TIS (*A5*). Purification and analysis was performed on a semipreparative HPLC system to give the final products as colorless solids. All final compounds were characterized by ESI mass spectrometry. Compounds **A**, **C**, **D**, and **E** were additionally characterized by MALDI-TOF mass spectrometry.

TATE-(BBB1)$_1$ (**A**). 4-(*O*-Methylencarboranyl)-benzoic acid (**3**) was attached to TATE on the solid phase using *A3* with 4 h reaction time. The product was cleaved using *A5*. The pure product was obtained by HPLC purification (23.7 mg, 36%). t_R = 11.6 min. MS (MALDI-TOF) *m/z*: calcd for $C_{59}H_{80}B_{10}N_{10}O_{14}S_2$, 1325.6; found, 1326.1 $(M + H)^+$, 1348.5 $(M + Na)^+$. MS (ESI) *m/z*: found, 1326.6 $(M + H)^+$, 1348.5 $(M + Na)^+$, 1364.5 $(M + K)^+$.

TATE-(BBB2)$_1$ (**B**). 3,5-Bis-(*O*-methylencarboranyl)-benzoic acid (**6**) was attached to TATE on the solid phase using *A3* with 4 h reaction time. The product was cleaved using *A5*. The pure product was obtained by HPLC purification (6.9 mg, 9%). t_R = 13.2 min. MS (ESI) *m/z*: calcd for $C_{62}H_{92}B_{20}N_{10}O_{15}S_2$, 1497.8; found, 1498.9 $(M + H)^+$, 1520.9 $(M + Na)^+$, 1536.9 $(M + K)^+$.

TATE-(sarcosine)$_n$-glycine-(BBB1)$_1$ (**C**, **D**, **E**). The single sarcosine units (4 equiv/unit) and the glycine unit (4 equiv) were attached stepwise to TATE using *A1*−*A3*, the reaction time/unit was 30 min. After attachment of glycine, 4 equiv of compound **3** (59 mg, 0.2 mmol) were coupled to the resulting TATE derivative using *A3* with 4 h reaction time. The products were cleaved by using *A5*.

TATE-(sarcosine)$_1$-glycine-(BBB1)$_1$ (**C**). The pure product was obtained by HPLC purification (30.4 mg, 21%). t_R = 11.5 min. MS (MALDI-TOF) *m/z*: calcd for $C_{64}H_{88}B_{10}N_{12}O_{16}S_2$,

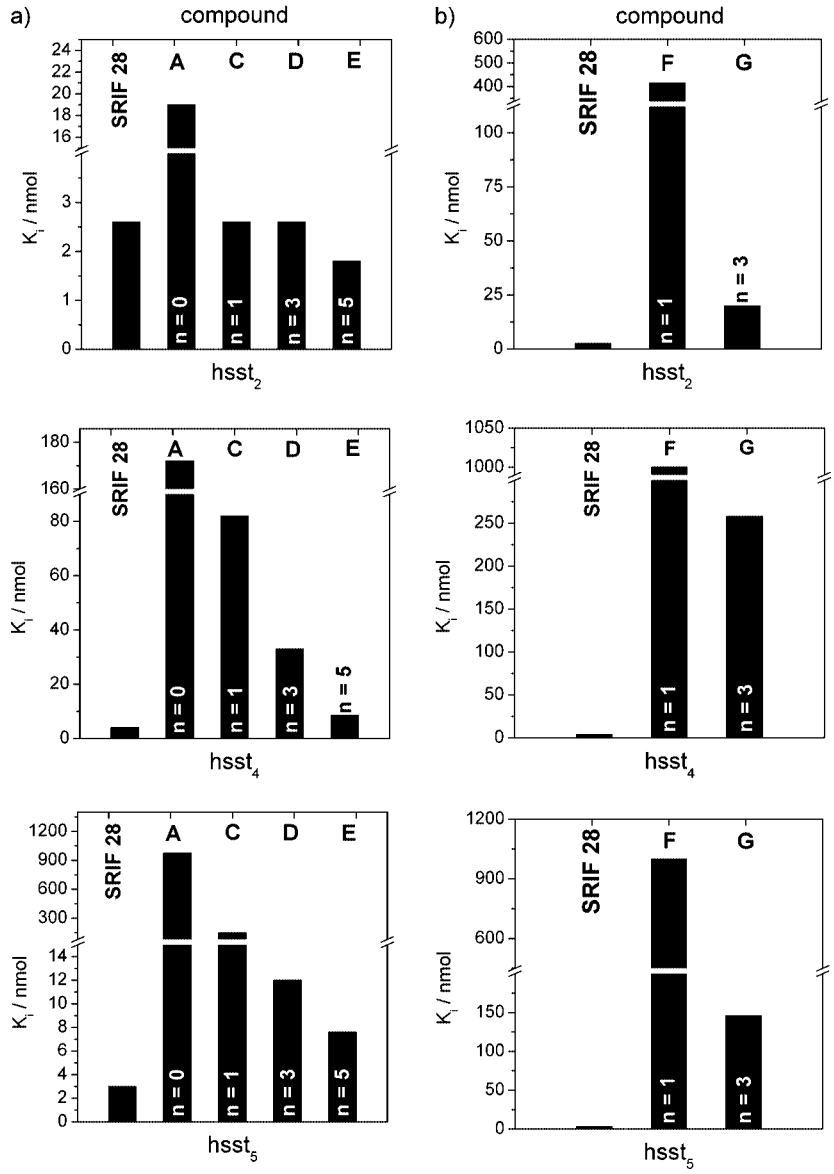


Figure 4. Structure−affinity correlation of compounds with (a) one unit of BBB1 and (b) two units of BBB1 toward $hsst_2$, $hsst_4$, and $hsst_5$.

1453.7; found, 1454.4 $(M + H)^+$, 1476.4 $(M + Na)^+$. MS (ESI) *m/z*: found, 1454.7 $(M + H)^+$, 1476.73 $(M + Na)^+$, 1492 $(M + K)^+$, 738.9 $(M + Na)^{2+}$, 746.9 $(M + K)^{2+}$.

TATE-(sarcosine)$_3$-glycine-(BBB1)$_1$ (**D**). The pure product was obtained by HPLC purification (17.5 mg, 22%). $t_R = 11.7$ min. MS (MALDI-TOF) *m/z*: calcd for $C_{70}H_{98}B_{10}N_{14}O_{18}S_2$, 1595.9; found, 1596.1 $(M + H)^+$, 1618.2 $(M + Na)^+$, 1634.2 $(M + K)^+$. MS (ESI) *m/z*: found, 1596.9 $(M + H)^+$, 1618.8 $(M + Na)^+$, 1634.8 $(M + K)^+$, 809.9 $(M + Na)^{2+}$, 817.9 $(M + K)^{2+}$.

TATE-(sarcosine)$_5$-glycine-(BBB1)$_1$ (**E**). The pure product was obtained by HPLC purification, (32.5 mg; 18%). $t_R = 11.7$ min. MS (MALDI-TOF) *m/z*: calcd for $C_{76}H_{108}B_{10}N_{16}O_{20}S_2$, 1738.0; found, 1739.4 $(M + H)^+$, 1761.4 $(M + Na)^+$, 1777.4 $(M + K)^+$. MS (ESI) *m/z*: found, 1738.9 $(M + H)^+$, 1760.9 $(M + Na)^+$, 1776.9 $(M + K)^+$, 880.4 $(M + Na)^{2+}$, 888.4 $(M + K)^{2+}$.

TATE-(sarcosine)$_n$-lysine-(BBB1)$_2$ (**F**, **G**). The single sarcosine units (4 equiv/unit) and the lysine unit (4 equiv) were attached stepwise to TATE using *A1*−*A3*, the reaction time/unit was 30 min. After attachment of lysine, compound **3** (4 equiv) was coupled to the resulting TATE derivative using *A3* with 4 h reaction time. The products were cleaved by using *A5*.

TATE-(sarcosine)$_1$-lysine-(BBB1)$_2$ (**F**). The pure product was obtained by HPLC purification (4.0 mg, 4%). $t_R = 13.6$ min. MS (ESI) *m/z*: calcd for $C_{78}H_{113}B_{20}N_{13}O_{18}S_2$, 1801.1; found, 1802.2 $(M + H)^+$, 1824.2 $(M + Na)^+$, 1840.1 $(M + K)^+$, 912.6 $(M + Na)^{2+}$.

TATE-(sarcosine)$_3$-lysine-(BBB1)$_2$ (**G**). The pure product was obtained by HPLC purification (2.7 mg, 3%). $t_R = 14.1$ min. MS (ESI) *m/z*: calcd for $C_{84}H_{123}B_{20}N_{15}O_{20}S_2$, 1943.3; found, 1943.2 $(M + H)^+$, 1966.2 $(M + Na)^+$, 1982.2 $(M + K)^+$, 983.6 $(M + Na)^{2+}$, 995.1 $(M + K)^{2+}$.

Binding Assay. All compounds were tested for their ability to bind to the five human sst receptor subtypes in complete displacement experiments using the universal SRIF radioligand [^{125}I]-[Leu^8, D-Trp^{22}, Tyr^{25}]-SRIF-28. CHO-K1 and CCL39 cells stably expressing the human sst_1−sst_5 receptors were grown as described previously (*14*). Cell membrane pellets were prepared and receptor autoradiography was done as described in detail previously (*14*). SRIF-28 was run in parallel as control. IC_{50} values were calculated after quantification of the data using

a computer-assisted image processing system and are given as a mean of 2 to 3 measurements.

RESULTS

***closo*-Borane Building Block Chemistry.** The first two steps of the reactions shown in Scheme 1 were accomplished in a fast and easy way and gave the desired compounds in high yields. In the final reaction, the methyl ester had to be cleaved. Here, a decomposition of the borane cluster under basic conditions was observed. Common ways of saponification of methyl esters, e.g., NaOH (0.005−5 N) as well as LiI/pyridine, were not successful. However, the use of equimolar amounts of LiOH in a mixture of THF and H_2O resulted in excellent yields. Therefore, this reaction scheme allows a practicable and sufficient synthesis of *closo*-borane conjugated linker molecules, containing 10 or 20 boron atoms.

Peptide Synthesis. The syntheses of the target compounds were performed on the solid phase, including cyclization, conjugation with spacer units, and coupling with linker molecules.

Coupling. In a first experiment, BBB1 was to be coupled to the TTV, which was conjugated with two, four, and six sarcosine units as spacer. In this trial, no coupling of the peptide conjugate and BBB1 was achieved. A possible explanation for this result is the importance of a primary amino group to couple the BBB. This led to the change of the spacer structure. Consequently, the final sarcosine unit was replaced by glycine to give conjugates with one, three, and five sarcosine units, followed by one glycine unit. The coupling of these compounds with the BBB was successful, which demonstrates the requirement of a primary amino function of the final amino acid. Alternatively, lysine was used as final amino acid to provide the possibility to connect two BBBs to one TTV by coupling a boron cluster to the N-terminus and to the ε-amino function in the side chain of lysine. Here, the final target compounds could be isolated as well. Figure 2 summarizes the derivatives synthesized.

Binding Assay. The data of the *in vitro* study prove that appropriate conjugation of TATE with a *closo*-borane compound provides derivatives still retaining biological affinity. Table 1 summarizes the results of the *in vitro* assays on the binding affinity of the derivatives to the five subtypes of the human somatostatin receptor. Furthermore, these data demonstrate a correlation between biological affinity and length of the inserted spacer.

There is almost any affinity if the boronated linker BBB1 or BBB2 is coupled to TATE directly (compound **A** and **B**). Except for sst_1 (there is no affinity toward the sst_1 subtype for almost all the octreotide and octreotate derivatives known in the literature), the affinities improve with an increase of spacer length. Similarly, the affinity increases by the use of spacer units even when two units of BBB1 are coupled to the same TATE molecule. However, a higher number of *closo*-borane clusters attached to the TTV has a negative influence on the affinity of the peptide. These findings are illustrated in Figure 3. Nevertheless, there are three compounds (**C**, **D**, **E**) that show equal or better affinity toward $hsst_2$, the most frequently overexpressed sst (*21*), than endogenous SRIF-28. Therefore, these three boronated compounds are extremely promising candidates for tumor targeting. The impact of the spacer is most obvious for $hsst_4$ for derivatives containing one or two units of BBB1.

DISCUSSION

We established a synthesis route directed to different *closo*-borane linker compounds in high yields, which contain 10 and 20 boron atoms per *closo*-borane building block. Saponification of the methyl esters could be performed by the use of LiOH in THF/water without decomposition of the borane clusters, which is essential for the synthesis of the *closo*-borane building blocks *p*-(*O*-methylencarboranyl)-benzoic acid (BBB1) and 3,5-bis-(*O*-methylencarboranyl)-benzoic acid (BBB2). These BBBs can directly be coupled to the N-terminus of TATE or to the N-terminus of other peptides. Furthermore, the BBB can be coupled to a primary amino function in the side chain of a peptide, e.g., to the ε-amino function of lysine.

Obviously, the introduction of a spacer is necessary to retain the high affinity of the peptidic targeting vector to the receptor. In the case of a directly coupled *closo*-borane building block to the TTV (e.g., compound **B**), the affinity to the somatostatin receptor may be lost completely. In contrast, various spacer units containing sarcosine, glycine, and lysine guarantee retaining affinity. This indicates a structure−affinity relation in a way that with increasing chain length of the spacer unit ($n = 1$, 3, 5 for compounds **C**, **D**, **E**) the affinities of the peptidic targeting vector to the transmembrane tumor receptor are being improved. These relationships of type affinity versus spacer length can be identified both for mono-*closo*-borane (Figure 3a) as well as di-*closo*-borane (Figure 3b) conjugated compounds for the four relevant human somatostatin receptor subtypes 2, 3, 4, and 5. In the case of the human sst_4 and sst_5 subtypes, the binding affinities of compounds **C**, **D**, and **E** successively improve by about 1 order of magnitude. This is graphically illustrated for $hsst_2$, $hsst_4$, and $hsst_5$ in Figure 4.

The affinity data demonstrate that the use of an appropriate spacer is the option to avoid a loss of biological affinity of *closo*-borane conjugated regulatory peptides. In fact, potentially useful affinities have been obtained for mono- and di-*closo*-borane linkers with 10 or 20 boron atoms per building block.

The use of amino acids with a primary amino group, such as glycine or lysine, is essential as final spacer units in the coupling step with the BBB. In the case of the N-methylated amino acid sarcosine, coupling was not successful.

The introduction of lysine as terminal spacer unit, in addition, allows the coupling of two BBBs to one TATE molecule. Even in the case of 20 boron atoms per TATE (cf. compound **G**), the binding affinities of the conjugates are still in the 20 nM range for sst_2. Continuing this concept, compounds with up to 60 boron atoms per peptide may be synthesized in the case of two tri-*closo*-borane building blocks. The approach of introducing one or two mono- or di-*closo*-borane building blocks into various candidates for BNCT by realizing the potential of appropriate spacer units may be directly converted to other relevant regulatory peptides.

There are many modified octreotide derivatives, such as ^{111}In-labeled DTPA-octreotide (OctreoScan), which is used as the gold standard for scintigraphic imaging of neuroendocrine tumors. This compound and alternative octreotide-based radioligands for PET/CT or radionuclide therapy, such as ^{68}Ga-DOTA-TOC and ^{90}Y-DOTA-TOC, show affinities to the somatostatin receptor in the ranges 2.5−22 nM for $hsst_2$ and 57−37 nM for $hsst_5$ (*14*). The best *closo*-borane conjugated TATE derivatives described in this study offer high affinities toward their transmembrane tumor receptor in the ranges 1.8−20 nM for $hsst_2$ and 7.6−146 nM for $hsst_5$. This justifies the assumption that these new *closo*-borane conjugated TATE derivatives could be potentially useful and may stimulate new directions for BNCT.

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Supporting Information Available: Solvent gradients for HPLC. This material is available free of charge via the Internet at http://pubs.acs.org.

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