Structural patterns enhancing the antibacterial activity of mettallacarborane-based antibiotics

Jakub Cebula 1, Krzysztof Fink 1, Waldemar Goldeman 2, Bożena Szermer-Olearnik 1, Anna Nasulewicz-Goldeman 3, Mateusz Psurski 3, Monika Cuprych 3, Anna Kędziora 4, Bartłomiej Dudek 4, Gabriela Bugla-Płoskońska 4, Tomasz M. Goszczyński 1

1. Laboratory of Biomedical Chemistry, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences
2. Department of Organic and Medicinal Chemistry, Faculty of Chemistry, Wrocław University of Science and Technology
3. Laboratory of Experimental Anticancer Therapy, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Science
4. Department of Microbiology, Faculty of Biological Sciences, University of Wrocław

* Corresponding author: Tomasz M. Goszczyński, goszczynski@hirszfeld.pl

Abstract

Each year, the number of infections caused by multidrug-resistant bacteria is increasing, making new public health crises likely in the future. To prevent this, novel drugs capable of overcoming current resistances are needed. Boron cluster-based antibiotics offer a valuable possibility to create a new class of antibiotics and expand antibiotic’s chemical space beyond conventional carbon-based molecules. In this work, we identified the two most promising structural patterns providing cobalta bis(dicabrollide)(COSAN)-based compounds with potent and selective activity toward Staphylococcus aureus (including clinical strains): introduction of the α-amino acid amide to the molecule and the addition of iodine directly to the metallacarborane cage. Furthermore, we found that proper hydrophobic-hydrophilic balance is crucial for the selective activity of the tested compounds toward S. aureus over mammalian cells. The patterns proposed in this paper can be useful in the development of metallacarborane-based antibiotics with potent antibacterial properties and low cytotoxicity.

Keywords: metallacarboranes; boron clusters, COSAN, Staphylococcus aureus, antibiotics, antibacterial activity

Introduction

Antibiotics are among the most important groups of drugs and are essential to the modern healthcare system. Initially, most of the antibiotics were of natural origin and were obtained from microorganisms. 1 With the spread of antibiotic resistance among bacteria, the source of new antibacterial drugs has shifted to the screening of small-molecule libraries and medicinal chemistry methods to modify the existing lead structures. However, in recent years, we have observed decreasing effectiveness of these approaches with the drop in new antibiotics both in the pipeline and entering the market each year. Although iterative enhancements of already existing antibiotics have many benefits (e.g., smaller risk of toxicity, already defined molecular target), they are less likely to
deliver a compound that will be able to help with bacterial resistance in the long run.\textsuperscript{1} Thus, it is crucial to expand the chemical space of antibiotic development beyond the biofamiliar, carbon-based area. Compounds based on inorganic, abiotic pharmacophores may be a rich source of new antibiotics.

A promising source of new pharmacophores for antimicrobial therapy are boron clusters.\textsuperscript{2,3} Courtesy of boron self-catentation, boron clusters constitute a large family of abiotic, polyhedral boron hydrides and their heteroatom derivatives.\textsuperscript{4-6} This structural diversity can be further combined with the ease of modification of boron clusters with organic substituents, resulting in an almost infinite number of derivatives. The properties of boron clusters such as delocalized charge and hydridic hydrogens\textsuperscript{7,8}, as well as the arrangement of organic substituents on a 3D scaffold, result in different modes of interactions of boron cluster derivatives with biomolecules including proteins\textsuperscript{9-15} and lipid membranes\textsuperscript{16-20}. Additionally, no enzymatic system capable of boron cluster degradation has been identified as of yet.\textsuperscript{19-22} Thus, boron clusters provide both tremendous structural diversity and foreignness to living organisms which should help to fight off bacterial resistance\textsuperscript{5}.

Derivatives of metallacarborane cobalta bis(dicarbollide) (1, COSAN, [3,3'-Co-(C\textsubscript{2}B\textsubscript{9}H\textsubscript{11})\textsubscript{2}]),\textsuperscript{23,24} a member of the boron cluster family, show promising antimicrobial activities.\textsuperscript{2} The derivatives of 1 modified with simple organic compounds attached through ether linker were active against Gram-positive bacteria: \textit{Staphylococcus aureus}, methicillin resistant \textit{S. aureus} (MRSA), \textit{Enterococcus faecalis} and \textit{Streptococcus pyogenes}; Gram-negative bacteria: \textit{Pseudomonas aeruginosa} and \textit{Escherichia coli}; fungi: \textit{Candida albicans} and \textit{Trichosporon cutaneum}.\textsuperscript{25-27} Additionally, primary and secondary amino derivatives of 1 showed activity against Gram-positive bacteria and fungi, with the remaining tertiary and quaternary ammonium derivatives inactive.\textsuperscript{27,28} Finally, an iodoalkoxy derivative of 1 – [8-O-CH\textsubscript{2}CH\textsubscript{3}-8'I-3,3'-Co(1,2-C\textsubscript{2}B\textsubscript{9}H\textsubscript{10})] showed high activity against MRSA and ability to maintain its activity even while tested for resistance generation.\textsuperscript{29} The abovementioned pioneering studies on derivatives of 1 showed the high potential of metallacarborane-based antibiotics. Therefore, it is important to expand the number of tested derivatives of 1 and identify structural patterns improving the antibacterial properties of those derivatives.

Herein, our goal was to gain more insight into the relationship between the structures of derivatives of 1 and their antibacterial activity and host cytotoxicity. For the preparation of derivatives of 1, a versatile nucleophilic ring opening reaction of oxonium salts of 1 and 2 (I-COSAN, [8-I-3,3'-Co(1,2-C\textsubscript{2}B\textsubscript{9}H\textsubscript{10})(1',2'-C\textsubscript{2}B\textsubscript{9}H\textsubscript{11})]) was used\textsuperscript{30-32}. We studied modifications directly at the metallacarborane cage and in organic parts attached to the cluster via the –(O(CH\textsubscript{2}CH\textsubscript{3})OCH\textsubscript{2}CH\textsubscript{3})– linker. The first set consists of compounds derived from the oxonium salt of 1 and the following nucleophiles: N-heterocycles, aromatic and aliphatic amines, and an \alpha-amino acid amide – L-lysine amide. This set was supposed to
identify the relationship between organic substituents and the biological activity of corresponding derivatives. The second set consists of derivatives of 1 iodinated at the 8-position with attached aliphatic amines and α-amino acid amides. These compounds were synthesised to determine if iodination of 1 in the 8-position could increase the activity of the derivatives and to indicate which parts of the most active molecule are crucial towards its biological activity. Antibacterial activity was evaluated on Gram-positive *Staphylococcus aureus* ATCC 6538 and Gram-negative *Pseudomonas aeruginosa* ATCC 27853. Both *S. aureus* and *P. aeruginosa* are also listed by the World Health Organization (WHO) as pathogens of critical and high concern due to their rising antibiotic resistance. For this reason, the best compounds were also evaluated on 6 multidrug-resistant clinical strains of *S. aureus*. The host cytotoxicity of the compounds was assessed in 5 eukaryotic cell lines: two normal (Balb/3T3, MCF10A) and 3 cancer (A549, MV4-11, UMUC3) cell lines.

**Results and Discussion**

The synthesis scheme and the scope of the synthesized compounds are outlined in [Scheme 1](#). This work is based on compounds prepared from oxonium salts of 1 and 2. The greatest advantage of using 1 and 2 derivatives as a starting point is their versatility, as they can react with various nucleophiles (e.g., O-, N-, S-, P-, C-, halogen nucleophiles), making it possible to obtain a broad spectrum of compounds. While there are many examples of compounds synthesised from oxonium salts of 1, only a few of them have been screened for antibacterial activities. Kvasničková et al. worked with a series of 8-O(CH$_2$)$_2$O(CH$_2$)$_2$-NH$_m$X$_n$ (X = Me, Et, m = 1,2,3, n = 3-m) derivatives. Compounds containing multiple boron clusters were studied by Romero et al. and Popova et al. While pioneering, those works did not identify any potential antimicrobial leads. This work is supposed to address this gap.

[Table 1](#) contains results obtained from initial screening of antibacterial and cytotoxic properties of all synthetized compounds (3-16) and parent unmodified clusters (1 and 2). Among the tested compounds, we identified several metallacarborane derivatives with good activity against *S. aureus*, while none showed activity against *P. aeruginosa*. Parent compounds – unmodified metallacarborane 1 and its iodinated derivative 2 – were ineffective against both tested bacterial strains and were mildly or noncytotoxic, depending on the tested cell line.
Structural screening started with the monoderivatives of 1 (compounds 3-10), where only one vertex is modified. The first pair of synthesised derivatives was prepared from aromatic N-heterocycles – pyridine and pyridine N-oxide. These two compounds differ only by the additional oxygen attached to the heterocycle through a dative bond in the pyridine N-oxide. However, such a minor change was enough to cause a significant difference in antibacterial activity. The N-oxide derivative 4 lacked any antibacterial activity, whereas the pyridine derivative 3 had high bacteriostatic activity against S. aureus ATCC 6538 (minimal inhibitory concentration, MIC = 3.91 µM; minimal bactericidal concentration, MBC > 1000 µM). Importantly, both 3 and 4 had similar, high toxicity to all tested cell lines. Thus, the addition of oxygen led to the loss of antibacterial activity but did not change the toxicity toward cell lines.

The next pair of compounds was obtained through reactions with aromatic amines: aniline (5) and 9-aminoacridine (6). Both derivatives were inactive toward the tested bacteria. The aniline derivative 5 was highly cytotoxic, while the 9-aminoacridine derivative 6 showed surprisingly low
cytotoxicity, despite being derived from fairly toxic 9-aminoarcridine \(^3\). Thus, arene-based organic substituents resulted in derivatives with no antibacterial activity but different toxicities.

Derivatives 7 and 8 contain hydrophobic moieties of different shapes: 7 has a bulky adamantyl-moiety, and 8 has a long, linear C8 aliphatic chain. Both derivatives show similar, moderate antibacterial activity against \(S. \text{aureus}\) (MIC = 7.81 – 15.63 \(\mu\)M) and high toxicity toward cell lines (IC\(_{50}\) values in the low micromolar range for all tested cell lines). The calculated therapeutic index (TI) values for both derivatives are below 1, which shows that these derivatives are more toxic toward mammalian cells than bacterial cells. Thus, attachment of hydrophobic moieties to the metallacarborane resulted in compounds that had moderate antibacterial activity but lacked selectivity over mammalian cells.

Derivative 9 is an expansion of 8, where the long n-hexyl chain is terminated with an amino group introducing a positive charge into the terminal part of the molecule. This modification increases antibacterial activity against \(S. \text{aureus}\) ATCC 27853 (MIC of 9 drops to 3.91 \(\mu\)M from MIC = 15.63 \(\mu\)M for 8) and decreases the cytotoxicity toward cell lines. As a result, the TI increased from 0.30 for 8 to 4.58 for 9. Thus, the addition of a cationic amino group increased both the activity against \(S. \text{aureus}\) of the derivative and its selectivity.

Encouraged by these results, we decided to synthesize a metallacarborane derivative with a proteinogenic, alkaline amino acid, L-lysine, which structurally resembles 9. To avoid an additional negative charge, apart from the COSAN, we decided to make the carboxyl group of L-lysine in the form of neutral amide 10. This modification decreases the activity of 10 against \(S. \text{aureus}\) (MIC = 7.81 \(\mu\)M) in comparison to 9, but the cytotoxicity toward cell lines decreases significantly, resulting in higher TI (TI = 6.90). Thus, the addition of the amide group slightly decreased the activity of 10 against \(S. \text{aureus}\) but improved its selectivity. Importantly, for all derivatives containing the cationic amino group (8, 9, and 10), MBC values equal MIC values, which suggests that these derivatives have a bactericidal mechanism of action.

Table 1. Antibacterial and cytotoxic properties of compounds 1 - 16. MIC - minimal inhibitory concentration, MBC - minimal bactericidal concentration, IC\(_{50}^{72h}\) - half maximal inhibitory concentration of proliferation, TI – therapeutic index, n.a. – not applicable. Van – vancomycin, Cef – ceftazidime, Mem – meropenem. MCF 10A – human mammary epithelial cell line, Balb/3T3 – mouse embryo fibroblast cell line, A549 – human lung carcinoma, MV-4-11 – human acute myeloid leukaemia cell line, UMUC-3 – human transitional bladder carcinoma cell line. Additional figures for the IC\(_{50}^{72h}\) data are available in the Supplementary Information in Figs. S1 to S16.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>IC(_{50}^{72h}) (95% CI) [(\mu)M]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td>ATCC 6538</td>
<td></td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>ATCC 27853</td>
<td></td>
</tr>
</tbody>
</table>
In the first series of metallacarborane derivatives presented above (3-10), we identified the L-Lys amide (derivative 10) motif as the best. Based on that, we synthesized an iodinated analogue of 10 – derivative 11 – to test whether the introduction of an iodine atom in the 8-position of the metallacarborane cage will improve the antibacterial activity. Indeed, compound 11 shows the highest activity against *S. aureus* ATCC 6538 (MIC = 0.98 µM) among the tested compounds, while its cytotoxicity remains low, similar to its noniodinated analogue 10, resulting in a therapeutic index of 56.5. This modification also closed the gap between 11 and both positive controls in terms of their antibacterial activity. Thus, iodination of the metallacarborane cage increased the activity and selectivity against *S. aureus* ATCC 6538.
Next, we wanted to test 2 different compounds that allow us to further identify crucial structural motifs of highly active and selective 11. They were derived from two amino acid amides: L-norleucine (L-Nle) 12 and glycine (Gly) 13. Nle lacks the ε-amine group of Lys, leaving only a hydrophobic, aliphatic side chain, whereas Gly consists of only an amide backbone. The L-Nle derivative 12 showed slightly lower but still high activity against S. aureus (MIC = 1.95 µM) and higher cytotoxicity in comparison to 11, causing the therapeutic index to decrease to 19.9. This observation is in agreement with the results obtained for compounds 7 and 8, suggesting that the attachment of hydrophobic substituents results in good antibacterial activity of the derivatives but poor selectivity over mammalian cells. Gly derivative 13 had the same activity against S. aureus as 11 (MIC = 0.98 µM) and slightly higher toxicity, resulting in a therapeutic index of 49.4. This result suggests that a positively charged side chain is not crucial for either the high antibacterial activity or selectivity of metallacarborane derivatives. Instead, the amide backbone seems to be the necessary structural pattern for highly active metallacarborane derivatives. Furthermore, these results imply that for high selectivity of metallacarborane derivatives, proper hydrophobic-hydrophilic balance is needed. Additional data gathered from the haemolysis assay further support this statement (Table 2). Compounds 11 and 13, which are more balanced in terms of hydrophobicity/hydrophilicity, were less cytotoxic toward red blood cells than 12, which has a hydrophobic side chain.

Table 2. Haemolysis assay results conducted for compounds 11-13. EC<sub>50</sub> - half maximum effective concentration. Additional figures for the EC<sub>50</sub> data are available in Supplementary Information on Fig. S17.

<table>
<thead>
<tr>
<th>Compound</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM) (95% CI)</td>
<td>71.4 (68.2-74.5)</td>
<td>38.4 (36.4-40.5)</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

To evaluate whether the beneficial effect of the addition of iodine in the 8-position of the metallacarborane cage can be observed for metallacarborane derivatives other than the 10/11 compound pair, compounds 14-16 were synthesised. These compounds are complementary to the metallacarborane derivatives containing secondary, tertiary, and quaternary amines already published by Kvasničková et al. 27. The only difference that distinguishes 14-16 from previously published compounds is the introduction of iodine. The noniodinated derivatives were inactive against S. aureus DBM 3178 and two strains of P. aeruginosa, NRRL B-59188 and DBM 3081, at the tested concentrations (minimum inhibitory concentrations that inhibit 80% of growth, MIC<sub>80</sub> ≥ 90 mg/L). However, iodinated secondary- and tertiary-amine derivatives – compounds 14 and 15, respectively – showed high activity
against *S. aureus* ATCC 6538 with MIC values of 1.9 µM for 14 and 7.8 µM for 15. Thus, the addition of iodine in the 8-position to amine oxonium salt derivatives of 1 activated these compounds against *S. aureus*. However, further research should be conducted to verify whether this effect can be observed in other strains of *S. aureus*, especially *S. aureus* DBM 3178, which was used in the original work.

Finally, we wanted to check how three of the most selective and active compounds (11-13) perform against clinical isolates of *S. aureus*. Strains used in the last rounds of tests are resistant to antibiotics of multiple classes, including clinically important methicillin and vancomycin (Table S1, Supplementary Information). Multidrug-resistant strains usually possess multiple resistance mechanisms \(^36\). It was hypothesised that metallacarborane-based antibiotics could overcome resistances due to their unique structure and abiotic nature \(^5\). The results presented in Table 3 clearly show that our compounds maintain some of their antibacterial activity when tested on multidrug-resistant *S. aureus* strains. All tested compounds preserved activity against *S. aureus* 2, which is the only strain susceptible to vancomycin. Compound 11 maintains activity against all remaining strains, but it is significantly lower than that against *S. aureus* ATCC 6538 and *S. aureus* 2, decreasing its selectivity towards bacteria (Table 1 and Table 3).

Table 3. Antibacterial activity of compounds 11-13 tested against multidrug-resistant *S. aureus*. Resistance profiles of *S. aureus* 2 and *S. aureus* N1-N5 strains are available in Table S1.

<table>
<thead>
<tr>
<th></th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
</tr>
<tr>
<td></td>
<td>[µM]</td>
<td>[µM]</td>
<td>[µM]</td>
</tr>
<tr>
<td><em>S. aureus</em> 2</td>
<td>1.95</td>
<td>3.9</td>
<td>1.95</td>
</tr>
<tr>
<td><em>S. aureus</em> N1</td>
<td>62.5</td>
<td>62.5</td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>S. aureus</em> N2</td>
<td>62.5</td>
<td>62.5</td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>S. aureus</em> N3</td>
<td>62.5</td>
<td>62.5</td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>S. aureus</em> N4</td>
<td>62.5</td>
<td>62.5</td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>S. aureus</em> N5</td>
<td>125</td>
<td>125</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

Since compound 11 was identified as the best antibacterial compound, we performed a kill time assay at two concentrations corresponding to the MIC and MBC values (Figure 1). The kill time kinetics of compound 11 was fairly quick, as it was capable of reducing colony forming units (CFU) by over 99.9% in 3 hours (using MBC concentration) and by 100% by 24 hours. At lower concentrations (MIC value), compound 11 was also capable of killing bacteria but was slower, as it reached an MBC threshold of 99.8% only after 24 h of incubation. Nevertheless, these data show that the mode of action of compound 11 is bactericidal.
Figure 1. Kill time assay for compound 11 at concentrations of 0.9 and 1.9 µM conducted against the S. aureus ATCC 6538 strain. The dotted line depicts the minimal bacterial concentration (MBC) threshold (99.9% of initial CFU killed). The results are statistically significant (P < 0.0001, repeated measurements two-way ANOVA).

All synthesised compounds were also studied as potential cytostatic drugs, as metallacarborane derivatives can exhibit exceptionally high cytotoxic properties. For this purpose, all compounds were tested on three cancer cell lines: leukaemia MV-4-11, lung cancer A549, and bladder cancer UMUC3 cell lines (Table 1). Most of the derivatives lacked any selectivity toward cancer cell lines over non-malignant cell lines, with two exceptions: compounds 11 and 16. Compounds 11 and 16 showed selective activity against the MV-4-11-cell line (IC\textsubscript{50} = 2.09 µM and 4.58 µM, respectively) over non-malignant human MCF 10A (IC\textsubscript{50} = 55.2 µM and >100 µM, respectively) and mouse Balb/3T3 (IC\textsubscript{50} = 28.9 µM and 34.8 µM, respectively) cell lines. Thus, compounds 11 and 16 are over 26 and 22 (for the calculation IC\textsubscript{50} = 100 µM was assumed for compound 16) times more cytotoxic toward MV-4-11 than to MCF 10A, respectively. Derivative 11 has already been identified in our studies as the best antibacterial agent. However, compound 16 – the quaternary-amine derivative – was completely inactive against the tested bacterial strains. These results show the potential of metallacarborane derivatives as anticancer agents, especially for leukaemia.

Conclusions

Metallacarboranes and other boron clusters constitute attractive pharmacophores for new antibiotics. Since boron clusters are novel additions to the antibiotic pharmacophore list, it is important...
to identify structural rules that govern their biological activities. Herein, we identified several structural patterns for metallacarborane derivatives that improve their antibacterial activity against *S. aureus* ATCC 6538 and selectivity over mammalian cells. We showed that a proper hydrophobic-hydrophilic balance of organic substituents is crucial for the selective activity of metallacarborane derivatives against bacteria. Among the tested organic substituents, modification with α-amino acid amides results in derivatives with high antibacterial activity and selectivity. Furthermore, iodination of the metallacarborane cage enhances or even in some cases induces antibacterial activity. Compound 11, which consists of an iodinated metallacarborane cage and L-Lys amide, showed high bactericidal activity against *S. aureus* ATCC 6538, with fast killing kinetics and low toxicity against non-malignant mammalian cell lines and erythrocytes. This compound was also active against multidrug-resistant isolates of *S. aureus*. We believe that the dependencies identified in this work will be useful for the future development of metallacarborane-based antibiotics.

**Experimental Section**

**Materials**

Cs[1] was purchased from Katchem spol. s r. o (Prague, Czech Republic), Nε-Boc-L-lysine amide hydrochloride was purchased from Fluorochem Ltd. (Glossop, United Kingdom), and pyridine was purchased from Acros Organics (Geel, Belgium). Norleucine amide was prepared using a literature procedure39. The remaining compounds used for the synthesis and chemical characterization of the compounds were purchased from Merck (Merck Sp. z o.o., Poznan, Poland). All reagents were used without further purification.

**LC-MS Measurements**

HPLC analyses were carried out using the Ultimate 3000 RS HPLC system (Dionex, Sunnyvale, CA, USA) equipped with a DAD detector. The purity of the compounds was assessed at 280 nm using full gradient separation. Column: Hypersil Gold 50×2.1 (Thermo Scientific, s/n 0110796A6); mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in CH₃CN; gradient: Time/% of B: 0/5, 1/5, 16/95, 20/95; flow rate: 0.5 mL/min; injection 1µL (0.4 mM in CH₃CN). Purity of all compounds that was determined by HPLC was better than 95%. The UV-VIS spectra were recorded using an acetonitrile-water mixture (55:45 v/v for compound 10, 60:40 v/v for compound 9, 70:30 v/v for compounds remaining compounds) containing 0.1% formic acid. High-resolution mass spectrometry experiments were carried out on a MicrOTOF-Q II spectrometer (Bruker Daltonic, Bremen, Germany) equipped with an electrospray ion source. The instrument was operated in the negative-ion mode and calibrated with a sodium formate solution (10 mM).
NMR spectroscopy

The NMR spectra were recorded on a 400 MHz Jeol ECZ 400S (1H, 13C{1H} and 11B{1H} NMR) or 600 MHz Bruker Avance (1H and 13C{1H} NMR) spectrometer in acetone-d$_6$ (Merck cat. No. 151793) or DMSO-d$_6$ (Merck cat. No. 441392) as solvent in Wilmad® quartz NMR tubes (Merck cat. No. Z562262). In selected cases, DEPT135, COSY and HMQC/HSQC spectra were also measured. Chemical shifts (δ) were expressed in parts per million (ppm), while multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sext = sextet, hept = heptet, nonet = nonet, m = multiplet (complex pattern), br = broad. For clarity, the set of broad multiplets of B-H protons at ca. 1-4 ppm has been omitted in the 1H NMR description.

Synthesis of substrates

Substrate A, [8-0(CH$_2$CH$_2$)$_2$O-3,3'-Co(1,2-C$_2$B$_9$H$_{10}$)(1',2'-C$_2$B$_9$H$_{11}$)], and Substrate B, [8,8'- µ-1,3,3'-Co(1,2-C$_2$B$_9$H$_{10}$)$_2$], were synthetised according to previously published procedures 38, 40-42. Substrate A was not purified and used crude for further syntheses. Substrate C, [8-O(CH$_2$CH$_2$)$_2$O-8'-I-3,3'-Co(1,2-C$_2$B$_9$H$_{10}$)(1',2'-C$_2$B$_9$H$_{11}$)], was synthetized as follows: approximately 1 mL of 1,4-dioxane (Merck cat. No. 296309) was added to a 300 mg suspension of Substrate B in anhydrous cyclohexane (40 mL), homogenized in an ultrasonic bath and heated under reflux for 60 min. After cooling to room temperature, the orange precipitate was centrifuged, washed with 10 mL of cyclohexane and petroleum ether, and dried under vacuum. This synthesis gives a higher yield (88% compared to 57%) and has a friendlier workup procedure than the one reported earlier$^{42}$. Yield: 315 mg (88%).

Synthesis of 3 - [8-C$_3$N-CH$_2$OCH$_2$CH$_2$O-3,3'-Co(1,2-C$_2$B$_9$H$_{10}$)(1',2'-C$_2$B$_9$H$_{11}$)]

A total of 135 mg (0.329 mmol) of crude Substrate A was dissolved in 12 mL of acetonitrile. Then, 26 μL (0.362 mmol) of pyridine (Acros cat. No. 364425000) dissolved in 3 ml acetonitrile was briefly added. The solution was mixed for 22 hours at room temperature under an Ar atmosphere. To react with the remaining 10% of the metallacarborane substrate, the mixture was refluxed for 1 h under an Ar atmosphere. Next, acetonitrile was partially removed under reduced pressure until 4 mL of the mixture was left in the flask. The product was precipitated with 20 mL of 1 M HCl$_{aq}$, and the supernatant was discarded. The precipitate was dissolved in 3 mL of MeOH and precipitated with 10 mL of 1M HCl$_{aq}$, and the supernatant was discarded. MeOH - 1 M HCl$_{aq}$ precipitation was additionally repeated twice. The final precipitate was washed 3 times with 10 mL of water and dried under vacuum. Yield: 97.4 mg (60%). 1H NMR (400 MHz, acetone-d$_6$) δ: 3.61 (s, 2×CH$_2$, 4H), 3.95 (br s, 2×BCH, 2H), 4.08 (m, 2×BCH + CH$_2$, 4H), 4.95 (m (AA'XX' spin system), CH$_2$, 2H), 8.21 (t, J=7.2 Hz, m-PyH, 2H), 8.73 (tt, J=8.0 Hz, J=1.2 Hz, p-PyH, 1H), 9.25 (d, J=7.2 Hz, o-PyH, 2H); 11B{1H} NMR (128 MHz, acetone-d$_6$) δ: 23.93 (s, 1B), 5.73 (br s, 1B), -0.21 (br s, 1B), -3.28 (br s, 1B), -5.48 (br s, 2B), -7.41 and -7.84 (2×br s, total integration 4B),
-9.41 (br s, 2B), -17.96 (br s, 2B), -20.75 (br s, 2B), -22.98 (br s, 1B), -29.32 (br s 1B); $^{13}$C($^1$H) NMR (100 MHz, acetone-$d_6$): δ: 46.61 (br s, 2×CH), 52.24 (br s, 2×CH), 61.62, 68.98, 69.19, 72.49, 128.08, 145.84, 145.98. $^1$H and $^{13}$C($^1$H) NMR spectra are in good agreement with literature data. $^1$H and $^{13}$C($^1$H) NMR spectra of the reaction solution that was further treated with the addition of 10 mL 1 M HCl were recorded. A precipitate was obtained. The supernatant was discarded, and the precipitate was further processed. The precipitate was dissolved in 2 mL of MeOH and treated with 2 mL of 1 M HCl. After being removed from the flask to leave 2 mL of solution in the flask. The solution was treated with 10 mL of 1 M HCl. The reaction flask. The mixture was refluxed for 1 h under an Ar atmosphere. Acetonitrile was partially removed under reduced pressure to leave 2.5 mL of reaction solution that was further treated with the addition of 10 mL 1 M HCl. The precipitate was formed. The supernatant was discarded, and the precipitate was further purified. A precipitate was dissolved in 4 mL of MeOH and treated with 8 mL of 1 M HCl, forming a new precipitate. The precipitate was discarded, and the supernatant was further purified. The precipitate was dissolved in 2 mL of MeOH and treated with 8 mL of 1 M HCl, forming a new precipitate. The precipitate was washed 3 times with 10 mL of water and dried under vacuum. Yield: 90.0 mg (70%) $^1$H NMR (400 MHz, acetone-$d_6$): δ: 3.52-3.54 (m, CH$_2$, 2H), 3.58-3.60 (m, CH$_2$, 2H), 3.94 (m (AA'XX' spin system), CH$_2$, 2H), 4.00 (br s, 2×CH, 2H), 4.10 (br s, 2×CH, 2H), 4.92 (m (AA'XX' spin system), CH$_2$, 2H), 8.33 (tt, J = 8.0 Hz, J = 2.4 Hz, m-PyH, 2H), 8.77 (tt, J = 7.6 Hz, J = 1.2 Hz, p-PyH, 1H), 9.51 (m, o-PyH, 2H); $^{11}$B($^1$H) NMR (128 MHz, acetone-$d_6$): δ: 23.81 (s, 1B), 5.52 (br s, 1B), -0.31 (br s, 1B), -3.20 (br s, 1B), -5.35 (br s, 2B), -7.41 and -7.90 (2×br s, total integration 4B), -9.49 (br s, 2B), -17.88 (br s, 2B), -20.79 (br s, 2B), -22.83 (br s, 1B), -29.62 (br s 1B); $^{13}$C($^1$H) NMR (100 MHz, acetone-$d_6$): δ: 46.59 (br s, 2×CH), 52.45 (br s, 2×CH), 68.61, 72.37, 82.66, 129.48, 142.69, 145.66 (signal of one CH$_2$ carbon not observed due to overlap). HPLC purity: 95.1%, ESI-MS: [M+Na]$^+$ m/z (calculated/found) 529.3556/529.3537

Synthesis of 4 - [8-C$_6$H$_5$N-O-CH$_2$CH$_2$OCH$_2$CH$_2$O-3,3'-Co(1,2-C$_5$H$_5$)$_2$] (1',2'-C$_2$H$_4$)$]$

A total of 104.5 mg (0.254 mmol) of crude Substrate A and 35.7 mg of pyridine N-oxide (Merck cat. No. 131652) (0.375 mmol) were dissolved in 15 mL of acetonitrile and refluxed for 1 h under an Ar atmosphere. Next, acetonitrile was partially removed under reduced pressure to leave 2.5 mL of reaction solution that was further treated with the addition of 10 mL 1 M HCl. The precipitate was further processed, and the supernatant was discarded. The precipitate was dissolved in 4 mL of MeOH and treated with 20 mL of 1 M HCl, forming a new precipitate that was further processed; the supernatant was discarded. The 4 mL MeOH – 20 mL 1 M HCl precipitation scheme was additionally repeated 3 times. The final precipitate was washed 3 times with 10 mL of water and dried under vacuum. Yield: 90.0 mg (70%) $^1$H NMR (400 MHz, acetone-$d_6$): δ: 3.52-3.54 (m, CH$_2$, 2H), 3.58-3.60 (m, CH$_2$, 2H), 3.94 (m (AA'XX' spin system), CH$_2$, 2H), 4.00 (br s, 2×CH, 2H), 4.10 (br s, 2×CH, 2H), 4.92 (m (AA'XX' spin system), CH$_2$, 2H), 8.33 (tt, J = 8.0 Hz, J = 2.4 Hz, m-PyH, 2H), 8.77 (tt, J = 7.6 Hz, J = 1.2 Hz, p-PyH, 1H), 9.51 (m, o-PyH, 2H); $^{11}$B($^1$H) NMR (128 MHz, acetone-$d_6$): δ: 23.81 (s, 1B), 5.52 (br s, 1B), -0.31 (br s, 1B), -3.20 (br s, 1B), -5.35 (br s, 2B), -7.41 and -7.90 (2×br s, total integration 4B), -9.49 (br s, 2B), -17.88 (br s, 2B), -20.79 (br s, 2B), -22.83 (br s, 1B), -29.62 (br s 1B); $^{13}$C($^1$H) NMR (100 MHz, acetone-$d_6$): δ: 46.59 (br s, 2×CH), 52.45 (br s, 2×CH), 68.61, 72.37, 82.66, 129.48, 142.69, 145.66 (signal of one CH$_2$ carbon not observed due to overlap). HPLC purity: 95.1%, ESI-MS: [M+Na]$^+$ m/z (calculated/found) 529.3556/529.3537
that was processed further. To obtain the final product, 4 mL MeOH – 1.5 mL 1 M HClaq precipitation followed by precipitation with 20 mL 1 M HClaq was repeated two more times. The final precipitate was washed 3 times with 10 mL of water and dried under vacuum. Yield: 67.7 mg (51%), \(^1\)H NMR (400 MHz, acetone-d\(_6\) \(\delta\) : 3.70 (m, (AA'XX' spin system), CH\(_2\), 2H), 3.77 (m, (AA'XX' spin system), CH\(_2\), 2H), 3.86 (m, CH\(_2\), 2H), 3.92-3.94 (m, 2\times BCH + CH\(_2\), 4H), 4.02 (br s, 2\times BCH, 2H), 7.55-7.63 (m, ArH, 3H), 7.69-7.72 (m, ArH, 2H), (NH\(_2\)) protons not observed); \(^{11}\)B\(^{1\text{H}}\) NMR (128 MHz, acetone-d\(_6\) \(\delta\) : 23.50 (s, 1B), 6.50 (br s, 1B), 0.07 (br s, 1B), -3.07 (br s, 1B), -5.72, and -7.00 (2\times br s, total integration 6B), -9.81 (br s, 2B), -17.77 (br s, 2B), -20.69 (br s, 2B), -23.00 (br s, 1B), -29.13 (br s 1B); \(^{13}\)C\(^{1\text{H}}\) NMR (100 MHz, acetone-d\(_6\) \(\delta\) : 46.98 (br s, 2\times BCH), 50.74, 51.62 (br s, 2\times BCH), 63.41, 69.55, 70.70, 123.85, 130.16, 130.22, 134.00, HPLC Purity: 95.0%, ESI-MS [M] \(m/z\) (calculated/found) 503.3788/503.3797

Synthesis of 6 - \([8\text{-C}_{13}\text{H}_{17}\text{N}_{2}\text{-CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{O}-3,3'\text{-Co(1,2-C}_2\text{B}_3\text{H}_10)\{1',2'-C}_2\text{B}_3\text{H}_{11}\}]\)

A total of 134 mg (0.327 mmol) of crude Substrate A and 182 mg of 9-aminoacridine (Merck cat. No. 92817) (0.935 mmol) were suspended in 15 mL of acetonitrile (solubility problems occurred only with 9-aminoacridine) in a reaction flask and refluxed for 1.5 h under an Ar atmosphere. Acetonitrile was removed under reduced pressure. The crude product was partially dissolved in 6 mL of MeOH and treated with 12 mL of 1 M HCl\(_aq\). The supernatant was discarded, and the precipitate was once again partially dissolved in 6 mL of MeOH and treated with 12 mL of 1 M HCl\(_aq\). The supernatant was discarded, and the precipitate was washed with 1.5 mL of MeOH with agitation in an ultrasonic washer for 5 mins, followed by centrifugation. The supernatant containing mostly 9-aminoacridine was discarded. MeOH washing/centrifugation was additionally repeated 4 times (until there was no sign of 9-aminoacridine on HPLC) to obtain the final product. It was then washed 3 times with 10 mL of water and dried under vacuum. Problems with purification might be avoided if no or only a small excess of 9-aminoacridine is used. Here, it was used in excess to avoid the formation of the bis-form. Yield: 93.0 mg (47%), \(^1\)H NMR (400 MHz, DMSO-d\(_6\) \(\delta\) : 3.42 (s, 2\times CH\(_2\), 4H), 3.87 (br s, 2\times BCH, 2H), 3.99 (t, J=5.2 Hz, CH\(_2\), 2H), 4.04 (br s, 2\times BCH, 2H), 4.94 (t, J=5.2 Hz, CH\(_2\), 2H), 7.61 (t, J=7.6 Hz, ArH, 2H), 8.03 (t, J=8.0 Hz, ArH, 2H), 8.20 (d, J=9.2 Hz, ArH, 2H), 8.62 (d, J=8.0 Hz, ArH, 2H), 10.01 (br s, NH\(_2\))\(^\text{(-)}\) (2H); \(^{11}\)B\(^{1\text{H}}\) NMR (128 MHz, DMSO-d\(_6\) \(\delta\) : 23.55 (s, 1B), 4.87 (br s, 1B), -1.70, -4.92 and -8.51 (overlapping broad signals from ca. -2 to -15 ppm, total integration 10B), -18.90, -21.29 and -30.60 (overlapping broad signals from ca. -15 to -39 ppm, total integration 6B); \(^{13}\)C\(^{1\text{H}}\) NMR (100 MHz, DMSO-d\(_6\) \(\delta\) : 46.63 (br s, 2\times BCH), 47.76, 52.00 (br s, 2\times BCH), 68.83, 68.96, 72.64, 113.21, 118.73, 124.46, 125.94, 136.82, 141.19, 158.84, HPLC purity: 96.7% + 1.24% bis-form \([3,3'-\text{Co(1,2-C}_2\text{B}_3\text{H}_{10})\{1',2'-C}_2\text{B}_3\text{H}_{11}\}]\)-8-OCH\(_2\)CH\(_2\)OCH\(_2\)CH\(_2\)-[C\(_{13}\)H\(_{17}\)N\(_2\)]-8-CH\(_2\)CH\(_2\)OCH\(_2\)CH\(_2\)O-3,3'-Co(1,2-C\(_2\)B\(_3\)H\(_{10}\))\{1',2'-C\(_2\)B\(_3\)H\(_{11}\)\}], ESI-MS [M] (calculated/found) 604.4058/604.4066, bis-form ESI-MS [M] \(m/z\) (calculated/found) 1015.7462/1015.7371.
Synthesis of 7 - [8-C_{10}H_{12}NH-CH_{2}CH_{2}OCH_{2}CH_{2}O-3,3'-Co(1,2-C_{2}B_{9}H_{10})(1',2'-C_{2}B_{9}H_{12})]

A total of 141 mg (0.343 mmol) of crude Substrate A and 151 mg of 1-adamantylamine (Merck cat. No. 138576) (0.996 mmol) were dissolved in CH_{2}Cl_{2} and refluxed for 1.5 h under an Ar atmosphere. CH_{2}Cl_{2} was removed under reduced pressure. The reaction mixture was dissolved in 3 mL of acetonitrile, and the obtained precipitate was filtered off. The acetonitrile solution was treated with 15 mL of 1 M HCl, the solution was discarded, and the precipitate was dissolved in 4 mL of acetone and again treated with 15 mL of 1 M HCl. Acetone-HCl (4-15 mL) precipitation was additionally repeated 4 times. The obtained oily residue was washed 3 times with 10 mL of water and dried under vacuum. Yield: 152 mg (78.7%), \(^1\)H NMR (400 MHz, DMSO-d_{6}) \(\delta\): 1.54 (d, J=12.2 Hz, 3×CH_{2}H_{6}^{Ad}, 3H), 1.61 (d, J=12.2 Hz, 3×CH_{2}H_{6}^{Ad}, 3H), 1.79 (s, 3×CH_{2}^{Ad}, 6H), 2.07 (br s, 3×CH^{Ad}, 3H), 3.01 (br s, CH_{2}, 2H), 3.52 (t, J=5.2 Hz, CH_{2}, 2H), 3.60 (t, J=5.2 Hz, CH_{2}, 2H), 3.66 (t, J=4.8 Hz, CH_{2}, 2H), 3.90 (br s, 2×CH{B}, 2H), 4.04 (br s, 2×CH{B}, 2H), 8.19 (br s, NH_{2}^{(+), 2H}); \(^11\)B\(^{1}\)H NMR (128 MHz, DMSO-d_{6}) \(\delta\): 24.05 (s, 1B), 6.00 (br s, 1B), -1.86, -4.80 and -8.15 (overlapping broad signals from ca. 2 to -14 ppm, total integration 10B), -18.95, -21.29 and -30.34 (overlapping broad signals from ca. -14 to -37 ppm, total integration 6B); \(^{13}\)C\(^{\text{1}}\)H NMR (100 MHz, DMSO-d_{6}) \(\delta\): 28.94 (CH^{Ad}), 35.65 (CH_{2}^{Ad}), 37.92 (CH^{Ad}), 39.78, 46.66 (br s, 2×CH{B}), 51.63 (br s, 2×CH{B}), 56.90 (CNH), 66.67, 68.79, 72.02, HPLC purity: 97.1%, ESI-MS [M] (calculated/found) 561.4573/561.4572.

Synthesis of 8 - [8-CH(3)(CH_{2})-NH-CH_{2}CH_{2}OCH_{2}CH_{2}O-3,3'-Co(1,2-C_{2}B_{9}H_{10})(1',2'-C_{2}B_{9}H_{12})]

A total of 109 mg (0.264 mmol) of crude Substrate A was dissolved in 10 mL of acetonitrile. Then, 131 \(\mu\)L (0.793 mmol) of 1-octylamine (Merck cat. No. 74988) was added to the reaction flask. The mixture was refluxed for 1 h under an Ar atmosphere. Acetonitrile was partially removed under reduced pressure until approximately 2 mL of solution was left in a flask. The remaining solution was treated with 15 mL of 1 M HCl. The supernatant was discarded, and the precipitate was further processed. The precipitate was dissolved in 2 mL of MeOH and treated with 15 mL of 1 M HCl. The supernatant was discarded, and the precipitate was further processed. The 2 mL MeOH - 15 mL 1 M HCl precipitation process was additionally repeated 4 times. Next, the precipitate was dissolved in 2 mL MeOH and again treated with 1 M HCl, but this time gradually, in a much lower volume, until the first visible precipitate appeared. Then, the precipitate was discarded, and the solution was further treated with 15 mL of 1 M HCl. The gradual precipitation method was repeated 5 times to obtain the final precipitate, which was washed 3 times with water and dried under vacuum. Yield: 27.4 mg (19%), \(^1\)H NMR (400 MHz, acetone-d_{6}) \(\delta\): 0.84 (t, J=6.4 Hz, CH_{3}, 3H), 1.24-1.37 (m, 4×CH_{2}, 8H), 1.42 (quint, J=7.6 Hz, CH_{2}, 2H), 1.89 (quint, J=8.0 Hz, CH_{2}, 2H), 3.33 (m, CH_{2}, 2H), 3.48 (quint, J=5.6 Hz, CH_{2}, 2H), 3.61 (m (AA’XX’ spin system), CH_{2}, 2H), 3.71 (m, CH_{2}, 2H), 3.89 (m (AA’XX’ spin system), CH_{2}, 2H), 3.94 (br s, 1×CH{B}, 3H), 4.04 (br s, 2×CH{B}, 2H), 6.00 (br s, 1B), -1.94, -4.80 and -8.15 (overlapping broad signals from ca. 2 to -14 ppm, total integration 10B), -18.95, -21.29 and -30.34 (overlapping broad signals from ca. -14 to -37 ppm, total integration 6B); \(^{13}\)C\(^{\text{1}}\)H NMR (100 MHz, DMSO-d_{6}) \(\delta\): 28.94 (CH^{Ad}), 35.65 (CH_{2}^{Ad}), 37.92 (CH^{Ad}), 39.78, 46.66 (br s, 2×CH{B}), 51.63 (br s, 2×CH{B}), 56.90 (CNH), 66.67, 68.79, 72.02, HPLC purity: 97.1%, ESI-MS [M] (calculated/found) 561.4573/561.4572.
2×BCH, 2H), 4.04 (br s, 2×BCH, 2H), 7.95 (br s, NH₂(+), 2H); 11B[1H] NMR (128 MHz, acetone-d₆) δ: 23.64 (s, 1B), 5.94 (br s, 1B), -0.63 (br s, 1B), -3.60 (br s, 1B), -5.95 and -7.56 (2×br s, total integration 6B), -10.44 (br s, 2B), -18.31 (br s, 2B), -21.52 (br s, 2B), -23.27 (br s, 1B), -29.66 (br s 1B); 13C[1H] NMR (100 MHz, acetone-d₆) δ: 13.38 (CH₂), 22.44, 25.98/26.02/26.05 (CH₃), 26.33, 28.91, 31.63, 46.81 (br s, 2×BCH), 46.96/47.04/47.12 (CH₂), 47.69/47.83/47.93 (CH₃), 51.74 (br s, 2×BCH), 64.99/65.00 (CH₂), 69.55, 71.44 (signal of one CH₂ carbon of the n-octyl chain not observed due to overlap). HPLC purity: 97.2%, ESI-MS [M] (calculated/found) 539.4729/539.4738.

Synthesis of 9 - [8-NH₂(CH₃)₂-NH-CH₂CH₂OCH₂CH₂O-3,3'-Co(1,2-C₂B₉H₁₀)(1',2'-C₂B₉H₁₁)]

A total of 102 mg (0.248 mmol) of crude Substrate A was dissolved in 10 mL of acetonitrile. A total of 576 mg (4.96 mmol) of 1,6-diaminohexane (Merck cat. No. H11696) was dissolved in 5 mL of acetonitrile until complete dissolution at 40°C and added to Substrate A solution. The mixture was refluxed for 1 h under an Ar atmosphere. Acetonitrile was partially removed under reduced pressure. The remaining 2 mL of reaction mixture was treated with 20 mL of 1 M HClaq. The supernatant was discarded, and the precipitate was dissolved in 4 mL MeOH and precipitated with 20 mL of 1 M HClaq.

A 4 mL MeOH – 20 mL 1 M HClaq precipitation scheme was performed two more times. Next, the precipitate was dissolved in 4 mL MeOH and treated with 2.9 mL 1 M HClaq. The precipitate was discarded, and the solution was further treated with an additional 20 mL of HClaq. The 4 mL MeOH – 2.9 mL 1 M HClaq followed by 20 mL 1 M HClaq precipitation scheme was additionally repeated twice. The final precipitate was washed 3 times with 10 mL of water and dried under vacuum. Yield: 75.2 mg (58%), 1H NMR (400 MHz, acetone-d₆) δ: 1.56 (m, 2×CH₂, 4H), 1.85-1.96 (m, 2×CH₂, 4H), 3.17 (m, CH₂, 2H), 3.25 (m, CH₂, 2H), 3.36 (m, CH₂, 2H), 3.61 (m, CH₂, 2H), 3.73 (m, CH₂, 2H), 3.91 (t, J=5.0 Hz, CH₂, 2H), 3.99 (br s, 2×BCH, 2H), 4.08 (br s, 2×BCH, 2H), 8.11 (br s, NH₂, 2H), 8.59 and 8.64 (bimodal br s, NH₂(-)), actual integration ~1.5H); 13B[1H] NMR (128 MHz, acetone-d₆) δ: 23.42 (s, 1B), 5.43 (br s, 1B), -0.40, -3.71 and -7.80 (overlapping broad signals from ca. 2 to -14 ppm, total integration 10B), -18.14 and -21.23 (2×br s, total integration 5B), -29.39 (br s 1B); 13C[1H] NMR (100 MHz, acetone-d₆) δ: 25.37, 25.47, 25.56, 26.70, 39.56, 46.79 (br s, 2×BCH), 47.01, 47.33, 52.14 (br s, 2×BCH), 65.29, 69.05, 71.75. NMR spectra are in good agreement with literature data⁴⁴, HPLC purity: 95.1%, ESI-MS [M] (calculated/found) 526.4523/526.4515.

Synthesis of 10 - (HCOOH[8-(NH₂(CH₃)₂)-(CONH₂)-CH-NH-CH₂CH₂OCH₂CH₂O-3,3'-Co(1,2-C₂B₉H₁₀)(1',2'-C₂B₉H₁₁)]

Hydrochloride was removed from Ne-Boc-L-Lysine amide hydrochloride (Fluorochem cat. No. 492845) on Amberlite IRA4000 resin. Approximately 5 mL of resin suspended in water was placed in the column and then it was flushed with water (300 mL), 1 M NaOH (200 mL) and water (200 mL). 363 mg of Ne-
Boc-L-lysine amide hydrochloride was dissolved in 1 mL of water and applied to the column. The collected solution was frozen and lyophilised, receiving 282 mg of Nε-Boc-L-lysine amide. Yield: 94%.

A total of 161 mg (0.391 mmol) of crude Substrate A and 190 mg of Ne-Boc-L-lysine amide (0.774 mmol) were dissolved in 15 mL of acetonitrile and refluxed for 1.5 h under an Ar atmosphere. Acetonitrile was completely removed under reduced pressure, and the the remaining residue was dried under vacuum. After drying, the crude product was dissolved in 2 mL MeOH and treated with 10 mL of 1 M HClaq; the supernatant was discarded, and the precipitate was further purified. Dissolution in 2 mL of MeOH and precipitation with 10 mL of HClaq were additionally repeated 5 times. The precipitate was resuspended in 4 mL of CHCl3. Next, 4 mL of 1,1,1,3,3,3-hexafluoro-2-propanol and 160 µL 35% HCl were added to the mixture. The whole mixture was sonicated for 15 min and then stirred overnight at room temperature. CHCl3 and 1,1,1,3,3,3-hexafluoro-2-propanol were removed under reduced pressure. The remaining part was dissolved in 2 mL of MeOH and treated with 15 mL of 1 M HClaq; the supernatant was discarded, and the precipitate was further purified. This procedure was repeated once more. To obtain the final high-purity compound, the precipitate was dissolved in acetonitrile and purified by HPLC using a Hypersil GOLD column (5 µm, 150 mm x 10 mm) in a H2O (0.5% formic acid) – acetonitrile (0.5% formic acid) gradient. Fractions containing pure product were pulled together and precipitated with 1 M HCl. The precipitate was washed 3 times with 10 mL of water and dried under vacuum. Yield: 64.2 mg (27%), 1H NMR (400 MHz, DMSO-d6) δ: 1.24-1.36 (m, CH3, 2H), 1.37-1.53 (m, 2×CH2, CH2, 4H), 2.47-2.52 (m, CH3, 1H), 2.58-2.64 (m, CH3, 1H), 2.73 (t, J=7.4 Hz, CH2, 2H), 2.92 (t, J=6.4 Hz, CH2, 1H), 3.39 (t, J=5.6 Hz, CH2, 2H), 3.42 (t, J=5.2 Hz, CH2, 2H), 3.51 (t, J=5.6 Hz, CH2, 2H), 3.96 (br s, 2×CH2, 2H), 4.08 (br s, 2×CH2, 2H), 7.01 (br d, J=2.0 Hz, C(O)NH2, 1H), 7.33 (br d, J=2.0 Hz, C(O)NH2, 1H), ~5.6-9.3 (br s, NH2, 3H) (signal of the α-amino group not observed); 13C{1H} NMR (128 MHz, DMSO-d6) δ: 23.11 (s, 1B), 4.51 (br s, 1B), -1.66, -4.80 and -8.58 (overlapping broad signals from ca. 1.5 to -15 ppm, total integration 10B), -18.37, -21.41 and -30.21 (overlapping broad signals from ca. -15 to -35 ppm, total integration 6B); 13C{1H} NMR (100 MHz, DMSO-d6) δ: 22.82 (CH3), 27.46 (CH2), 32.79 (CH2), 39.20, 46.65 (br s, 2×CH2), 47.59 (CH3, 1H), 52.29 (br s, 2×CH2), 62.06 (CH), 68.76, 70.46, 71.74, 175.86 (C=O). Note: in the 1H and 13C{1H} NMR spectra, additional signals of residual formic acid are present at 8.17 and 164.30 ppm, respectively. HPLC purity: 98.4%, ESI-MS [M] (calculated/found) 555.4425/555.4427.

Synthesis of 11 - [8-(NH2CH2)4-(CONH2)-CH-NH-CH2CH2OCH2CH2O-8'-I-3,3'-Co(1,2-C2B10H10)(1',2'-C2B10H10)]

Hydrochloride was removed from Ne-Boc-L-Lysine amide hydrochloride (Fluorochem cat. No. 492845) on Amberlite IRA4000 resin. A suspension of 92 mg (0.17 mmol) of Substrate C and 84 mg of Ne-Boc-
L-lysine amide (0.34 mmol) in 11 mL acetonitrile was stirred for 24 hours at room temperature. After approximately one hour, the suspension turned to a clear solution. Acetonitrile was removed under reduced pressure, and the remaining residue was dried under vacuum. After drying, the crude product was dissolved in 2 mL of MeOH and treated with 10 mL of 1 M HCl\textsubscript{aq}. The precipitating yellow solid was centrifuged, and the supernatant was discarded. This precipitation procedure was repeated three times to obtain a pure product containing Ne-Boc-L-Lysine amide. Then, the precipitate was resuspended in 4 mL of MeOH, and 2 mL of concentrated HCl\textsubscript{aq} was added. The resulting solution was left at room temperature overnight. Finally, 20 mL of cold water was added, and the precipitating yellow solid was centrifuged, washed with 3 mL of cold water and petroleum ether, and dried under vacuum. Yield: 93 mg (76%). \textsuperscript{1}H NMR (600 MHz, DMSO-d\textsubscript{6}) \(\delta\): 1.24-1.37 (m, CH\textsubscript{3}\textsuperscript{13}N, 2H), 1.47-1.58 (m, CH\textsubscript{2}\textsuperscript{13}N, 2H), 1.70-1.82 (m, CH\textsubscript{2}\textsuperscript{13}N, 2H), 2.71 (m, CH\textsubscript{2}\textsuperscript{13}N, 2H), 2.96 (m, CH\textsubscript{2} 2H), 3.40-3.43 (m, CH\textsubscript{2} 2H), 3.44-3.46 (m, CH\textsubscript{2} 2H), 3.60 (t, \(J=5.4\) Hz, CH\textsubscript{2} 2H), 3.71 (m, CH\textsubscript{2}\textsuperscript{13}N, 1H), 4.10 (br s, 2×BCH, 2H), 4.34 (br s, 2×BCH, 2H), 7.70 (br s, C(O)NH\textsubscript{2}, 1H), 7.87 (br s, NH\textsubscript{2}, 1H), 8.01 (br s, C(O)NH\textsubscript{2}, 1H), 8.80 (br m, NH\textsubscript{2}, 1H), 9.02 (br m, NH\textsubscript{2}, 1H); \textsuperscript{13}C\textsuperscript{1}H NMR (128 MHz, DMSO-d\textsubscript{6}) \(\delta\): 21.19 (s, 1B), -1.34 and -6.69 (overlapping broad signals from ca. 12 to -14 ppm, total integration 11B), -19.88 (overlapping broad signals from ca. -14 to -34 ppm, total integration 6B); \textsuperscript{15}C\textsuperscript{1}H NMR (151 MHz, DMSO-d\textsubscript{6}) \(\delta\): 21.70 (CH\textsubscript{3}\textsuperscript{13}N), 27.11 (CH\textsubscript{2}\textsuperscript{13}N), 29.45 (CH\textsubscript{2}\textsuperscript{13}N), 38.84 (CH\textsubscript{2}\textsuperscript{13}N), 45.62, 54.50 (br s, 2×BCH), 56.41 (br s, 2×BCH), 59.74 (CH), 66.02, 68.28, 71.55, 169.60 (C=O). HPLC purity: 96.3%, ESI-MS [M]+/z (calculated/found) 681.3392/681.3385

**Synthesis of 12 - [8-CH\textsubscript{2}(CH\textsubscript{2})\textsubscript{3}-(CONH\textsubscript{2})-CH-NH-CH\textsubscript{2}CH\textsubscript{2}OCH\textsubscript{2}CH\textsubscript{2}O-8'-I-3',3'-Co(1,2-C\textsubscript{2}B\textsubscript{9}H\textsubscript{10})[1',2'-C\textsubscript{2}B\textsubscript{9}H\textsubscript{10}]]**

A suspension of 140 mg (0.26 mmol) of **Substrate C** and 68 mg of norleucine amide (0.52 mmol) in 16 mL of acetonitrile was stirred for 24 hours at room temperature. After approximately one hour, the suspension turned to a clear solution. Acetonitrile was removed under reduced pressure, and the remaining residue was dried under vacuum. After drying, the crude product was dissolved in 3 mL of MeOH and treated with 20 mL of 1 M HCl\textsubscript{aq}. The precipitating yellow solid was centrifuged, and the supernatant was discarded. This precipitation procedure was repeated three times. Next, the precipitate was dissolved in 4 mL of MeOH and treated with 2.5 mL of 1 M HCl\textsubscript{aq}. The precipitate was discarded, and the solution was further treated with an additional 20 mL of HCl\textsubscript{aq}. The final precipitate was washed with 2 mL of cold water and dried under vacuum. Yield: 80.6 mg (74%). \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) \(\delta\): 0.83 (t, \(J=6.8\) Hz, CH\textsubscript{3}N\textsuperscript{13}, 3H), 1.15-1.31 (m, 2×CH\textsubscript{3}N\textsuperscript{13}, 4H), 1.60-1.81 (m, CH\textsubscript{2}N\textsuperscript{13}, 2H), 2.96 (m, CH\textsubscript{2}, 2H), 3.43 (m, 2×CH\textsubscript{2}, 4H), 3.57 (t, \(J=5.2\) Hz, CH\textsubscript{2}, 2H), 3.67 (m, CH\textsubscript{2}N\textsuperscript{13}, 1H), 4.10 (br s, 2×BCH, 2H), 4.35 (br s, 2×BCH, 2H), 7.68 (br s, C(O)NH\textsubscript{2}, 1H), 7.91 (br s, C(O)NH\textsubscript{2}, 1H), 8.62 (br m, NH\textsubscript{2}, 1H), 8.77 (br m, NH\textsubscript{2}, 1H); \textsuperscript{13}B\textsuperscript{1}H NMR (128 MHz, DMSO-d\textsubscript{6}) \(\delta\): 20.96 (s, 1B), -1.34, -6.80 and -7.70 (overlapping...
broad signals from ca. 12 to -13 ppm, total integration 11B), -18.94 and -20.11 (overlapping broad signals from ca. -13 to -35 ppm, total integration 6B); $^{13}$C$^1$H NMR (100 MHz, DMSO-d$_6$) δ: 13.92 (CH$_3$), 22.25 (CH$_2$Nle), 26.56 (CH$_2$Nle), 29.70 (CH$_2$Nle), 45.59, 54.51 (br s, 2×BCH), 56.60 (br s, 2×BCH), 59.74 (CH), 66.36, 68.30, 71.45, 169.77 (C=O). HPLC purity: 95.5%, ESI-MS [M]$^+$ m/z (calculated/found) 666.3282/666.3280

Synthesis of 13 - [8-{CONH$_2$}-CH$_2$-NH-CH$_2$CH$_2$OCH$_2$CH$_2$O-8'-I-3,3'-Co(1,2-C$_2$B$_9$H$_{10}$)(1',2'-C$_2$B$_9$H$_{10}$)]

Hydrochloride was removed from glycinamide hydrochloride (Merck cat. No. 50070) on Amberlite IRA4000 resin. A suspension of 126 mg (0.23 mmol) of Substrate C and 58 mg of glycinamide (0.79 mmol in 10 mL of acetonitrile) was stirred for 24 hours at room temperature. After approximately one hour, the suspension turned to a clear solution. Acetonitrile was removed under reduced pressure, and the remaining residue was dried under vacuum. After drying, the crude product was dissolved in 2 mL of MeOH and treated with 20 mL of 1 M HCl$_{aq}$. The precipitating yellow solid was centrifuged, and the supernatant was discarded. This precipitation procedure was repeated three times. Next, the precipitate was dissolved in 5 mL of MeOH and treated with 5.5 mL of 1 M HCl$_{aq}$. The precipitate was discarded, and the solution was further treated with an additional 20 mL of HCl$_{aq}$. The final precipitate was washed with 2 mL of cold water and dried under vacuum. Yield: 67 mg (46.9%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ: 3.06 (m, CH$_3^{Gly}$, 2H), 3.40-3.45 (m, 2×CH$_2$, 4H), 3.58 (t, J=5.6Hz, CH$_2$, 2H), 3.63 (t, J=5.6Hz, CH$_2$, 2H), 4.10 (br s, 2×BCH, 2H), 4.34 (br s, 2×BCH, 2H), 7.52 (br s, C(O)NH$_2$, 1H), 7.76 (br s, C(O)NH$_2$, 1H), 8.66 (br s, NH$_2^{(s)}$, 2H); $^{13}$B$^1$H NMR (128 MHz, DMSO-d$_6$) δ: 20.96 (s, 1B), -1.47, -6.67 and -7.74 (overlapping broad signals from ca. 13 to -14 ppm, total integration 11B), -18.93 and -20.32 (overlapping broad signals from ca. -14 to -36 ppm, total integration 6B); $^{13}$C$^1$H NMR (100 MHz, DMSO-d$_6$) δ: 46.74 (CH$_2^{Gly}$), 48.22, 54.50 (br s, 2×BCH), 56.42 (br s, 2×BCH), 66.15, 68.37, 71.49, 167.01 (C=O). HPLC purity: 95.0%, ESI-MS [M]$^+$ m/z (calculated/found) 610.2654/610.2627

Synthesis of 14 - [8-CH$_3$-CH$_2$-NH-CH$_2$CH$_2$OCH$_2$CH$_2$O-8'-I-3,3'-Co(1,2-C$_2$B$_9$H$_{10}$)(1',2'-C$_2$B$_9$H$_{10}$)]

A suspension of 120 mg (0.23 mmol) of Substrate C and 1 mL (12.6 mmol) of ethylamine solution (Merck, 70% in water, cat. No. 471208) in 10 mL of acetonitrile was stirred overnight at room temperature. After approximately 10 minutes, the suspension turned to a clear orange solution. Acetonitrile was removed under reduced pressure, and the remaining residue was dried under vacuum. After drying, the crude, oily product was dissolved in 3 mL of MeOH and treated with 20 mL of 1 M HCl$_{aq}$. The precipitating red oil was centrifuged, and the supernatant was discarded. This precipitation procedure was repeated three times. Finally, the obtained red oil was washed with 5 mL of cold water and freeze-dried under vacuum. Yield: 127 mg (95.7%). $^1$H NMR (400 MHz, acetone-d$_6$) δ: 1.42 (t, J=7.6 Hz, CH$_3$, 3H), 3.44 (m, CH$_2$CH$_3$, 2H), 3.47-3.54 (m, 3×CH$_2$, 6H), 3.83 (m [AA’XX’ spin
system), CH$_2$, 2H), 4.18 (br s, 2×BCH, 2H), 4.36 (br s, 2×BCH, 2H), 7.94 (br s, NH$_2^{+}$, 2H); $^{11}$B($^1$H) NMR (128 MHz, acetone-d$_6$) δ: 20.64 (s, 1B), -1.42 (br s, 2B), -5.64, -6.51 and -8.21 (3×br s, total integration 9B), -18.87 (br s, 2B), -20.79 (br s, 2B), -24.51 (br s, 1B), -28.47 (br s 1B); $^{13}$C($^1$H) NMR (151 MHz, acetone-d$_6$) δ: 10.77 (CH$_3$), 43.67 (CH$_3$), 47.60 (CH$_3$), 54.22 (br s, 2×BCH), 56.58 (br s, 2×BCH), 65.50 (CH$_2$), 68.04, 71.86. HPLC purity: 96.3%, ESI-MS [M]+ m/z (calculated/found) 581.2752/581.2748

Synthesis of 15 - [8-(CH$_2$-CH$_2$)$_2$N-NH-CH$_2$CH$_2$OCH$_2$CH$_2$O-8'-I-3,3'-Co(1,2-C$_2$B$_9$H$_{10}$)(1',2'-C$_2$B$_9$H$_{10}$)]

A suspension of 100 mg (0.19 mmol) of Substrate C and 0.5 mL (4.8 mmol) of diethyamine (Merck, cat. No. 386456) in 10 mL of acetonitrile was stirred overnight at room temperature. After approximately 10 minutes, the suspension turned to a clear orange solution. Acetonitrile was removed under reduced pressure, and the remaining residue was dried under vacuum. After drying, the crude, oily product was dissolved in 3 mL of MeOH and treated with 20 mL of 1 M HCl$_{aq}$. The precipitating red oil was centrifuged, and the supernatant was discarded. This precipitation procedure was repeated three times. Finally, the obtained red oil was washed with 5 mL of cold water and freeze-dried under vacuum. Yield: 109 mg (96%). $^1$H NMR (400 MHz, acetone-d$_6$) δ: 1.42 (t, J=7.2 Hz, CH$_3$, 3H), 3.45-3.61 (m, 2×CH$_2$CH$_3$ + 3×CH$_2$, 10H), 3.89 (m (AA’XX’ spin system), CH$_2$, 2H), 4.19 (br s, 2×BCH, 2H), 4.36 (br s, 2×BCH, 2H), 8.01 (br s, NH$_2^{+}$, 1H); $^{11}$B($^1$H) NMR (128 MHz, acetone-d$_6$) δ: 20.66 (s, 1B), -1.14 and -1.54 (2×br s, 2B), -5.62, -6.46 and -8.22 (3×br s, total integration 9B), -18.87 (br s, 2B), -20.72 (br s, 2B), -24.39 (br s, 1B), -28.14 (br s 1B); $^{13}$C($^1$H) NMR (151 MHz, acetone-d$_6$) δ: 8.34 (CH$_3$), 47.92 (CH$_3$), 51.80 (CH$_2$), 54.07 (br s, 2×BCH), 56.61 (br s, 2×BCH), 64.22 (CH$_2$), 68.11, 72.15. HPLC purity: 95.4%, ESI-MS [M-H]$^-$ m/z (calculated/found) 609.3066/609.3057

Synthesis of 16 - [8-(CH$_2$-CH$_2$)$_3$N-CH$_2$CH$_2$OCH$_2$CH$_2$O-8'-I-3,3'-Co(1,2-C$_2$B$_9$H$_{10}$)(1',2'-C$_2$B$_9$H$_{10}$)]

A suspension of 120 mg (0.23 mmol) of Substrate C and 0.5 mL (3.6 mmol) of triethylamine (Merck, cat. No. 90335) in 10 mL of acetonitrile was stirred overnight at room temperature. After approximately 10 minutes, the suspension turned to a clear orange solution, but several hours later, an orange precipitate was formed. From this mixture, acetonitrile was removed under reduced pressure, and the remaining residue was dried under vacuum. After drying, the crude product was resuspended in 3 mL of MeOH and treated with 20 mL of 1 M HCl$_{aq}$. The precipitating solid was centrifuged, washed with 10 mL of cold water, and freeze-dried under vacuum. Yield: 140 mg (93.3%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ: 1.13 (t, J=7.2 Hz, 3×CH$_3$, 9H), 3.25 (q, J=7.2 Hz, 3×CH$_2$CH$_3$, 6H), 3.35 (m (AA’XX’ spin system), CH$_2$, 2H), 3.43 (s, 4H, 2×CH$_2$), 3.69 (m (AA’XX’ spin system), CH$_2$, 2H), 4.11 (br s, 2×BCH, 2H), 4.32 (br s, 2×BCH, 2H); $^{11}$B($^1$H) NMR (128 MHz, DMSO-d$_6$) δ: 21.02 (s, 1B), -1.80, -6.67 and -8.17 (overlapping broad signals from ca. 11 to -14 ppm, total integration 11B), -18.16, -20.22 and -27.92 (overlapping broad signals from ca. -14 to -33 ppm, total integration 6B); $^{13}$C($^1$H) NMR (151 MHz,
DMSO-d$_6$ δ: 7.79 (CH3), 53.42, 54.43 (br s, 2×CH3), 56.33, 56.42 (br s, 2×CH3), 63.99, 68.16, 72.01. HPLC purity: 95.4%, ESI-MS [M+CF$_3$COO]$^-$ m/z (calculated/found) 751.3311/751.3294.

**Cell culture lines and culture conditions**

A549 (human lung carcinoma) cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC; Porton Down, UK). UM-UC-3 (human urinary bladder transitional cell carcinoma), MCF 10A (non-tumorigenic human mammary gland cells), MV 4 11 (human biphenotypic leukemia) and Balb/3T3 (non-tumorigenic murine fibroblasts) cells were purchased from the American Type Culture Collection (ATCC; Rockville, USA). All cell lines were maintained at the Hirsfeld Institute of Immunology and Experimental Therapy (HIIET), Wroclaw, Poland. The Balb/3T3 and UM-UC-3 cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Scotland) supplemented with 10% (v/v) foetal bovine serum (FBS; GE Healthcare HyClone, Logan, USA) and 2 mM L-glutamine (Sigma–Aldrich, Poznań, Poland). The A549 cell line was cultured in OptiMEM (HIIET, PAS, Wroclaw, Poland) supplemented with 5% (v/v) FBS (GE Healthcare HyClone, Logan, USA) and 2 mM L-glutamine. The MV-4-11-cell line was cultured in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% (v/v) FBS, 2 mM L-Gln and 1 mM sodium pyruvate (Sigma–Aldrich, Poznań, Poland). The MCF 10A cell line was cultured in Ham’s F12 medium with glutamine (Corning) supplemented with 5% (v/v) FBS, 5% (v/v) horse serum, 10 µg/mL insulin, 0.05 µg/mL cholera toxin, 0.5 µg/mL hydrocortisone, and 20 ng/mL hEGF (all from Sigma–Aldrich). All culture media were supplemented with antibiotics – 100 µg/mL streptomycin (Polfa Tarchomin, Warsaw, Poland) and 100 U/mL penicillin (Sigma–Aldrich, Poznań, Poland). The cells were grown at 37°C in a humid atmosphere saturated with 5% CO2.

**Antiproliferative activity assessment by sulforhodamine B assay**

The cells were seeded on 384-well plates (Greiner Bio One, Kremsmünster, Austria) at 1×10$^3$ cells/well density for the A-549 and UM-UC-3 cell lines and 2×103 cells/well for the MCF-10A and Balb/3T3 cell lines. After overnight incubation, compounds were applied at various concentrations (ranging from 50 µM to 0.01 nM). After 72 h of incubation, the sulforhodamine B (SRB) assay based on Skehan et al. was carried out with slight modifications. In brief, 50 µL of medium was replaced with 30 µL/well of 25% (w/v) trichloroacetic acid (Avantor Performance Materials, Gliwice, Poland). After 1 h of incubation at room temperature, the plates were washed three times with tap water, and 20 µL of a 0.1% (w/v) solution of sulforhodamine B (Sigma–Aldrich, Poznan, Poland) in 1% (v/v) acetic acid (Avantor Performance Materials, Gliwice, Poland) was added to each well. After 30 min of incubation at room temperature, unbound dye was washed out with 1% (v/v) acetic acid. Bound dye was solubilized with 70 µL of 10 mM unbuffered TRIS (Avantor Performance Materials, Gliwice, Poland)
solution. The procedure was performed using a BioTek EL-406 washing station (BioTek Instruments, USA). Absorbance was read using a Biotek Hybrid H4 reader (BioTek Instruments, USA) at 540 nm wavelength. Crude absorbance data were used to calculate proliferation inhibition using the following formula:

$$\% \text{Inh} = \left[ \frac{(A_p - A_m)}{(A_k - A_m)} \times 100 \right] - 100$$  \hspace{1cm} (1)

where:

- $A_m$ - absorbance for cell-free wells,
- $A_k$ - absorbance for vehicle-treated, control wells,
- $A_p$ - absorbance for compound-treated wells;

The %Inh was next used for IC$_{50}$ calculations performed in GraphPad Prism 7.05 (GraphPad Software, Inc.) utilizing the ‘[Inhibitor] vs. response – Variable slope (four parameters)’ model.

**Antiproliferative activity assessment by MTT assay**

MV-4-11 cells were seeded on 384-well plates (Greiner Bio-One, Kremsmünster, Austria) at a density of $1 \times 10^3$ cells/well. After overnight incubation, compounds were applied at various concentrations (ranging from 100 µM to 0.03 nM). After 72 h of incubation, the MTT assay based on Mosmann, T. 46 was carried out with slight modifications. In brief, 20 µL of MTT (Sigma–Aldrich, Poznań, Poland) solution in PBS (5 mg/mL) was added to each well, and after 4 h of incubation, the plates were gently centrifuged (rt, 5 min, 300×g). The culture medium was gently removed using a BioTek EL-406 washing station, and the remaining formazan was solubilized in DMSO (Avantor Performance Materials, Gliwice, Poland; 75 µL/well). After 1 h of incubation, the absorbance was read using a Biotek Hybrid H4 reader (BioTek Instruments, USA) at a wavelength of 570 nm. Crude absorbance data were used to calculate proliferation inhibition using the following formula (1)

**Haemolytic Assay**

The haemolytic activity of the metallacarborane derivatives was evaluated as the amount of hemoglobin released by the disruption of mouse red blood cells (mRBCs) using a modified method described by Oddo et al. 47. Erythrocytes were collected by centrifugation at 1500 × g for 15 min, washed two times with PBS (10 mM, pH 7.4) and suspended to a final concentration of 5% v/v. Then, an equal volume of erythrocyte suspension and peptide solution with various concentrations was mixed in 96-well plates and incubated for 1 h at 37°C. After centrifugation at 1000×g for 5 min, the absorbance of the supernatant was measured at 490 nm to monitor the release of hemoglobin using a microplate reader (Biotek SynergyH4 Hybrid Reader). Untreated erythrocytes and erythrocytes
treated with 1% Triton X-100 were employed as negative and positive controls, respectively. The hemolysis percentage was calculated from the following equation:

\[
\text{Hemolysis rate (\%) = } \frac{OD_{490\text{nm}} (\text{compounds}) - OD_{490\text{nm}} (\text{negative control})}{OD_{490\text{nm}} (\text{positive control}) - OD_{490\text{nm}} (\text{negative control})}
\]  

(2)

Half maximal effective concentrations (EC_{50}) were defined as the compound concentrations causing 50% hemolysis. The experiments were performed in triplicate.

**Bacterial strains and culture conditions**

The bacterial strains used were *Staphylococcus aureus* ATCC 6538, clinical strains of methicillin-resistant *Staphylococcus aureus* S. aureus 2, S. aureus N1, S. aureus N2, S. aureus N3, S. aureus N4, S. aureus N5, and *Pseudomonas aeruginosa* ATCC 27853 stored at -70°C. Clinical strain MRSA 2 was provided by the Strain Collection of the Department of Microbiology of Wroclaw University, and clinical strains MRSA N1-N5 were provided by the Strain Collection of the Department of Pharmaceutical Microbiology and Parasitology of Wroclaw Medical University. The following materials were used in the studies: Mueller Hinton Broth (MHB, Sigma–Aldrich), Mueller Hinton Agar (MHA, Sigma–Aldrich), 0.9% NaCl solution (Chempur), the antibiotics vancomycin (MIP Pharma Poland), ceftazidime (Fresenius Kabi Poland), and meropenem (Ranbaxy), and the antibiotic discs cefoxitin (30 μg/disc), erythromycin (15 μg/disc), clindamycin (2 μg/disc), ciprofloxacin (5 μg/disc), gentamycin (2 μg/disc), tetracycline (30 μg/disc), amoxicillin/clavulanic acid (20/10 μg/disc), cefuroxime (30 μg/disc), cefotaxime (5 μg/disc), amikacin (30 μg/disc), imipenem (10 μg/disc), meropenem (10 μg/disc), trimethoprim-sulfamethoxazole (1.25-23.75 μg/disc), penicillin (10 μg/disc), and chloramphenicol (30 μg/disc) (Oxoid).

**Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays.**

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined to analyse the sensitivity of bacterial strains to metallacarboranes according to Clinical and Laboratory Standards Institute (CLSI) methods with our slow modification^48. Prior to each investigation, the revitalization of bacterial strains was performed by spreading the inoculum on an agar plate (MHA) and incubating at 37°C for 19 h. Before an experiment, a stock of metallacarboranes (at concentrations from 0.5 mM to 976.6 nM) in MHB with a final volume of 200 μL was prepared in microtitration plates. Next, the preincubated bacterial strains were established at 0.5 McFarland standard and added at a final concentration of 1.5x10^5 CFU/mL to each metallacarborane sample concentration. The following controls were established: pure medium, medium with metallacarboranes sample stock and medium with bacterial strains. Antibiotic susceptibility was used as an internal experimental control. Break points of the antibiotics sensitivity were read according to European Committee on Antimicrobial
Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters. Minimal inhibitory concentration (MIC) values were read within 16-19 h. Original methods are based on the optical density. To verify bactericidal and bacteriolytic effects with MBC determination (that is, more than 99.9% of killed cells), bacteria were inoculated on MHA agar plates (at a volume of 10 µL), and the number of colonies was counted. Comparison of MIC and MBC values gave us some preliminary information about the force of metallacarborane activity and made it possible to select the appropriate trials. The experiment was repeated at least 3 times.

**Antibiotic Susceptibility Testing**

The sensitivity of the tested bacterial strains to antibiotics was determined with disc diffusion methods. The criteria for the selection of antimicrobials were based on the EUCAST recommendation. Bacteria (0.5 McFarland) were inoculated, and antibiotic discs were placed on the MHA plate. Plates were incubated at 37°C for 18 h, followed by zone diameter measurement and breakpoint estimation.

**Kill-time kinetics**

The antimicrobial activity of compound 11 was investigated by analysing the survival of bacteria treated with the compound at given exposure times. *S. aureus* ATCC 6538 (3.9 x 10^4 CFU/mL) was treated with compound 11 at 0.98 and 1.9 µM concentrations (1x and 2x MIC). After various time periods (0.5, 1, 2, 3 and 24 h), equal volumes of samples were diluted and plated on agar plates. Bacterial colonies were counted after 24 h of incubation at 37°C. The results were logarithmically transformed (logarithm base – 10) and evaluated using RM 2-way ANOVA implemented in GraphPad Prism 7.02.

**Therapeutic index calculation**

The therapeutic index was calculated according to the formula below using data from previous experiments:

\[
TI = \frac{IC_{50\ MCP-10A}}{MIC\ S.\ aureus} \tag{3}
\]

**Author information**

*Corresponding Author:

email: goszczynski@hirszfeld.pl (TMG)

**Acknowledgement**
The authors thank A. Junka (Department of Pharmaceutical Microbiology and Parasitology of Wroclaw Medical University, Wroclaw, Poland) for the MRSA strains from the Strain Collection of the Department of Pharmaceutical Microbiology and Parasitology of Wroclaw Medical University. This work was supported by the National Science Centre, Poland, grant Nos. 2016/23/D/NZ1/02611, 2019/35/O/NZ7/03764 and 2019/32/C/NZ7/00510.

Conflict of Interest Disclosure

The authors declare no competing financial interest.

Supporting Information

$^1$H NMR, $^{13}$C($^1$H) NMR, $^{11}$B($^1$H) NMR, HRMS, HPLC traces, additional in vitro data.

References

Uncategorized References


(33) Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis.; World Health Organization, 2017.


I-COSAN \[\rightarrow\] amino amide \[\xrightarrow{3h} \text{1.9 \(\mu\)M} S. aureus\] variable side chain