Synthesis of boron cluster analogs of penicillin and their antibacterial activity

Daria Różycka a, Zbigniew J. Leśnikowski b, Agnieszka B. Olejniczak a,∗

a Screening Laboratory, Institute of Medical Biology, Polish Academy of Sciences, 106 Lodowa St, Lodz, 93-232, Poland
b Laboratory of Molecular Virology and Biological Chemistry, Institute of Medical Biology, Polish Academy of Sciences, 106 Lodowa St, Lodz, 93-232, Poland

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A B S T R A C T
Antimicrobial resistance (AMR) is an exceptional increasing challenge for human health; it is urgently need to develop novel leads that can be developed to clinically useful drugs. The idea to modify old class of antibiotics that has been a cornerstone of medical has been dramatically refreshed searching for ways to overcome antibiotic-resistant bacteria. A very interesting development is the implementation of carboranes in design of pharmacologically active molecules. A series of novel penicillin G analogs bearing lipophilic 1,2-dicarba-closo-dodecarborane (ortho-carborane), 1,7-dicarba-closo-dodecarborane (meta-carborane), or 1,12-dicarba-closo-dodecarborane (para-carborane) boron clusters, instead of the phenyl ring, were synthesized. The boron-cluster penicillin G analogs were obtained via amidation of 6-aminopenicillanic acid (6-APA) with N-succinimidyl active esters containing ortho-, meta-, or para-carborane. Alternatively, analogs containing ortho- or para-carborane were synthesized using ortho- or para-carborane cluster acid chlorides. The compounds thus obtained were tested in vitro against gram-positive bacteria Staphylococcus aureus and gram-negative bacteria Klebsiella pneumoniae, Enterobacter cloacae, Acinetobacter baumannii, and Pseudomonas aeruginosa. The most potent inhibitor of gram-positive bacterial growth was compound 9, bearing a para-carborane cluster. Compounds 7 and 8 bearing ortho- or metha-carborane, respectively, were less active against S. aureus.

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1. Introduction

β-Lactam antibiotics are among the most commonly prescribed drugs. They share the same structural feature, the β-lactam ring, and the same mode of action, inhibition of bacterial cell-wall synthesis by acting as suicide substrates for penicillin-binding proteins (PBPs), which are transpeptidases or carboxypeptidases essential for cell-wall synthesis [1,2]. They form stable covalent adducts with PBPs, acylating the cysteine residue of PBPs at an active site and blocking transpeptidation reaction even in the presence of high concentrations of β-lactam antibiotics, and a decrease of the production of outer membrane proteins which allow the transfer of β-lactams through the outer membrane thereby lowering the effective concentration of antibiotics in the periplasm. Moreover, in gram-negative bacteria, efflux pumps which can export β-lactams outside the bacterial cells through the outer membrane can decrease the effective concentration of drugs in the periplasm [1]. As many as 40 structurally different β-lactams in 73 formulations are currently available for medical use [2]. The preservation and improved efficacy of this class of antimicrobial agents has been an ongoing challenge due to the continuous emergence of multidrug-resistant bacterial strains [2].

The focal point of our research was to recognize the potential of boron clusters as prospective building blocks [3] in the design of antibacterial agents. This article reports studies of the preparation and antibacterial activity of carborane cluster conjugates of 6-
aminopenicillanic acid (6-APA) — analogs of penicillin G.

We proposed to use dicarba-closo-dodecaboranes (C_{2}B_{10}H_{12}) — which are leads for the design and synthesis of novel molecules with potential antibacterial activity. The dicarba-closo-dodecaboranes are iicosahedral carbon-containing boron clusters that exist in three isomeric forms — ortho-, meta-, or para — depending on the mutual orientation of two carbon atoms within the boron cage [4]. The literature contains numerous examples of bioactive compounds in which carboranes are used as surrogates for lipophilic organic groups — most popularly, as a substitute for the phenyl ring. One of potential advantages of closo-carborane over the phenyl group is the increased hydrophobicity and larger surface area, both of which may facilitate hydrophobic contacts with nonpolar regions of the target protein [5]. The fact that compounds based on abiotic, polyhedral, boron-cluster scaffolds will be foreign to living organisms is of potential advantage, because it may be expected that enzymatic systems of living organism will be less efficient in metabolizing these molecules, thereby resulting in their higher stability in the biological environment.

Anti-infective drugs bearing essential boron components form an area of medicinal chemistry that is explored. Among antibiotics, Kerydin (tavaborate) — containing a single boron atom and being an approved topical solution for the treatment of onychomycosis caused by Trichophyton — is one example [6]. Vaborbactam (formerly RPX 7009) is a new peptidoglycan stem-peptides metabolite (Data). The FT-IR spectra of all of the conjugates were obtained by treatment of 6-APA conjugates with 1H, 13C, 11B NMR, FT-IR, and MS. Signals in 1H NMR showed the characteristic resonance band of the penicillin lactam at around 1735 cm⁻¹, carbonyl of the amide bond, and 1H NMR was observed the diagnostic, broad, BH stretching band at 2604, 2594, and 2604 cm⁻¹; furthermore, we observed the diagnostic, broad, BH stretching band at 2604, 2594, and 2604 cm⁻¹ for the ortho-, meta-, and para-isomers.

During the synthesis of conjugate 7, we observed a slow transformation of the electronneural closo-carborane cage into a negatively charged nido-cage, resulting in the formation of a derivative with nido-carborane with approximately 5% yield. The presence of this form of carborane was confirmed by 1H NMR and MS spectra. Changing reaction conditions such as an excess of TEA, a prolonged reaction time, or higher temperature led to formation of a mixture of closo-nido-derivatives of conjugate 7 that were difficult to separate. The synthesis of a conjugate with a fully opened form of cluster was unsuccessful and was abandoned. The formation of the nido-form of the carborane cluster during synthesis of compound 8 containing a meta-carborane cluster was not observed.

Originally, the nido-carborane monoanion (7,8-C_{2}B_{9}H_{12})⁻ was obtained by treatment of ortho-carborane or its substituted derivatives with methanolic potassium hydroxide as described by Hawthorne [12]. Other deboronation reagents such as tertiary amines, hydrazine, ammonia, piperidine, pyrrolidine and fluoride ions have been also used [13]. Removal of the BH group occurs during the synthesis of compounds via acid chlorides.

We developed an alternative method for the synthesis of compounds 7 and 9. The method is based on a simple nucleophilic substitution of halogen in acid chlorides 10 and 11 that were obtained from 1 or 3 by the amino group of 6-APA (Scheme 3). The acid chlorides 10 and 11 have been synthesized from acids 1 and 3 in the reaction with thionyl chloride (SOCl₂) [14], and then condensed with 6-APA in 2% sodium bicarbonate (NaHCO₃) water/acetone solution at room temperature. It is of practical importance, however, that both methods provide the desired products; the active ester method with the use of 4 and 6 affords the expected conjugate 7 and 9 with much higher yield: 67% and 95% vs. 36% and 38%, using acid chlorides 10 and 11. Nonetheless, we did not observe the formation of the nido-form of the carborane cluster during the synthesis of compounds via acid chlorides.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of 6-APA boron cluster conjugates

We obtained 6-APA conjugates 7–9 containing boron clusters in a simple three-step procedure. In the first step, we synthesized carboxylic acids 1–3 derivatives of all three carborane isomers — ortho-, meta-, or para- [8,9]. In the second step, acids 1–3 were transformed into corresponding active N-succinimidy esters 4–6 by using a standard reaction with N-hydroxysuccinimide (NHS) and N,N-diisopropylcarbodiimide (DIC) in dichloromethane (DCM) (Scheme 1). The N-succinimidy esters 4–6 were purified by flash chromatography and characterized by 1H NMR, FT-IR, and MS. Signals in 1H NMR showed the characteristic resonance band of the penicillin lactam at around 1735 cm⁻¹, carbonyl of the amide bond, and 1H NMR was observed the diagnostic, broad, BH stretching band at 2604, 2594, and 2604 cm⁻¹.

![Scheme 1. Synthesis of active esters 4–6 containing boron clusters: a) NHS, DIC, DCM, at room temperature for 2 h.](image-url)
2.2 Biological investigations

2.2.1 Antibacterial activity of 6-APA derivatives 7–9

The antibacterial activities of conjugates 7–9, 6-APA, and penicillin G were expressed as minimal inhibitory concentrations (MIC) values [15]. For this study, we selected bacteria responsible for nosocomial infectious worldwide; these include: Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and the Enterobacter species. Most of these are multidrug-resistant isolates, which pose one of the greatest challenges in clinical practice and are considered among the top three threats to global public health.

The conjugates 7–9 revealed different antibacterial activity against gram-positive S. aureus bacteria (ATCC 25923 and ATCC 29213) and four clinical isolates – 11 S. aureus 21 (meticillin-sensitive S. aureus [MSSA]) as well as 12 and 20 (meticillin-resistant S. aureus [MRSA]) – depending on the carborane cluster isomer attached to 6-APA. The MIC data are presented in Table 1. Conjugates 8, containing meta-carborane, and 9, containing para-carborane, showed the highest antibacterial activity against

Table 1

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC (µg/mL)</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>6-APA</th>
<th>penicillin G</th>
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<td>S. aureus ATCC 25923</td>
<td>256</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>0.25</td>
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<tr>
<td>S. aureus ATCC 29213</td>
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<td>256</td>
<td>128</td>
<td>128</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S. aureus 11 (MSSA)</td>
<td>&gt;256</td>
<td>256</td>
<td>128</td>
<td>128</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>S. aureus 12 (MRSA)</td>
<td>&gt;256</td>
<td>256</td>
<td>128</td>
<td>&gt;256</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>S. aureus 20 (MRSA)</td>
<td>&gt;256</td>
<td>256</td>
<td>128</td>
<td>&gt;256</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>S. aureus 21 (MSSA)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>128</td>
<td>&gt;256</td>
<td>128</td>
<td></td>
</tr>
</tbody>
</table>

Scheme 2. Synthesis of 6-APA conjugates modified with the carborane cluster 7–9: a) 4 (for conjugate 7), 5 (for conjugate 8), and 6 (for conjugate 9), DCM, and TEA, at room temperature for 18 h.

Scheme 3. An alternative pathway for the synthesis of 7 and 9: a) 6-APA, 10 (for conjugate 7), 11 (for conjugate 9), acetone, and NaHCO3, at room temperature for 4 h.
S. aureus 25923 (MIC 64 μg/mL). The ortho-carborane–6-APA conjugate 7 exhibited lower activity against S. aureus 25923 (MIC 256 μg/mL). Conjugate 9 was more active than conjugates 7 and 8 against S. aureus ATCC 29213 and all clinical isolates. 6-APA exhibited the same activity as compounds 8 and 9 against S. aureus 25923, and the same activity as compound 9 against S. aureus ATCC 29213 and S. aureus 11 (MSSA). However, compound 9 was more active than 6-APA against S. aureus 12 (MRSA), 20 (MRSA), and 21 (MSSA). Conjugates 7–9 exhibited lower antibacterial activity than penicillin G and ciprofloxacin against S. aureus ATCC 25923, ATCC 29213, 11 (MSSA), and 20 (MRSA); however, conjugate 9 exhibited the same activity (MIC 128 μg/mL) as penicillin G and ciprofloxacin against S. aureus 12 (MRSA) and 21 (MSSA).

We hypothesized that the higher activity of conjugate 9, as compared to conjugates 7 and 8, against S. aureus could be attributable to the different lipophilicities of ortho-/meta-/para-carborane. Carboranes are characterized by exceptional lipophilicity or amphiphilicity. This property makes them particularly suitable for use as lipophilic compounds in biologically active molecules, and as an inorganic mimic of the phenyl group. The high lipophilicity of many boron clusters and their derivatives can be explained by the presence of a partial negative charge located on boron-bound hydrogen atoms in BH groups and their “hydride-like” character. This prevents them from forming classical hydrogen bonds, which imbues the boron cluster with a lipophilic character [16]. The lipophilicity of carborane isomers increases in the following order: ortho-carborane < -meta-carborane < para-carborane.

Recently, we published methods for the synthesis of novel thymine derivatives bearing lipophilic, electron-neutral anions of 1,2-dicarba-closo-dodecaborane, 1,12-dicarba-closo-dodecaborane, or 7,8-dicarba-nido-undecaborane anion [17]. We observed different activities (effect of these compounds on Mycobacterium tuberculosis thymidylate kinase (TMPKmt) and on the growth of M. smegmatis and M. tuberculosis) depending on the closo-/nido-status of the boron cage.

Compounds 7–9 were inactive against gram-negative bacteria (Table S1 Supplementary Data), which may be attributed to the different gram-negative cell-wall structure.

2.2.2. In vitro cytotoxicity assay

Cytotoxicity was compared in two cell lines: an adult human keratinocyte line (HaCaT) and human fetal lung fibroblasts (MRC-5). The cytotoxicity of compounds 7–9 was established by measuring the 50% cytotoxic concentration (CC50) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining as described previously [18,19]: this was then compared with the cytotoxicities of 6-APA and penicillin G.

In general, low toxicity in the cell line tested was observed for the compounds 7–9, 6-APA, and penicillin G, although toxicity varies in different cell lines (Table 2). The compounds 7–9 clearly demonstrated a slightly higher toxicity, although the cytotoxic effect still remained low (from 196 to 274 μM).

3. Conclusions

In summary, in our quest for innovative antibacterial agents, we developed a method for the synthesis of 6-APA modified with an ortho-/meta-/para-carborane cluster (compounds 7–9). The synthesis of these new molecules was conveniently achieved using active esters and the amine group of 6-APA. Compounds 7 and 9 were obtained through an alternative pathway using acid chlorides bearing the ortho-/para-carborane cluster. The antibacterial activity of these conjugates has been evaluated in vitro against seven reference bacterial strains: S. aureus ATCC 25923, ATCC 29213, K. pneumoniae ATCC 700603 (ESBL+), E. cloacae DSM 6234, A. baumannii ATCC 17987, and P. aeruginosa ATCC 27853, and against four clinical isolates of S. aureus (two MRSA and two MSSA). The activity of the 6-APA and carborane conjugates against gram-positive bacteria decreases in the following order 9 < 8 < 7, which may be associated with their lipophilicity. The conjugates obtained were inactive against gram-negative bacteria. Conjugate 9 had higher activity than 6-APA against S. aureus 12 (MRSA) and 21 (MSSA), and the same activity as penicillin G against these bacteria.

These preliminary findings and the new bioconjugates designs proposed in this article form the basis for developing a new class of penicillin analogs containing carborane cluster with antibacterial activity. These compounds can be used as lead structures for further investigations. Studies on further modifications and optimization of this class of molecules are underway in our laboratory.

4. Experimental

4.1. Chemistry

4.1.1. Materials and methods

Most of the chemicals were obtained from the Aldrich Chemical Company and were used without further purification unless otherwise stated. Flash chromatography was performed using silica gel 60 (230–400 mesh, ASTM, Aldrich Chemical Company). Rf values refer to analytical TLC performed using pre-coated silica gel 60 F254 plates purchased from Sigma-Aldrich (Steinheim, Germany) and developed in the indicated solvent system. Carborane was purchased from KATCHEM spol. s r.o. (Rež/Prague, Czech Republic). Compounds were visualized by use UV light (254 nm), 0.5% acidic solution of PdCl2 in HCI/methanol for boron-containing derivatives or iodine vapor. The yields were not optimized. 1H NMR, 13C NMR, and 11B NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer equipped with a direct AT probe. The spectra for 1H, 13C, and 11B nuclei were recorded at 600.26 MHz, 150.94 MHz, and 192.59 MHz, respectively. Deuterated solvents were used as standards. For NMR, the following solvents were used: CDCl3 (δH = 7.25, δC = 39.70 ppm) DMSO-d6 (δH = 2.50, δC = 39.70 ppm). All chemical shifts (δ) are quoted in parts per million (ppm) relative to the external standards. The following abbreviations are used to denote the multiplicities: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet, quint = quintet, bs = broad singlet, and m = multiplet. J values are given in Hz. Mass spectra were performed on a Purlon S (Teledyne ISCO, USA). The ionization was achieved by electrospray ionization in the positive ion mode (ESI+) and negative ion mode (ESI–). The capillary voltage was set to 2.5 kV. The source temperature was 200 °C, and the desolvation temperature was 350 °C. Nitrogen was used as a desolvation gas (35 L/min, purity >99%, nitrogen
4.1. General procedure for the synthesis of 3-(1,2-dicarbocloso-
dodecaboran-1-yl)propanoic acid N-succinimidyl ester (4), 3-(1,7-
dicarbocloso-dodecaboran-1-yl)propanoic acid N-succinimidyl
ester (5), 3-(1,12-dicarbocloso-dodecaboran-1-yl)propanoic acid N-
succinimidyl ester (6) and 3-(1,12-dicarbocloso-dodecaboran-1-yl)
proponionic acid (1), 3-(1,7-dicarbocloso-dodecaboran-1-yl)propionic
acid (2), 3-(1,12-dicarbocloso-dodecaboran-1-yl)propionic acid (2) and
3-(1,12-dicarbocloso-dodecaboran-1-yl)propionic acid (3) (20–127 mg,
0.09–0.59 mmol) was dissolved in dry CH2Cl2 (6.5–40.7 mL). Next,
N-hydroxysuccinimide (1eq. for 1 and 3, 2 eq. for 2) and N,N-
diisopropylcarbodiimide (1eq. for 1 and 3, 2 eq. for 2) were added.
The solution was stirred at RT. After 2 h the reaction mixture was
concentrated in vacuo. The crude product was purified by chromatography
on silica gel (230–400 mesh) using CHCl3 as the eluant to give product 4–6.

4.1.2. General procedure for the synthesis of 3-(1,2-dicarbocloso-
dodecaboran-1-yl)propanoic acid N-succinimidyl ester (4), 3-(1,7-
dicarbocloso-dodecaboran-1-yl)propanoic acid N-succinimidyl
ester (5), 3-(1,12-dicarbocloso-dodecaboran-1-yl)propanoic acid N-
succinimidyl ester (6)

4.1.3. General procedure for the synthesis of (25S,5R,6R)-6-(3’-(1,2-
dicarbocloso-dodecaboran-1-yl)propanamido)-3,3-dimethyl-7-
oxo-4-thia-1-azacyclo[3.2.0]heptane-2-carboxylic acid (7),
(25S,5R,6R)-6-(3’-(1,7-dicarbocloso-dodecaboran-1-yl)
propanamido)-3,3-dimethyl-7-oxy-4-thia-1-azacyclo[3.2.0]
heptane-2-carboxylic acid (8), (25S,5R,6S)-6-(3’-(1,12-
dicarbocloso-
dodecaboran-1-yl)propanamido)-3,3-dimethyl-7-oxy-4-thia-
azacyclo[3.2.0]heptane-2-carboxylic acid (9)

6-APA (21.6–28.8 mg, 0.1–0.14 mmol) and TEA (56–108 μL) was
shaken with CH2Cl2 (0.40–0.7 mL) until the mixture was homogen-
ous. The mixture was cooled in ice and a N-succinimidyl ester
(1.2 eq.) was added in one portion with stirring. The reaction
mixture was allowed to warm to RT and left overnight. The reaction
was quenched by evaporation of the solvents. The crude product
was purified by column chromatography on silica gel (230–400
mesh) with a gradient of CH2O (0–10%) in CH2Cl2 as the eluent.
Chromatographically purified compound was dissolved in CH2Cl2
(5 mL) and washed with 3% HClaq. The organic phase was separated,
dried over MgSO4, filtered, and evaporated to dryness. The residue
was dissolved in CH2Cl2 (0.2 mL) and resultant solution was added
to a vigorously stirred petroleum ether (20 mL). A precipitate was
isolated by centrifugation. Precipitation was done twice. Then solid
was triturated with n-hexane (20 mL) to afford the title compounds
7–9.

Conjugate 7: pale white solid, yield 67%. TLC (CH2Cl2/MeOH,
90:10 v/v); Rf = 0.15; 1H NMR (DMSO-d6, 600.26 MHz): δ (ppm) =
11.70 (br s, 1H, COOH), 8.45 (d, 1H, NH, JH-NH = 6.00), 5.13
(br s, 1H, NH, CH-carborane), 4.91 (d, 1H, H-5, JH-H = 6.00), 4.42
(q, 1H, H-6), 3.63 (s, 1H, H-2), 2.43–2.40 (m, 4H, 2 x CH2-linker),
1.52 (s, 3H, CH3), 1.19 (s, 3H, CH3); 13C NMR (DMSO-d6, 175.95 MHz):
δ (ppm) = 170.29 (C7), 169.83 (COOH), 75.77 (C2), 72.16 (C5), 56.96
(C6, 51.91 (C8), 34.19 (C9), 22.88 (CH-linker), 27.27 (CH3),
26.75 (CH2); 11B{H BB} NMR (DMSO-d6, 224.50 MHz): δ (ppm) =
-3.18 (s, 2B), -6.23 (s, 2B), -9.83 (s, 4B), -11.58 to
-12.71 (m, 2B); FT-IR: ν (cm−1) = 2579 (BH), 1732 (C = O ben-
cyclic), 1667 (C = O ester), 722 (BB); ESI-MS: m/z: 415 [M++], calcd

Conjugate 8: pale white solid, yield 75%. TLC (CH2Cl2/MeOH,
90:10 v/v); Rf = 0.16; 1H NMR (DMSO-d6, 600.26 MHz): δ (ppm) =
8.41 (d, 1H, NH, JH-NH = 6.00), 4.92 (d, 1H, H-5, JH-H = 6.00),
4.43–4.40 (m, 4H, H-6, H-5), 4.02 (br s, 1H, NH, CH-carborane),
3.63 (s, 1H, H-2), 2.27–2.26 (m, 2H, CH2-linker), 2.21–2.20 (m, 2H, CH2-linker),
1.51 (s, 3H, CH3), 1.19 (s, 3H, CH3); 13C NMR (DMSO-d6, 175.95 MHz):
δ (ppm) = 170.32 (C7), 170.05 (COOH), 72.13 (C5), 56.91 (C6), 56.29
(CH-carborane), 51.88 (C2), 34.87 (CH-linker), 31.72 (CH2,
27.26 (CH2), 26.75 (CH); 11B{H BB} NMR (DMSO-d6, 224.50 MHz):
δ (ppm) = -4.49 (s, 2B), -11.11 (s, 4B), -13.59 (s, 2B), -14.93 (s, 2B);
FT-IR: ν (cm−1) = 2594 (BH), 1732 (C = O lactam), 1660 (C = O
ester), 728 (BB); ESI-MS: m/z: 415 [M++], 447 [M++-MeOH],

Conjugate 9: pale white solid, yield 55%. TLC (CH2Cl2/MeOH,
90:10 v/v); Rf = 0.23; 1H NMR (DMSO-d6, 600.26 MHz): δ (ppm) =
12.23 (br s, 1H, COOH), 8.31 (d, 1H, NH, JH-NH = 6.00), 4.87
(d, 1H, H-5, JH-H = 6.00), 4.36 (q, 1H, H-6), 3.63 (s, 1H, H-2),
2.07–2.04 (m, 2H, CH2-linker), 1.89–1.87 (m, 2H, CH2-linker),
1.51 (s, 3H, CH3), 1.18 (s, 3H, CH3), (CH-carborane signal overlapped
with signal from H2O in DMSO); 13C NMR (DMSO-d6, 175.95 MHz):
δ (ppm) = 170.34 (C7), 170.05 (COOH), 72.17 (C5), 59.91 (C6), 56.92
(CH-carborane), 51.85 (C2), 34.42 (CH-linker), 33.74 (CH2-
linker), 27.52 (CH2), 26.74 (CH); 11B{H BB} NMR (DMSO-d6, 224.50 MHz):
δ (ppm) = -12.62 (s, 5B), -15.04 (s, 5B); FT-IR: ν (cm−1) = 2604
(BH), 1737 (C = O lactam), 1658 (C = O amide), 730 (BB); ESI-MS:
m/z: 415 [M+], 447 [M++-MeOH], 470 [M++-MeOH+Na+], calcd
further use. The acid chlorides 10, 11 were then treated with a solution of 6-APA (15–40 mg, 0.07–0.18 mmol) in 2% NaHCO₃ (2.8–7.4 mL) diluted with acetone (2.1–5.5 mL). The reaction mixture was stirred 4 h at RT and concentrated under reduced pressure. The aqueous layer was then acidified with HCl (0.1 M), extracted with ethyl acetate and then washed with water dried over anhydrous MgSO₄. The ethyl acetate was rotary evaporated and triturated with n-hexane and petroleum ether.

Conjugate 7: pale white solid, yield 36%. TLC (CH₂Cl₂/MeOH, 90:10 v/v): \( R_I = 0.15; ^1H \text{ NMR (DMSO-}d_6, 600.26 \text{MHz)}: \delta \text{(ppm) = 12.45 (br s, 1H, COOH), 8.77 (d, 1H, NH, J_HH = 6), 5.46 (t, 2H, H5, H6), 5.17 (br s, 1H, CH-carborane), 4.21 (s, 1H, H2), 2.50 (t, 2H, CH2-linker), 2.50–1.50 (m, 10H, B₁₀H₁₀), 2.43 (t, 2H, CH₂-linker), 1.61 (s, 3H, CH3), 1.47 (s, 3H, CH3); ESI-MS: m/z: 414 [M-H]⁻, calcd for C₁₃H₂₆B₁₀N₂O₄S 415.26.}

Conjugate 9: pale white solid, yield 38%. TLC (CH₂Cl₂/MeOH, 90:10 v/v): \( R_I = 0.23; ^1H \text{ NMR (DMSO-}d_6, 600.26 \text{MHz)}: \delta \text{(ppm) = 8.66 (d, 1H, NH, J_HH = 6), 5.42–5.38 (m, 2H, H5, H6), 4.18 (s, 1H, H2), 3.65 (br s, 1H, CH-carborane), 2.10–1.60 (m, 10H, B₁₀H₁₀), 2.11–2.09 (m, 2H, CH₂-linker), 1.89–1.87 (t, 2H, CH₂-linker), 1.60 (s, 3H, CH3), 1.46 (s, 3H, CH3); ESI-MS: m/z: 414 [M-H]⁻, calcd for C₁₃H₂₆B₁₀N₂O₄S = 415.26.}

4.2. Biological activity evaluation

4.2.1. Antibacterial activity evaluation

The antimicrobics spectrum of tested compounds was evaluated by the minimal inhibitory concentrations (MIC) method by using the serial twofold dilution method under standard conditions, as described in the Committee Laboratory Standards (CLSI) reference method M7-A10 [15]. Reference and clinical bacterial strains were cultivated on tryptic soy agar (TSA) according to the recommendation of the American Type Culture Collection (ATCC). All strains were incubated for 24 h at 37 °C. The reference method (broth microdilution susceptibility test) was as follows: The concentrations of 6-APA, 7–9, 6-APA, and penicillin G were dissolved in dimethyl sulfoxide (DMSO), and the final DMSO concentration was brought to a maximum of 0.5% DMSO. A series of the twofold dilution method under standard conditions, as measured by the minimal inhibitory concentrations (MIC) method by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, the cells were seeded at 1 × 10⁵ cells per well in 96-well microtiter plates and allowed to proliferate at 37 °C for 18 h. Confluent monolayers of cells were treated with different concentrations of the compounds (1–500 μM) in triplicate or replaced with fresh medium (untreated controls). The compounds were dissolved in DMSO to form drug solutions and then suspended in supplemented growth medium. After incubation for 24 h at 37 °C in 5% CO₂, the number of viable cells was determined by the formazan method based on the conversion of the tetrazolium salt MTT to formazan by living cells [19]. The CC₅₀ was defined as the concentration required to reduce the cell growth by 50%, as compared to untreated controls. The cell variability was evaluated as the mean value density resulting from six mock-treated cell controls. The CC₅₀ was calculated by linear regression analysis of dose–response curves obtained from the data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jorgchem.2018.11.037.

References