Journal of Organometallic Chemistry 881 (2019) 19–24

Contents lists available at ScienceDirect

Journal of Organometallic Chemistry

journal homepage: www.elsevier.com/locate/jorganchem

Synthesis of boron cluster analogs of penicillin and their antibacterial activity

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ARTICLE INFO

Article history:
Received 7 November 2018
Received in revised form
29 November 2018
Accepted 30 November 2018
Available online 1 December 2018

Keywords:
Boron cluster
Carborane
6-APA
β-Lactam antibiotic
Antibacterial activity

ABSTRACT

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*-dodecaborane (*ortho*-carborane), 1,7-dicarba-*closo*-dodecaborane (*meta*-carborane), or 1,12-dicarba-*closo*-dodecaborane (*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 baumanii*, 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*.

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 preventing the remodeling of the peptidoglycan layer. These PBPs are traditionally categorized into high-molecular-weight PBPs (HMW-PBPs) and low-molecular weight PBPs (LMW-PBPs). The HMW-PBPs – which are further divided into classes A and B – are essential to bacterial cell survival. Proteins belonging to class A of HMW-PBPs catalyze the formation of the glycan chains, and both classes A and B of HMW-PBPs catalyze the crosslinking of peptidoglycan stem-peptides [1,2]. Bacteria have developed various mechanisms to resist β-lactam antibiotics. These include the production of lactamases which catalyze hydrolysis of the β-lactam ring, the production of low-affinity PBPs which catalyze the 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-

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https://doi.org/10.1016/j.jorganchem.2018.11.037
0022-328X/

aminopenicillanic acid (6-APA) – analogs of penicillin G.

We proposed to use dicarba-*closo*-dodecaboranes ($C_2B_{10}H_{12}$) – which are members of the vast boron cluster family of compounds – as leads for the design and synthesis of novel molecules with potential antibacterial activity. The dicarba-*closo*-dodecaboranes are icosahedral 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 (tavaborole) – 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 β-lactamase inhibitor based on a cyclic boronic acid pharmacophore [7].

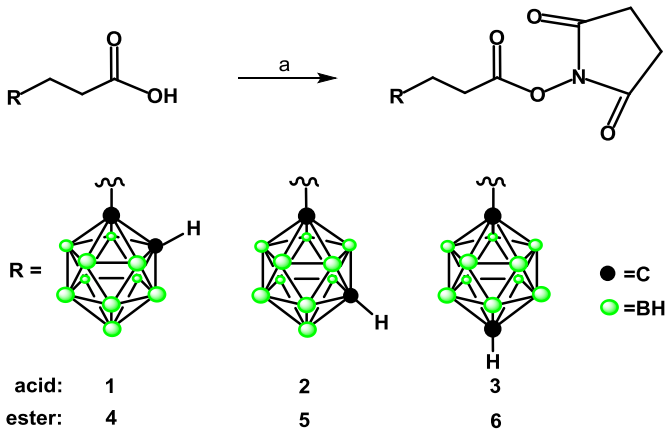
2. Results and discussion

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 carborane 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*-succinimidyl esters **4**–**6** by using a standard reaction with *N*-hydroxysuccinimide (NHS) and *N*,*N*′-diisopropylcarbodiimide (DIC) in dichloromethane (DCM) (Scheme 1).

The *N*-succinimidyl esters **4**–**6** were purified by flash chromatography and characterized by ^{1}H NMR, FT-IR, and MS (Supplementary Data). Compounds 3-(1,2-dicarba-*closo*-dodecaboran-1-yl) propanoic *N*-succinimidyl ester (**4**), 3-(1,7-dicarba-*closo*-dodecaboran-1-yl) propanoic *N*-succinimidyl ester (**5**), and 3-(1,12-dicarba-*closo*-dodecaboran-1-yl) propanoic *N*-succinimidyl ester (**6**) have been obtained with high yields (78–86%).



Scheme 1. Synthesis of active esters **4**–**6** containing boron clusters: a) NHS, DIC, DCM, at room temperature for 2 h.

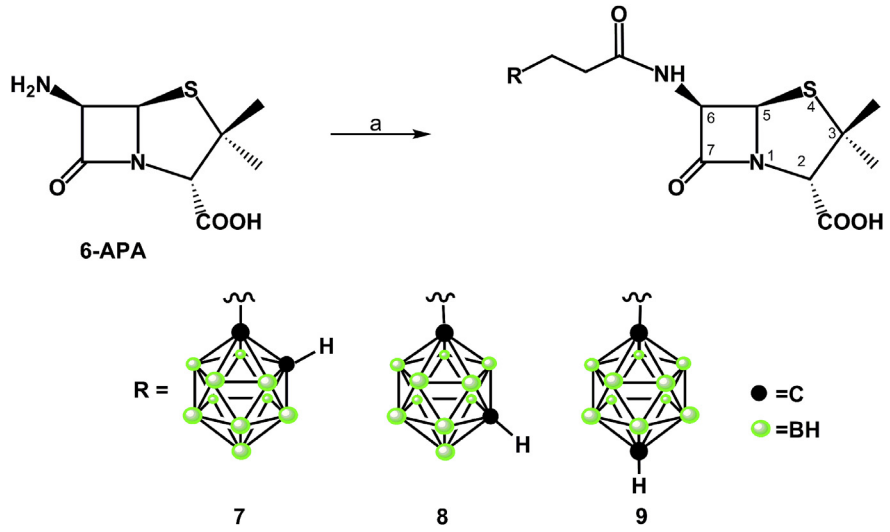
Using the novel, active ester, boron-cluster donors **4**–**6**, we obtained three 6-APA conjugates: (2S,5R,6R)-6-(3′-(1,2-dicarba-*closo*-dodecaboran-1-yl)propanamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (**7**), 2S,5R,6R)-6-(3′-(1,7-dicarba-*closo*-dodecaboran-1-yl)propanamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (**8**), and 2S,5R,6R)-6-(3′-(1,12-dicarba-*closo*-dodecaboran-1-yl)propanamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid (**9**) (Scheme 2). This was achieved simply in a one-step procedure by stirring 6-APA [10,11] with the active ester **4**, **5,** or **6**.

Briefly, 6-APA was dissolved in a mixture of DCM and trimethylamine (TEA), the obtained solution cooled in ice, and a suitable active ester was added. Reactions were conducted conveniently overnight at room temperature. After the reaction was completed, the crude products were purified by column silica gel chromatography. Conjugates **7**–**9** were obtained as white solids in good yields: 67% (**7**), 75% (**8**), and 55% (**9**). The structures of compounds **7**–**9** were confirmed by ^{1}H, ^{13}C, ^{11}B NMR, FT-IR, and MS. Signals in ^{1}H NMR showed the characteristic resonance band of the penicillin as well as of the carborane cluster (Figs. S10, S14, and S18 Supplementary Data). The FT-IR spectra of all of the conjugates **7**–**9** display a characteristic absorption band for the carbonyl of the β-lactam at around 1735 cm^{-1}, carbonyl of the amide bond, and carboxylate carbonyl between 1650 and 1680 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.

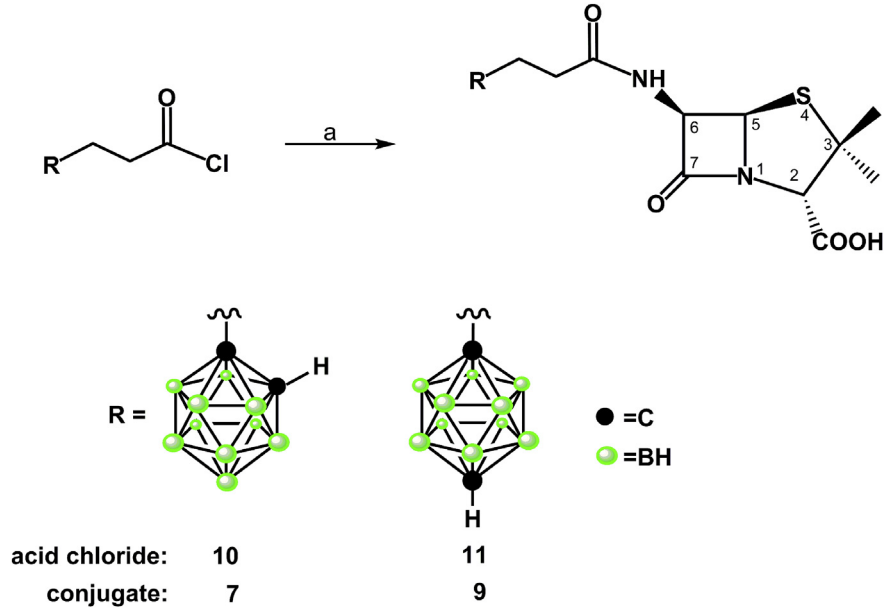
During the synthesis of conjugate **7**, we observed a slow transformation of the electroneutral *closo*-carborane cage into a negatively charged *nido*-cage, resulting in the formation of a derivative with *nido*-carborane with approximately 5% yield. The presence of this form of carborane was confirmed by ^{1}H 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-$C_2B_9H_{12}$)(−1) 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 regiospecifically at the most electropositive BH vertex B(3) or B(6) (8).

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 ($SOCl_2$) [14], and then condensed with 6-APA in 2% sodium bicarbonate ($NaHCO_3$) 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 conjugates **7** and **9** with much higher yield: 67% and 55% *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.



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 $NaHCO_3$, at room temperature for 4 h.

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 (methicillin-sensitive *S. aureus* [MSSA]) as well as 12 and 20 (methicillin-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
In vitro antibacterial activity of compounds 7–9, 6-APA, and penicillin G. against gram-positive bacteria.

Microorganism	MIC (μg/mL)				
	7	8	9	6-APA	penicillin G
S. aureus ATCC 25923	256	64	64	64	0.25
S. aureus ATCC 29213	>256	256	128	128	1
S. aureus 11 (MSSA)	>256	256	128	128	4
S. aureus 12 (MRSA)	>256	>256	128	>256	128
S. aureus 20 (MRSA)	>256	>256	128	>256	32
S. aureus 21 (MSSA)	>256	>256	128	>256	128

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* 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 lipophilicity of *ortho*-/*meta*-/*para*-carborane. Carboranes are characterized by exceptional lipophilicity or amphiphilicity. This property makes them particularly suitable for use as lipophilic components 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 lipopohilic, electron-neutral anions of 1,2-dicarba-*closo*-dodecaborane, 1,12-dicarba-*closo*-dodecaborane, or 7,8-dicarba-*nido*-undecaborate 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 (CC_{50}) 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 have been evaluated *in vitro* against seven reference bacterial strains: *S. aureus* ATCC 25923, ATCC 29213, *K. pneumoniae* ATCC 700603 (ESβL+), *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). R_f 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. (Reź/Prague, Czech Republic). Compounds were visualized by use UV light (254 nm), 0.5% acidic solution of $PdCl_2$ in HCl/methanol for boron-containing derivatives or iodine vapor. The yields were not optimized. ^{1}H NMR, ^{13}C NMR, and ^{11}B NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer equipped with a direct ATM probe. The spectra for ^{1}H, ^{13}C, and ^{11}B 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: $CDCl_3$ ($\delta_H = 7.25$, $\delta_C = 39.70$ ppm) DMSO-d_6 ($\delta_H = 2.50$, $\delta_C = 39.70$ 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, ddd = doublet of doublets of doublets, t = triplet, dt = doublet of triplets, q = quartet, quin = quintet, bs = broad singlet, and m = multiplet. J values are given in Hz. Mass spectra were performed on a PurIon 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

Table 2
Cytotoxicity of compounds 7–9, 6-APA, penicillin G.

Cell line	CC_{50} (μM)				
	7	8	9	6-APA	penicillin G
HaCaT	228.09 ± 6.55	256.14 ± 6.02	195.70 ± 6.80	248.95 ± 5.45	236.83 ± 7.31
MRC-5	274.38 ± 3.40	271.43 ± 3.71	246.45 ± 2.93	341.67 ± 1.84	381.65 ± 0.74

generator EURUS35 LCMS, E-DGSi SAS, France). The theoretical molecular masses of the compounds were calculated using the "Show Analysis Window" option in the ChemDraw Ultra 12.0 program. The calculated *m/z* corresponds to the average mass of the compounds consisting of natural isotopes. Infrared absorption spectra (IR) were recorded using a Nicolet 6700 Fourier-transform infrared spectrometer from Thermo Scientific equipped with a ETC EverGlo* source for the IR range, a Ge-on-KBr beam splitter, and a DLaTGS/KBr detector with a smart orbit sampling compartment and diamond window. The samples were placed directly on the diamond crystal, and pressure was added to make the surface of the sample conform to the surface of the diamond crystal.

Synthesis of acids **1**–**3** was prepared according to the literature [8,9].

Synthesis of acid chlorides **10**, **11** was prepared according to the literature [14].

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

3-(1,2-Dicarba-*closo*-dodecaboran-1-yl)propionic acid (**1**), 3-(1,7-dicarba-*closo*-dodecaboran-1-yl)propionic acid (**2**), 3-(1,12-dicarba-*closo*-dodecaboran-1-yl)propionic acid (**3**) (20–127 mg, 0.09–0.59 mmol) was dissolved in dry CH_2Cl_2 (6.5–40.7 mL). Next, *N*-hydroxysuccinimide (1eq. for **1** and **3**, 2 eq. for **2**) and *N,N′*-diisopropylcarbodiimid (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 column chromatography on silica gel (230–400 mesh) using $CHCl_3$ as the eluent to give product **4**–**6**.

N-succinimidyl ester **4**: white solid, yield 78%. TLC (CH_2Cl_2/MeOH, 95:5 v/v): $R_f = 0.58$; ^{1}H NMR ($CDCl_3$, 600.26 MHz): δ (ppm) = 3.73 (br s, 1H, CH-carborane), 2.91–2.87 (m, 6H, CH_2-linker, 2 × CH_2 succinimide), 2.70 (t, 2H, CH_2-linker), 2.75–1.70 (br m, 10H, $B_{10}H_{10}$); FT-IR: ν (cm^{-1}) = 2572 (BH), 1817 (C═O succinimide), 1779 (C═O succinimide), 1736 (C═O ester), 721 (BB); ESI-MS: *m/z*: 345 [*M*-H+MeOH]$^-$, calcd for $C_9H_{19}B_{10}NO_4 = 314.23$.

N-succinimidyl ester **5**: white solid, yield 78%. TLC (CH_2Cl_2/MeOH, 90:10 v/v): $R_f = 0.80$, (Et_2O): $R_f = 0.2$; ^{1}H NMR ($CDCl_3$, 600.26 MHz): δ (ppm) = 2.99 (br s, 1H, CH-carborane), 2.86 (br s, 4H, 2 × CH_2 succinimide), 2.74–2.72 (m, 2H, CH_2-linker), 2.43 (t, 2H, CH_2-linker), 2.75–1.75 (br m, 10H, $B_{10}H_{10}$); FT-IR: ν (cm^{-1}) = 2605 (BH), 1817 (C═O succinimide), 1780 (C═O succinimide), 1737 (C═O ester), 725 (BB); ESI-MS: *m/z*: 216 [$C_2B_{10}H_{11}CH_2CH_2COO$]$^-$, calcd for $C_9H_{19}B_{10}NO_4 = 314.23$.

N-succinimidyl ester **6**: white solid, yield 86%. TLC (CH_2Cl_2/MeOH, 90:10 v/v): $R_f = 0.42$; ^{1}H NMR ($CDCl_3$, 600.26 MHz): δ (ppm) = 2.84 (br s, 4H, 2 × CH_2 succinimide), 2.71 (br s, 1H, CH-carborane), 2.55–2.52 (m, 2H, CH_2-linker), 2.10 (t, 2H, CH_2-linker), 2-75-1.75 (br m, 10H, $B_{10}H_{10}$); FT-IR: ν (cm^{-1}) = 2611 (BH), 1818 (C═O succinimide), 1781 (C═O succinimide), 1738 (C═O ester), 727 (BB); ESI-MS: *m/z*: 345 [*M*-H+MeOH]$^-$, calcd for $C_9H_{19}B_{10}NO_4 = 314.23$.

*4.1.3. General procedure for the synthesis of (2S,5R,6R)-6-(3′-(1,2-dicarba-closo-dodecaboran-1-yl)propanamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (**7**), (2S,5R,6R)-6-(3′-(1,7-dicarba-closo-dodecaboran-1-yl) propanamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid (**8**), (2S,5R,6R)-6-(3′-(1,12-dicarba-closo-dodecaboran-1-yl)propanamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[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 CH_2Cl_2 (0.40–0.7 mL) until the mixture was homogenous. The mixture was cooled in ice a the *N*-succinimidyl ester **4**–**6** (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 compound was purified by column chromatography on silica gel (230–400 mesh) with a gradient of CH_3OH (0–10%) in CH_2Cl_2 as the eluent. Chromatographically purified compound was dissolved in CH_2Cl_2 (5 mL) and washed with 3% $HCl_{aq.}$ The organic phase was separated, dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was dissolved in CH_2Cl_2 (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 (CH_2Cl_2/MeOH, 90:10 v/v): $R_f = 0.15$; ^{1}H NMR (DMSO-d_6, 600.26 MHz): δ (ppm) = 11.70 (br s, 1H, COOH), 8.45 (d. 1H, NH, $J_{HH} = 6.00$), 5.13 (br s, 1H, CH-carborane), 4.91 (d, 1H, H-5, $J_{HH} = 6.00$), 4.42 (q, 1H, H-6), 3.63 (s, 1H, H-2), 2.43–2.40 (m, 4H, 2 x CH_2-linker), 1.52 (s, 3H, CH_3), 1.19 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 175.95 MHz): δ (ppm) = 170.29 (C7), 169.83 (COOH), 75.77 (C3), 72.16 (C5), 56.96 (C6), 51.91 (C2), 34.19 (CH-linker), 32.28 (CH-linker), 27.27 (CH_3), 26.75 (CH_3); ^{11}B{H BB} NMR (DMSO-d_6, 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 β-lactam), 1667 (C═O amide), 722 (BB); ESI-MS: *m/z*: 415 [*M*]$^+$, calcd for $C_{13}H_{26}B_{10}N_2O_4S = 415.26$.

Conjugate **8**: pale white solid, yield 75%. TLC (CH_2Cl_2/MeOH, 90:10 v/v): $R_f = 0.16$; ^{1}H NMR (DMSO-d_6, 600.26 MHz): δ (ppm) = 8.41 (d. 1H, NH, $J_{HH} = 6.00$), 4.92 (d, 1H, H-5, $J_{HH} = 6.00$), 4.43–4.40 (m, 1H, H-6), 4.02 (br s, 1H, CH-carborane), 3.63 (s, 1H, H-2), 2.27–2.26 (m, 2H, CH_2-linker), 2.21–2.20 (m, 2H, CH_2-linker), 1.51 (s, 3H, CH_3), 1.19 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6,175.95 MHz): δ (ppm) = 170.32 (C7), 170.05 (COOH), 72.13 (C3), 56.91 (C6), 56.29 (CH-carborane), 51.88 (C2), 34.87 (CH-linker), 31.72 (CH-linker), 27.26 (CH_3), 26.75 (CH_3); ^{11} B{H BB} NMR (DMSO-d_6, 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 β-lactam), 1660 (C═O amide), 728 (BB); ESI-MS: *m/z*: 415 [*M*]$^+$, 447 [*M*+MeOH]$^+$, 469 [*M*+MeOH+Na]$^+$, calcd for $C_{13}H_{26}B_{10}N_2O_4S = 415.26$.

Conjugate **9**: pale white solid, yield 55%. TLC (CH_2Cl_2/MeOH, 90:10 v/v): $R_f = 0.23$;^{1}H NMR (DMSO-d_6, 600.26 MHz): δ (ppm) = 12.23 (br s, 1H, COOH), 8.31 (d. 1H, NH, $J_{HH} = 6.00$), 4.87 (d, 1H, H-5, $J_{HH} = 6.00$), 4.36 (q, 1H, H-6), 3.63 (s, 1H, H-2), 2.07–2.04 (m, 2H, CH_2-linker), 1.89–1.87 (m, 2H, CH_2-linker), 1.51 (s, 3H, CH_3), 1.18 (s, 3H, CH_3), (CH-carborane signal overlapped with signal from H_2O in DMSO); ^{13}C NMR (DMSO-d_6,175.95 MHz): δ (ppm) = 170.34 (C7), 170.05 (COOH), 72.17 (C3), 59.91 (C6), 56.92 (CH-carborane), 51.85 (C2), 34.42 (CH-linker), 33.74 (CH-linker), 27.52 (CH_3), 26.74 (CH_3); ^{11}B{H BB} NMR (DMSO-d_6, 224.50 MHz): δ (ppm) = −12.62 (s, 5B), −15.04 (s, 5B); FT-IR: ν (cm^{-1}) = 2604 (BH), 1737 (C═O β-lactam), 1658 (C═O amide), 730 (BB); ESI-MS: *m/z*: 415 [*M*]$^+$, 447 [*M*+MeOH]$^+$, 470 [*M*+MeOH+Na]$^+$, calcd for $C_{13}H_{26}B_{10}N_2O_4S = 415.26$.

*4.1.4. General procedure for the synthesis of conjugates **7**, **9** using 3-(1,2-dicarba-closo-dodecaboran-1-yl)propionic acid chloride (**10**), 3-(1,12-dicarba-closo-dodecaboran-1-yl)propionic acid (**11**)*

3-(1,2-Dicarba-*closo*-dodecaboran-1-yl)propionic acid (**1**), 3-(1,12-dicarba-*closo*-dodecaboran-1-yl)propionic acid (**3**) (15–40 mg, 0.07–0.18 mmol) was refluxed with freshly distilled $SOCl_2$ for 4 h. After the completion of reaction, excess of $SOCl_2$ (27–41 mmol) was removed under reduced pressure to afford the acid chlorides **10**, **11** which were dissolved in anhydrous acetone for

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% $NaHCO_3$ (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 $MgSO_4$. The ethyl acetate was rotary evaporated and triturated with n-hexane and petroleum ether.

Conjugate **7**: pale white solid, yield 36%. TLC (CH_2Cl_2/MeOH, 90:10 v/v): $R_f = 0.15$; ^{1}H NMR (DMSO-d_6, 600.26 MHz): δ (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, CH_2-linker), 2.50–1.50 (m, 10H, $B_{10}H_{10}$), 2.43 (t, 2H, CH_2-linker), 1.61 (s, 3H, CH_3), 1.47 (s, 3H, CH_3); ESI-MS: *m/z*: 414 $[M\text{-H}]^-$, calcd for $C_{13}H_{26}B_{10}N_2O_4S = 415.26$.

Conjugate **9**: pale white solid, yield 38%. TLC (CH_2Cl_2/MeOH, 90:10 v/v): $R_f = 0.23$; ^{1}H NMR (DMSO-d_6, 600.26 MHz): δ (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, $B_{10}H_{10}$), 2.11–2.09 (m, 2H, CH_2-linker), 1.89–1.87 (t, 2H, CH_2-linker), 1.60 (s, 3H, CH_3), 1.46 (s, 3H, CH_3); ESI-MS: *m/z*: 414 $[M\text{-H}]^-$, calcd for $C_{13}H_{26}B_{10}N_2O_4S = 415.26$.

4.2. Biological investigation

4.2.1. Antibacterial activity evaluation

The antimicrobial 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 conjugates 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 compound dilutions were diluted with cation–adjusted Mueller–Hinton broth (CAMHB). We dispended 90 μL aliquots of this solution into microdilution sterile plates (Medlab Products). Then, 10 μL of bacteria inoculum was added. The final test concentration of bacteria was approximately 5×10^5 CFU mL^{-1} (5×10^4 CFU/well). The final concentration of compounds **7**–**9**, 6-APA, and penicillin G compounds ranged from 0.125 to 256 μg/mL all in twofold dilution steps. All experiments for each sample were conducted twice. These plates were incubated at 35 °C for 18 h.

The MIC was defined as the lowest drug concentration that completely inhibits growth of the organism in the microdilution wells, as detected by the unaided eye. Antimicrobial activities of the newly synthesized compounds were tested *in vitro* against seven reference bacterial strains (*S. aureus:* ATCC 25923, ATCC 29213, *K. pneumoniae* ATCC 700603, *E. cloacae* DSM 6234, *A. baumannii* ATCC 17987, and *P. aeruginosa* ATCC 27853) and four clinical isolates of *S. aureus* (two MRSA and two MSSA).

4.2.2. In vitro cytotoxicity assay

The MRC-5 (ATCC CCL-171, American Type Culture Collection, Rockville, MD, USA) and HaCaT (CLS, Cell Line Service GmbH, Eppelheim, Germany) cells were propagated in Eagle's minimal essential medium (MEM) and Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/mL penicillin G – 100 mg/mL streptomycin. Both cell lines were cultured at 37 °C in a humidified 5% CO_2 atmosphere. The cytotoxic activities of compounds were examined against cell lines by using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT). Briefly, the cells were seeded at 1×10^4 cells per well in 96-well microtiter plates and allowed to proliferate at 37 °C for 16 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% CO_2, 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 CC_{50} 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 CC_{50} was calculated by linear regression analysis of dose–response curves obtained from the data.

Acknowledgments

This study was supported by an internal grant from the Institute of Medical Biology, Polish Academy of Sciences, and in part by the National Science Centre, Poland (grant no. 2014/14/E/ST5/00577). A PurIon S mass spectrometer with CombiFlash Rf+ was purchased using funds from the National Science Centre, Poland (grant no. 2014/14/E/ST5/00577).

Appendix A. Supplementary data

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

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