Article

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Microwave-Facilitated SPOT-Synthesis of Antibacterial Dipeptoids.

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KEYWORDS. Peptoids, antibacterial, ampetoids, antimicrobials, MRSA, Staphylococcus aureus, SPOT-synthesis, cellulose, peptidomimetics, combinatorial library.

ABSTRACT: With microwave irradiation, the submonomer synthesis of dipeptoids on functionalized cellulose can be accelerated with good yields and purity. Optimization provided a library of 96 dipeptoids. From these, 29 compounds were found with an antibacterial activity against MRSA at a concentration of 25 μM. Large nonpolar residues, such as undecylamine and dehydroabiethylamine, are the key components engendering the observed antibacterial activity of these peptoids.

Introduction

The rapidly increasing resistance of bacteria to currently available antibiotics will be one of the main problems in the treatment of bacterial infections in the future. More than 70% of infections in US hospitals are already resistant to at least one of the conventional antibacterial drugs.[1-2] Antibacterial agents, including peptides (AMPs) and their corresponding peptidomimetics have been known since the 1920s.[3-4] Recent advances in the field of medicinal chemistry are leading to the fact that the modification of existing antibiotics and discovery of novel antibiotics classes is the focus of science today.

Helical, amphiphilic *N*-alkyl glycine oligomers, or "peptoids", were described by Barron *et al.* to imitate the effect of the natural antimicrobial peptide magainin. The authors succeeded in detecting the activity of these "ampetoids" (antimicrobial peptoid oligomers) against various pathogenic bacteria, as well as investigating their function depending on their structure.[6]

However, a trend is emerging towards the study of smaller "minimal" peptoid structures, which can be easily and quickly investigated using combinatorial solid-phase chemistry. The first minimal peptoids with efficacious antibacterial activities were found by screening a library containing 845 trimeric peptoids. A particularly important aspect was the introduction of a side chain with a terminal amino function, which had the greatest effect in the middle position.[7-8] Similar results were also obtained through the screening of a library with 10,000 trimers.[9] All active structures had a combination of cationic groups and hydrophobic side chains. Further study of minimal peptoids as antibacterial agents has slowed. In view of their straightforward synthesis and the dire need for new anti-infective agents, more research into minimal peptoids is needed to examine their efficacy as antibacterial compounds.

SPOT-Synthesis

Combinatorics is a well known approach in chemical biology and medical chemistry research.[10] Solid phase synthesis is one of the most frequent strategies for preparation of combinatorial libraries, including the synthesis of peptoid libraries.[11-12] However, the conventional polymeric supports (i.e. insoluble resin beads) have some disadvantages. These hydrophobic polymers are often expensive, fragile, difficult to manipulate and often incompatible with biological assays performed directly on the carrier. The disadvantages of conventional solid phases has motivated the development of new synthesis and screening platforms for combinatorial chemistry.

Frank *et al.* solved some of these carrier problems by development of the SPOT-synthesis technique[13] that was originally used in peptide synthesis.[14] The advantages of the SPOT-synthesis is based on the local addressed synthesis of small molecules/peptides using planar cellulose as a solid support, in order to form matrices of molecules.[15] In contrast to the polystyrene resins usually applied in solid-phase synthesis, the hydrophilic cellulosic membranes are easy to manipulate during the synthesis

and washing steps and have surprisingly high mechanical stability.[16] In addition, the hydrophilic cellulose is compatible with a range of biological screening methods directly on the carrier. The introduction of heating systems such as drying ovens and the unconventional microwave irradiation have also significantly reduced long reaction times.[17] Hence, the SPOT-synthesis provides a robust method for the synthesis and screening of large, parallel libraries of a range of molecules, including peptoids.[18-19]

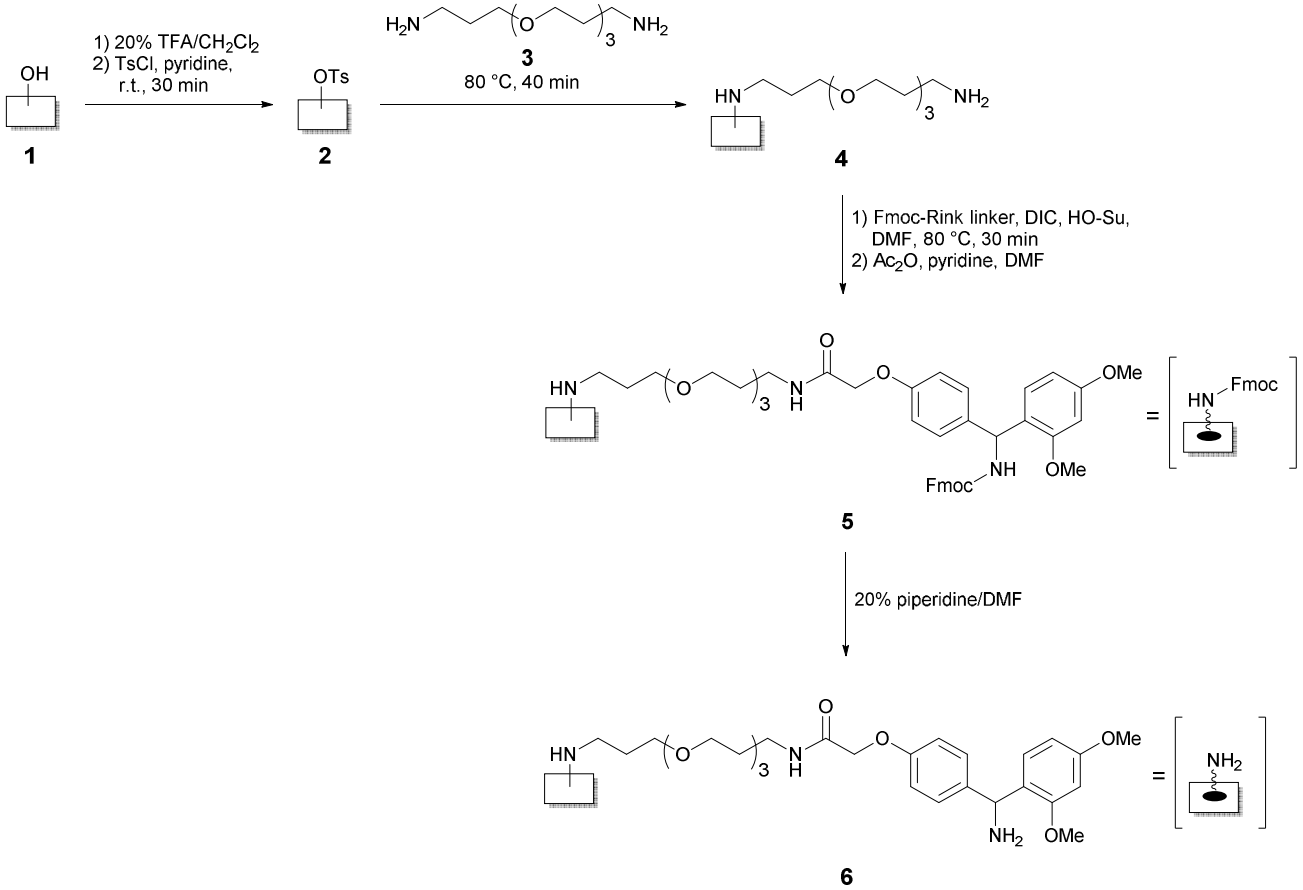
Herein, we report the microwave-assisted SPOT-synthesis of dipeptoids on functionalized cellulose as a carrier and its optimization using microwaves. Such new combinatorial method easily provided a library of dipeptoids in high purity. This library was subsequently tested for its antibacterial activity against methicillin-resistant *Staphylococcus aureus* strains (MRSA), and several active compounds were identified. Re-synthesis and testing of selected active agents served to validate their biological activity, and our method overall.

Results and Discussion

Preparation of the Cellulose Support

For SPOT-synthesis, Whatman 1 CHR cellulose chromatography paper was modified using the optimized method of Blackwell *et al.*[20] To activate cellulose, the primary alcohols of support were directly tosylated. The diamine **3** was introduced as a "spacer" element prior to coupling of the Rink-Amide linker, using a combination of *N,N'*-diisopropylcarbodiimide (DIC) and *N*-hydroxysuccinimide (HO-Su) as an activator. Thereafter, the support was treated to blanket acetylation to cap any free amino groups in order to avoid undesired side reactions (Scheme 1).

The loading level of support **5** was quantified as 400 - 1100 nmol/cm² by measuring the Fmoc-deprotection rate. The linker of support **5** was deprotected immediately prior to use by treatment with 20% piperidine in DMF.



Scheme 1. **Preparation of functionalized cellulose support** 6 **and linker installation.**

SPOT-Synthesis Optimization for Peptoids

In order to optimize the reaction conditions for peptoid synthesis on cellulose, different methods were evaluated.

Our studies were initiated through the synthesis and study of a small set of dipeptoids (**7** - **10**, Figure 1). This test set was prepared using the standard submonomer peptoid synthesis approach[21] in which we varied several reaction conditions, shown in Scheme 2. These submonomer syntheses all included microwave heating to accelerate each step, to expedite library generation.

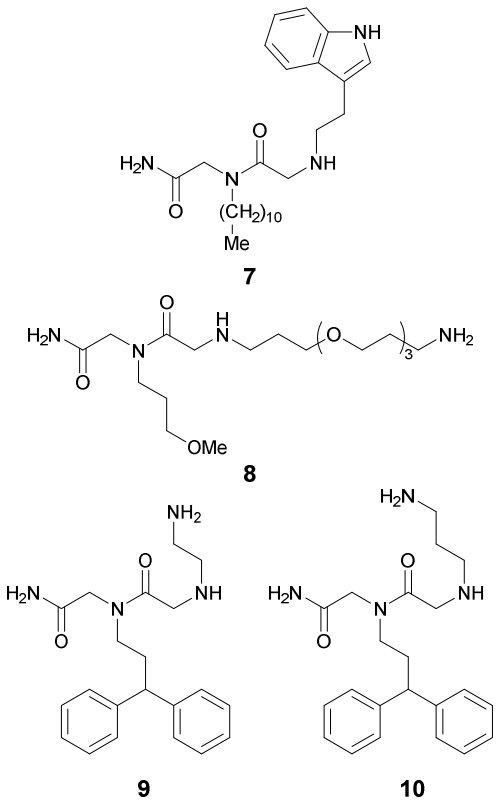
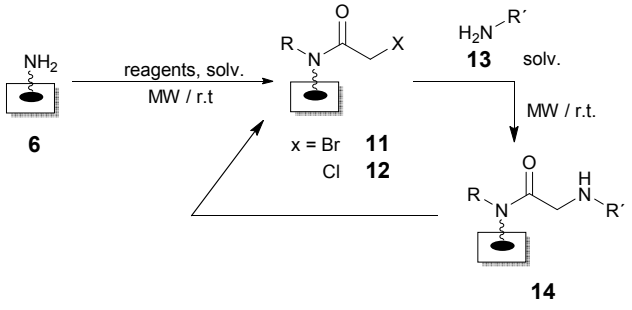


Figure 1. Dipeptoids 7 - 10 used for SPOT-synthesis optimization.



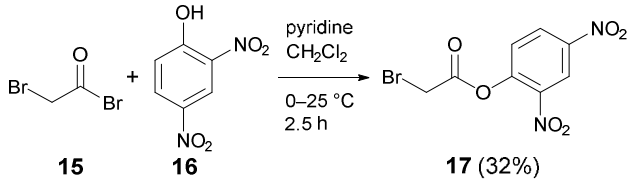
Scheme 2. General SPOT-synthesis of peptoids. R, R´ = amine residues (see Figure 3).

First, the free amine on the support was acylated by an active ester **17** or an activated haloacetic acid, such as bromoacetic acid **11** or cloroacetic acid **12**, with *N,N*-diisopropylcarbodiimide (DIC). 2,4-Dinitrophenyl bromoacetic acid ester (**17**) was synthesized from bromoacetic bromide (**10**) and 2,4-dinitrophenol (**16**), as shown in Scheme 3. In the synthesis of **17,** dry solvents as well as dry starting material **16** was found to play a crucial role. Under dry conditions, the product **17** can be easily purified by crystallization.

Thereafter we introduced frequently used primary amines (**13**) in peptoid chemistry such as pentan-1-amine, tryptamine, 3,3-diphenylpropylamine and 3-methoxypropan-1-amine, without changing their sequence in the peptoids backbone, to ensure reproducibility of the syntheses. Mono Boc-protected diamines[22] were used to avoid side reactions. The synthetic cycle of acetylation/amination was repeated to obtain dipeptoids. Cleavage from the support was carried out under TFA vapor in desiccator for 30 min, using the method of Blackwell et al.[20] The yields and purities of the synthesized dipeptoids **7** - **10** (Figure 1) were determined using HPLC and LCMS measurements, and the eight synthesis methods were systematically compared (Table 1).

The methods using bromoacetic acid (11) or chloroacetic acid (12) in DMF and microwave (entries 6 - 7, Table 1) did not provide satisfying purities. An overview of the best methods is given in

Table **2**.



Scheme 3. Synthesis of active ester **17**.

Table 1. Summary of the conditions for the SPOT-synthesis.

Entry	Solv.	Coupling reagent	Conditions
1	NMP	ester 17	r.t.; acylation 15 min; amination 45 min
2	NMP	DIC/bromoacetic acid (11)	MW; acylation 30 s, 500 W; amination 90 s, 550 W
3	NMP	DIC/bromoacetic acid (11)	r.t.; acylation 15 min; amination 45 min
4	NMP	ester 17	MW; acylation 30 s, 500 W; amination 90 s, 550 W
5	DMF	ester 17	MW; acylation 30 s, 500 W; amination 90 s 550 W
6	DMF	DIC/bromoacetic acid (11)	MW; acylation 30 s, 500 W; amination 90 s 550 W
7	DMF	DIC/chloroacetic acid (17)	MW; acylation 30 s, 500 W; amination 90 s 550 W
8	DMF	DIC/chloroacetic acid (12)	r.t.; acylation 15 min; amination 45 min

Table 2. Comparison of the purities of the SPOT-synthesis achieved by different methods. Purities were determined by HPLC (218 nm).

Entry	Conditions	7 [%]	8 [%]	9 [%]	10 [%]
1	ester 17/NMP/r.t.	89	23	60	77
2	11/DIC/NMP/MW	80	47	51	80
3	11/DIC/NMP/r.t.	71	45	71	70
4	ester 17/NMP/MW	80	23	12	4
5	ester 17/DMF/MW	81	61	59	68

The use of the ester **17** in combination with NMP and microwave irradiation does not appear to be particularly advantageous for the purities of peptoids **7** - **9** (**entry 4**). Ester **17**, on the other hand, reached similarly good yields (**entry 1**) at room temperature. These methods, described in entries 1 and 3, are not as fast and simple compared to carrying out the method with DIC and bromoacetic acid (**11**) in NMP and microwave irradiation (**entry 2**). In addition, the ester **17** must first be prepared, while all other starting materials are commercially available. Hence, the method with DIC and bromoacetic acid (**11**), 1-methyl-2-pyrrolidinone (NMP) as solvent and microwave irradiation (**entry 2,** Table 1) was selected for further work.

Identification of Suitable Amines for Peptoid Libraries

With the optimized method for microwave-assisted SPOT-synthesis in hand, a combinatorial library of dipeptoids was synthesized. However, prior to library synthesis, the amines that were suitable for such a library had to be identified. The amines should react well under the chosen conditions and provide the expected peptoids in high purity, since they would be used directly for biological tests without further purification.

The most promising amines were determined by SPOT-synthesis of two test libraries. In the first library, 3,3-diphenylpropylamine (**29**) was incorporated as the first amine in the peptoid synthesis and the amines **20** - **53** (**Error! Reference source not found.**) were varied in the second position. In the second library, amines **20** - **53** were varied in the first position while **29** was used as the second amine (Figure 2). The test libraries would thus report which amines can be installed in both positions of the dipeptoid scaffold.

After synthesis, the dipeptoids were cleaved from the membrane in TFA vapor and analyzed by HPLC and LCMS to determine their purities and confirm their existence. With the amines **20 – 37,** purities of more than 70% were achieved in both dipeptoid libraries. Therefore, they were selected for the final SPOT-synthesis dipeptoid library. The secondary amines **35**, **36**, **50** and **52** were selected for use only in the second position of the peptoids, as in the first position they did not undergo high yielding acylation reactions. The amines **34** and tryptamine (**24**) were not incorporated into the first position since their additional amino functional groups could be acylated as a side reaction.

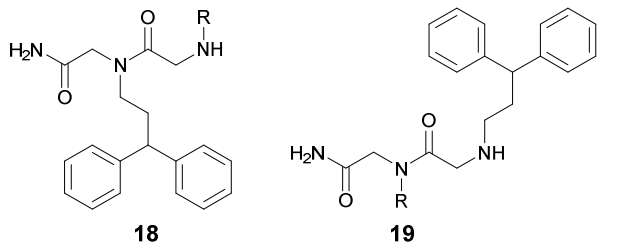


Figure 2. General description of synthesized peptoid libraries. R groups derived from amines **20** - **53**.

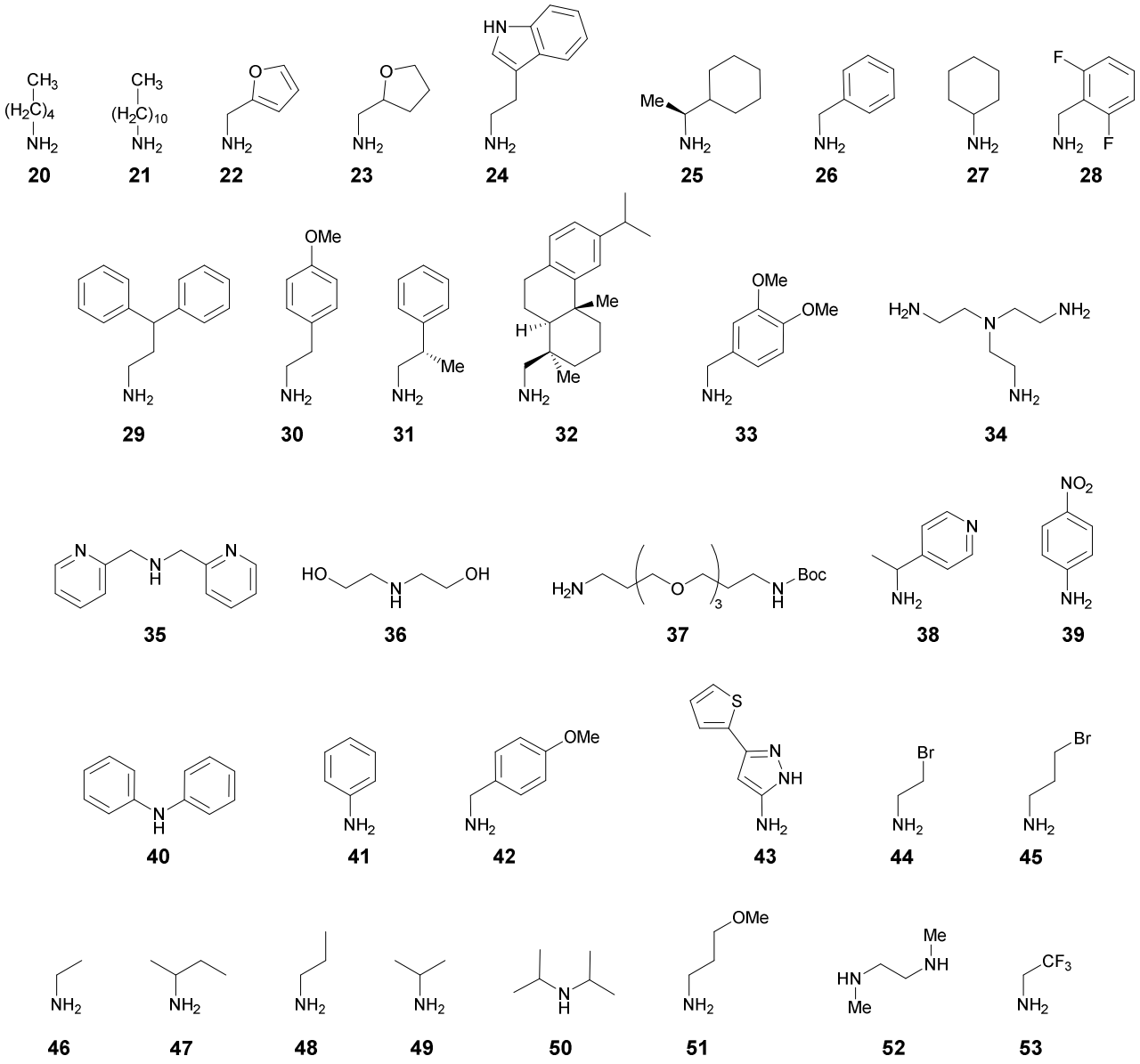


Figure 3. Amines used in the SPOT-synthesis of the test libraries. Amines **38** - **53** did not provide good purity and were not used in further testing.

Peptoids with the amines **38** - **53** did not achieve the minimum purity of 70%. These amines had several drawbacks: they reacted with themselves under those conditions (e.g. **44** and **45**), they were volatile (e.g. **46** - **49**), not sufficiently nucleophilic (e.g. **39** - **41** and **43**), sterically hindered (e.g. **40**, **47**, **49** and **50**) or did not survive the cleavage conditions (e.g. **42**). Therefore, they were not used for the final SPOT-synthesis of the dipeptoid library.

Synthesis of the Final Dipeptoid Library

With the amine screening data completed, a final 96-membered dipeptoid library was designed, with the final aim of being suitable for testing for antibacterial effects. The synthesis was designed in a way that the particularly reactive amines **20** - **37** (as revealed via out optimization studies above) could be combined to generate a 96-membered library (structures are shown in Table 4). Special focus was made on ensuring a high structural diversity. Amines **23**, **27**, **29**, **30**, **32**, **50** and **53** have already been incorporated into tripeptoids that display antibacterial activity.[7-9] Accordingly, we examined them in the current study to determine whether they continue to contribute to antibacterial activity in further minimized dipeptoids.

The synthesis was carried out on a freshly prepared cellulose membrane **5**. To obtain a representative overview of the purity, 30 selected representatives (Table 4) were analyzed by means of HPLC and MALDI-TOF after cleavage of the 96 synthesized dipeptoids.

Of the dipeptoids tested, ten (**55**, **57**, **58**, **62**, **64**, **94**, **96**, **97**, **105** and **107**) could be obtained with a purity of more than 80%. Purities above 70% were measured for peptoids **7**, **81**, **86**, **88**, **116**, **135**, **146**, and **148**. For the remaining compounds, the purity was less than 70% or could not be determined unequivocally since superimposing peaks prevented product classification.

To be able to interpret the results of the antibacterial tests, the corresponding concentrations of the tested compounds must be known. To determine the yield of the synthesized peptoids, the vacuum-dried peptoids were dissolved in acetonitrile and the concentrations were determined using the absorption integral at 218 nm. A dilution series of peptoid **7** was used as a reference.

Antibacterial Tests

The synthesized peptoids were tested for their antibacterial activity against MRSA strain ATCC 33591. A first series of experiments was carried out with a concentration of 50 µM in DMSO. Peptoids that displayed activity at 50 µM were subsequently tested again at 25 µM in DMSO.

The effects of the peptoids on bacterial growth were measured via simple absorbance assays in microtiter plates. In brief, 2 µL of a 5 mM solution of the dipeptoids was added to 200 µL of bacteria in Luria-Bertani (LB) growth medium to generate 50 µM solutions. After 18 h incubation at 37 °C with shaking at 200 rpm, the absorption of each well at 595 nm wavelength was measured.

Figure 4 shows the measured absorptions of the peptoids. With this test, 31 peptoids could be found that have an antibacterial effect at a 50 µM concentration. A structure-function pattern is especially striking. On closer examination of the active peptoids, the two particularly hydrophobic amines, undecylamine (**21**) and dehydroabietylamine (DHAA, **32**) were incorporated as side chains in all of the active substances. Compounds which contained neither of the two amines **21** and **32** showed no antibacterial effect.

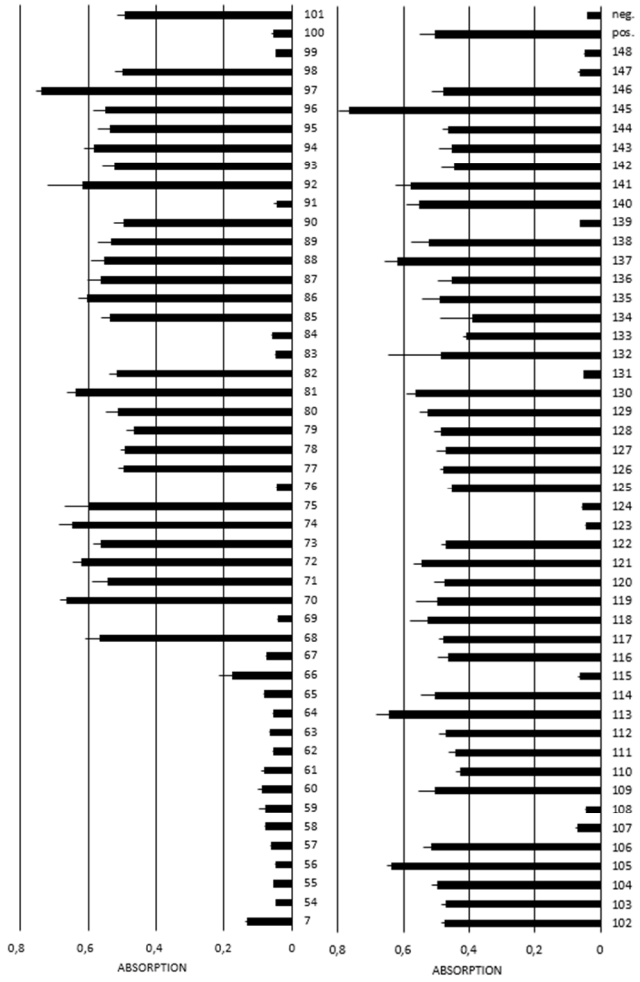


Figure 4. Antibacterial assay with MRSA and 50 µM of peptoids **7** and **65** - **148**. The bars correspond to absorption at 595 nm. Lower values indicate more potent antibacterial effect.

With the assumption that the amines **21** and **32** cause the actual activity of the peptoids, it is reasonable to conclude that homo dipeptoids containing these amines as side chains at both positions, must be particularly active. However, homo dipeptoid **68** carrying side chain **32** twice showed no antibacterial activity. Homo dipeptoid **61**, with two undecylamine (**21**) side chains, did show an antibacterial effect. Activity against MRSA by the peptoids **60** and **69** consisting of both amines **21** and **32** was also detected. In most dipeptoids with either variation as a side chain (**21** or **32**, respectively) antibacterial effects were detected (e.g. **7** and **76**, **54** and **62**, **55** and **63**, **56** and **64**, **57** and **65**, etc.). All dipeptoids containing DHAA (**32**) as a side chain were found as active compounds, whereas some analogous peptoids with undecylamine (**21**) were inactive (e.g., **91** was active and **92** inactive, **115** active and **116** inactive, **131** active and **132** inactive, etc.).

Since 50 µM is still a high concentration for antibacterial substances, a further series of tests with the concentration of 25 µM was performed. In this experiment, only the substances that already showed an antibacterial effect at 50 µM were tested. The measured absorption is plotted in Figure 5.

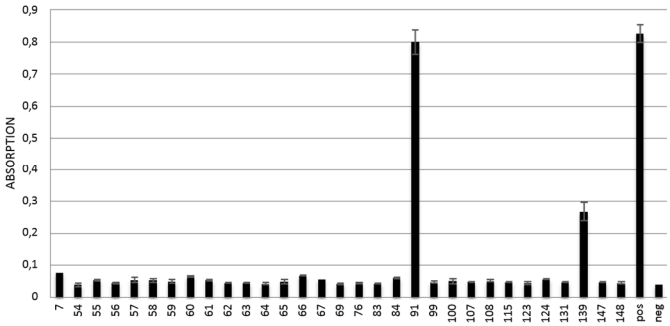


Figure 5. Antibacterial test with MRSA and a peptoid concentration of 25 μM. The bars correspond to the absorption at 595 nm.

In this experiment, it became clear that almost all active substances that already had an antibacterial effect at a concentration of 50 μM, retained this activity at 25 μM. The only exceptions were the dipeptoids **91** and **139**. While in the case of peptoid **139**, a small antibacterial effect could be seen, no activity was observed at **91**. The two peptoids **91** and **139** with dehydroabietylamine (**32**) as a side chain lost activity at a concentration of 25 μM. It is noteworthy that the dipeptoids **92** and **140** with undecylamine (**21**) as a side chain were already inactive at 50 μM, though having similar sequence structure. All other dipeptoids still had an antibacterial effect, regardless of which side chains were incorporated.

A subset of six representative dipeptoids was chosen for re-synthesis and re-screening to validate the library (See SI, Figure 2S). Authentic samples of the selected dipeptoids were synthesized and highly purified using RP-HPLC, lyophilized, and diluted to make 5 mM DMSO stock solutions. From the DMSO stocks, dilutions were prepared and the compounds were screened for antimicrobial activity at concentrations of 50, 25, 20, 15, 12.5, 10, and 5.0 μM as described above in LB medium as well as TSB (tryptic soy broth) medium, as TSB is a common medium for growth assays and antimicrobial susceptibility testing with *S. aureus*.[23-26] We used a MRSA strain (ATCC 33591) for all of these assays (see Methods). The results validate our preliminary assays of the SPOT library, except that compound **56** did not inhibit growth at 25 μM in LB medium (Table 3). In LB, several of the peptoids show inhibition of growth at concentrations as low as 10 μM. In TSB, only two peptoids show strong inhibition. **64** has an MIC of 20 μM, while **62** has an MIC of 12.5 μM, based on the collected data.

Table 3. MIC values for peptoids and control compounds in LB and TSB media

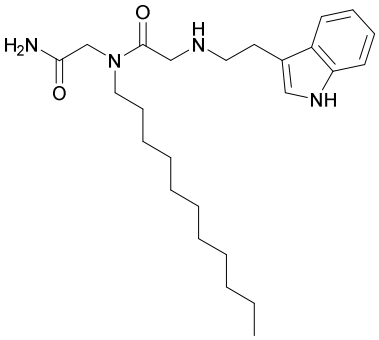
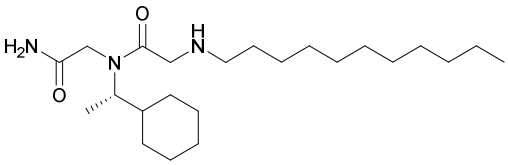
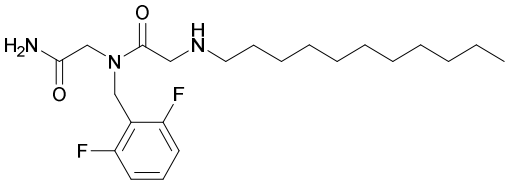
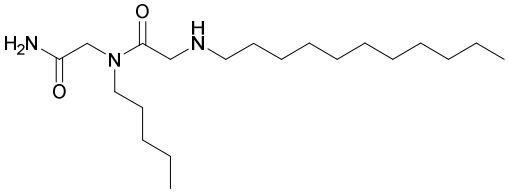
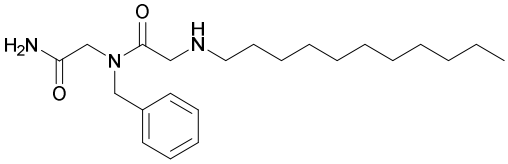
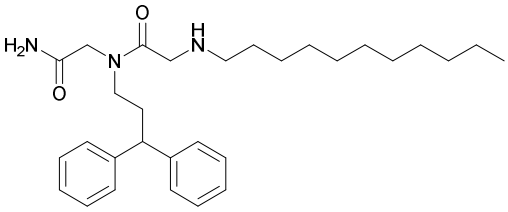
Compound	LB (μM)	TSB (μM)
54	**10**	-
56	**50**	-
60	**10**	-
62	**10**	**12.5**
64	**15**	**20**
69	**20**	-
DHAA	**20**	**25**
undecylamine	-	-

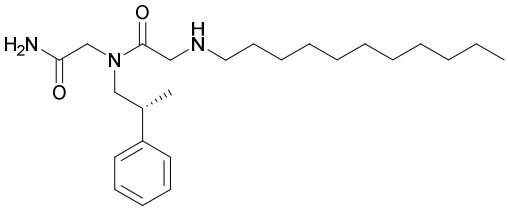
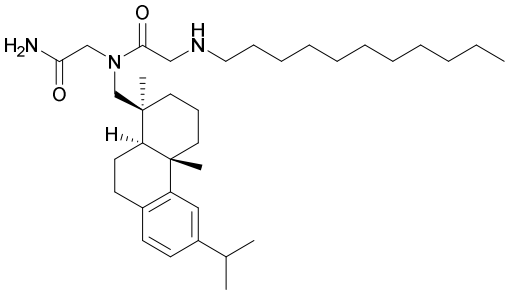
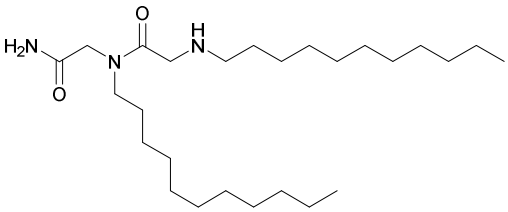
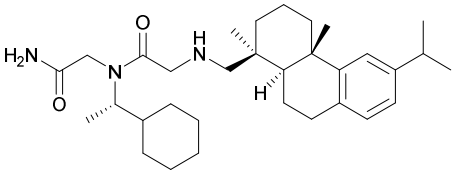
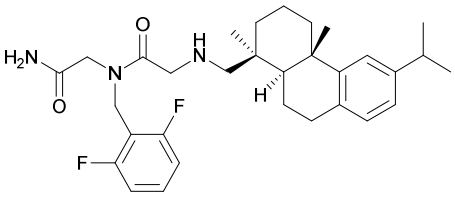
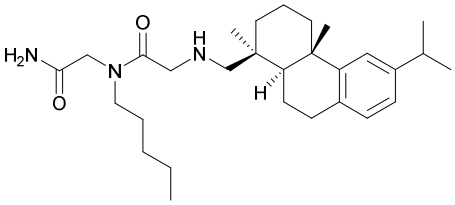
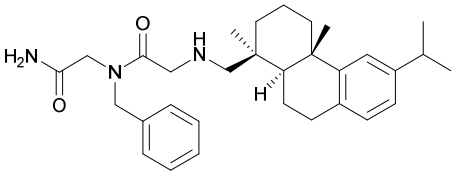
To ensure that the observed antibacterial effect for this series of active peptoids was not already produced by amines **21** and **32** alone, these amines were also tested. However, no significant antibacterial effect could be detected at these concentrations for undecylamine, the activity of DHAA was less than for all compounds in LB except **56**, and DHAA is less active than the two compounds with antimicrobial activity in TSB, compounds **62** and **64** (Figure 2S,

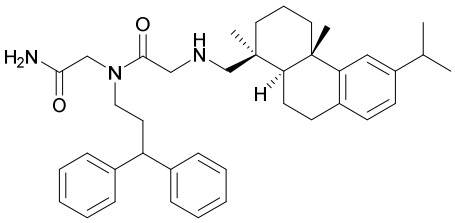
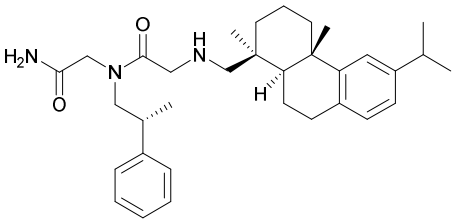
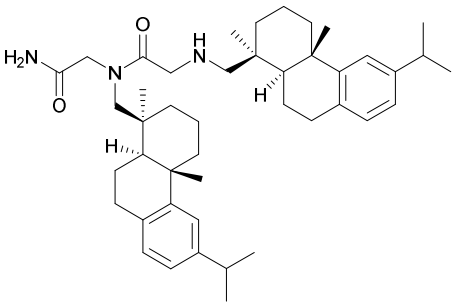
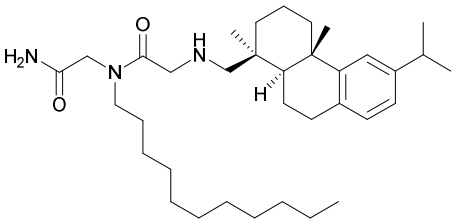
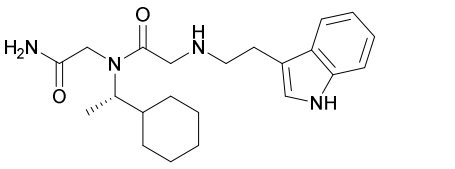
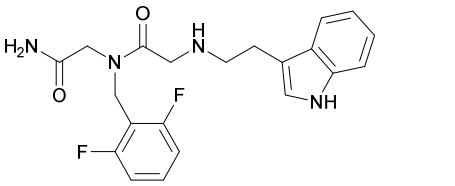
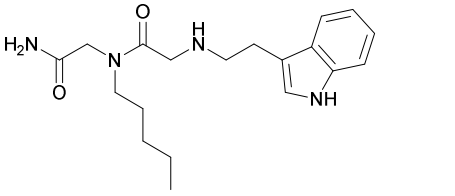
Table **3**).

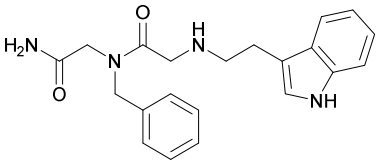
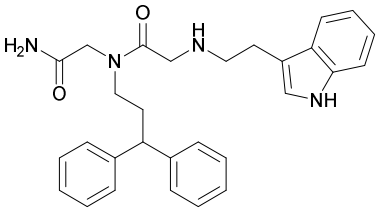
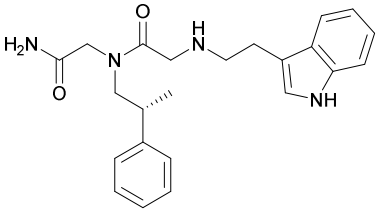
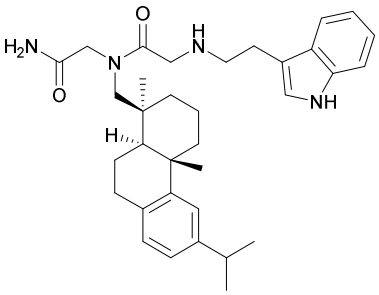
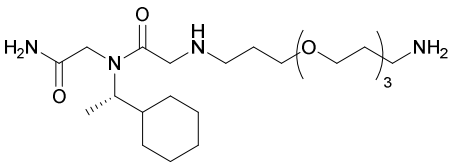
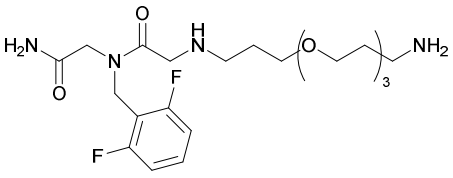
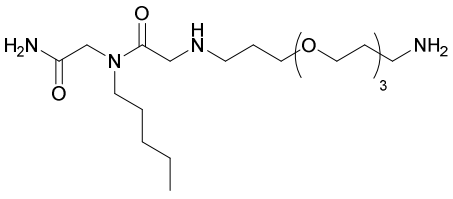
In future studies, these results need to be confirmed via the re-synthesis of all the active substances (on larger scale and in a quantifiable manner), and the effects of the active substances investigated with other bacteria. Nevertheless, these initial results for this dipeptoid library are compelling and suggestive of the minimized dipeptoid scaffold as a useful structural class for further study as antibacterials.

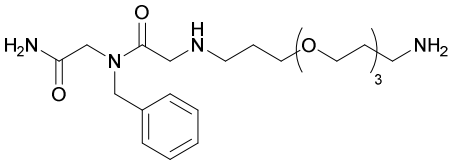
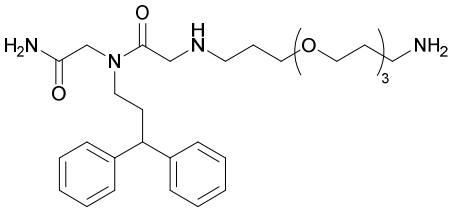
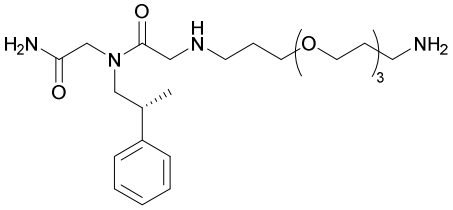
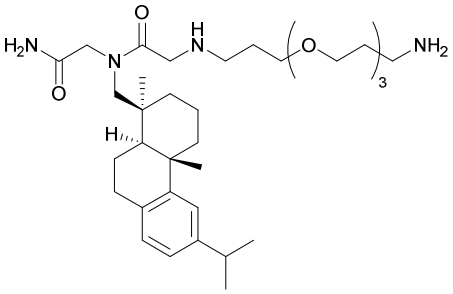
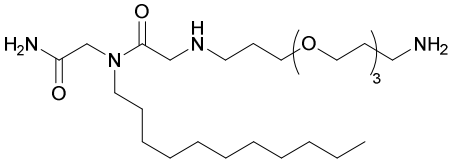
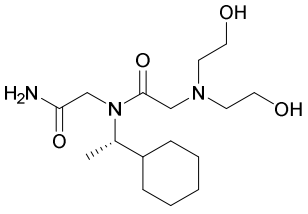
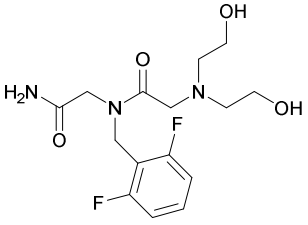
Table 4. Dipeptoids synthesized on cellulose, their activity against MRSA as well as their analyzed retention times, purities and masses.

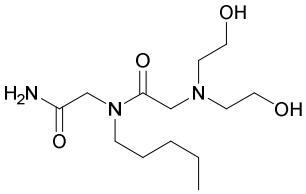
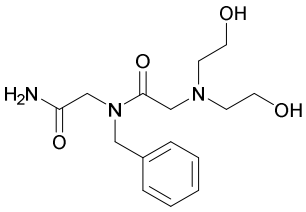
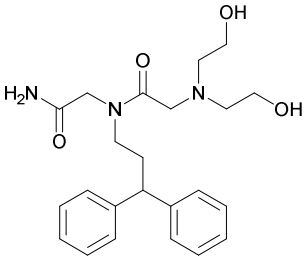
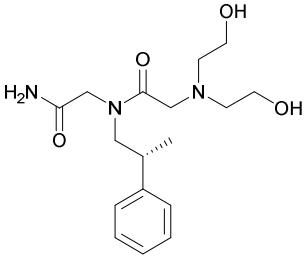
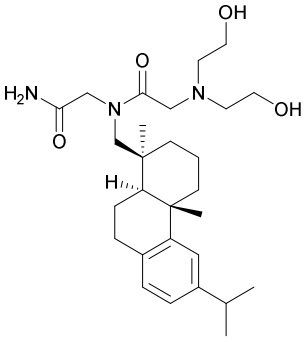
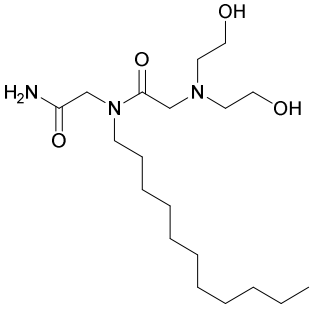
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
7		32.2	71	428.6	237: M^+ 2 Na/2	(+)	(+)
54		-	-	395.6	-	(+)	(+)
55		31.5	92	411.5	412.3	(+)	(+)
56		-	-	355.6	-	(+)	(–)
57		31.5	86	375.6	376.3	(+)	(+)
58		34.8	85	479.7	480.2	(+)	(+)

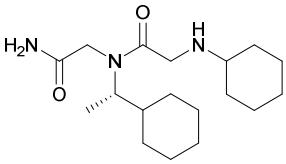
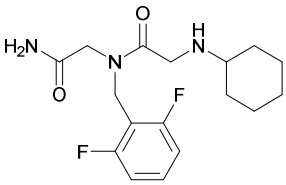
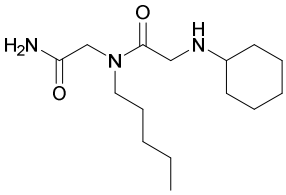
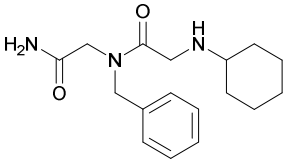
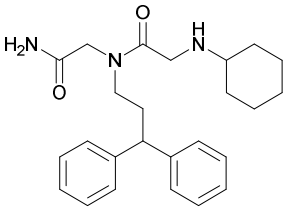
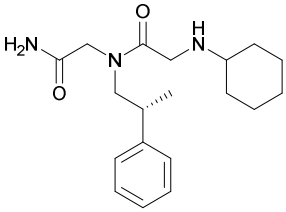
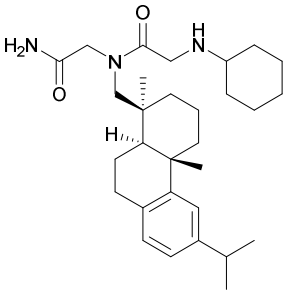
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 μM	Antibac. 25 μM
59		-	-	403.6	-	(+)	(+)
60		-	-	553.9	-	(+)	(+)
61		-	-	439.7	-	(+)	(+)
62		35.0	91	509.8	510.2	(+)	(+)
63		-	-	525.6	-	(+)	(+)
64		33.3	91	469.7	471.2	(+)	(+)
65		-	-	489.7	-	(+)	(+)

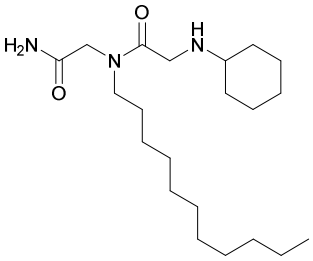
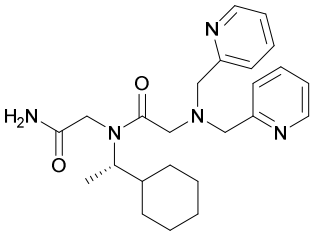
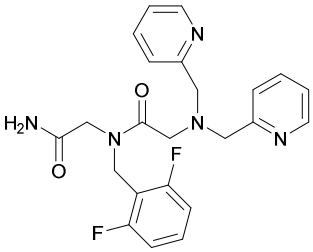
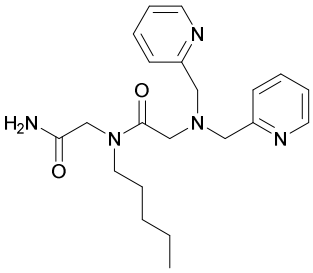
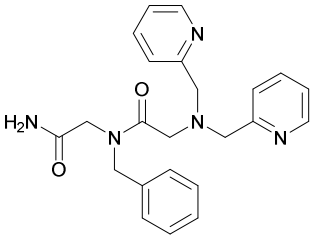
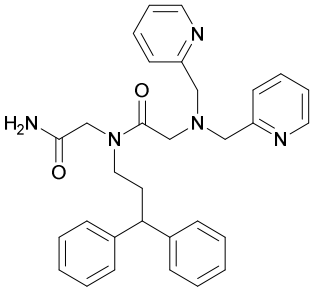
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 μM	Antibac. 25 μM
66		-	-	593.8	-	(+)	(+)
67		-	-	517.8	-	(+)	(+)
68		-	-	668.0	-	(–)	-
69		-	-	553.9	-	(+)	(+)
70		-	-	384.5	-	(–)	-
71		-	-	400.4	-	(–)	-
72		-	-	344.5	-	(–)	-

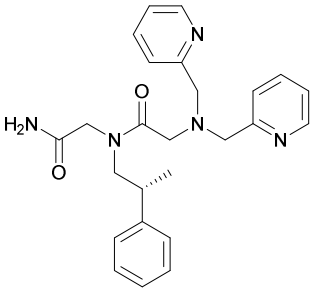
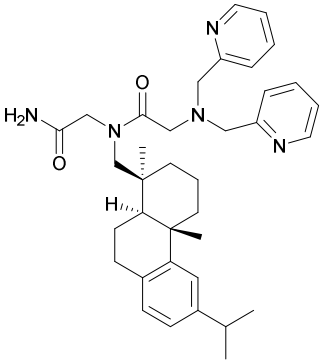
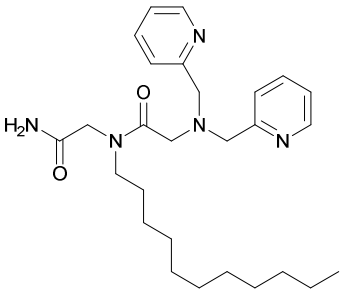
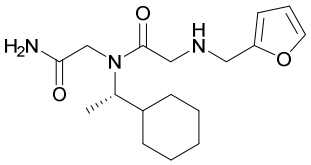
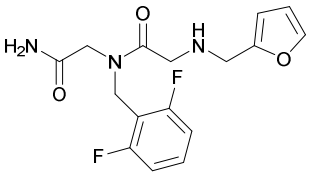
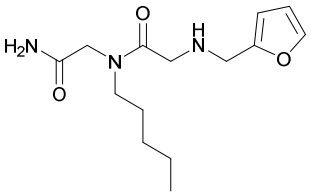
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
73		-	-	364.4	-	(–)	-
74		28.0	64	468.6	257: M + 2 Na/2	(–)	-
75		-	-	392.5	-	(–)	-
76		34.9	54	542.8	294: M + 2 Na/2	(+)	(+)
77		-	-	444.6	-	(–)	-
78		-	-	460.5	-	(–)	-
79		-	-	404.5	-	(–)	-

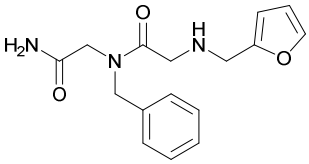
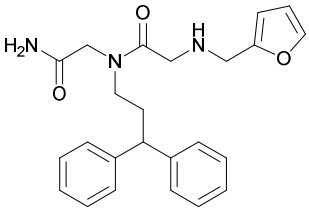
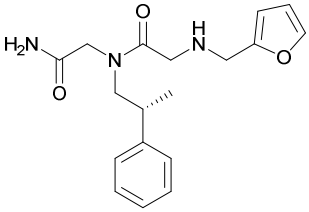
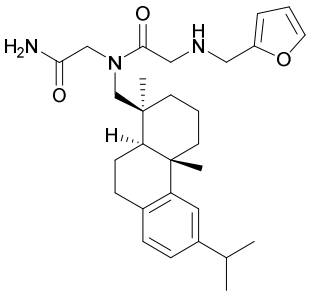
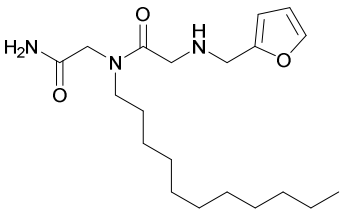
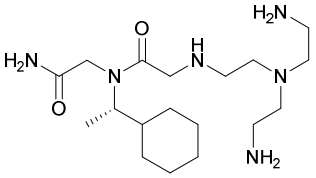
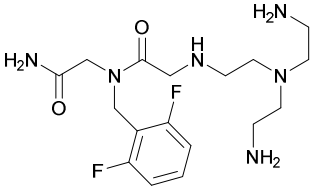
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
80		18.5	55	424.5	425.2	(–)	-
81		23.6	74	528.7	529.1	(–)	-
82		-	-	452.6	-	(–)	-
83		-	-	602.9	-	(+)	(+)
84		-	-	488.7	-	(+)	(+)
85		-	-	329.4	-	(–)	-
86		17.1	70	345.3	346.1	(–)	-

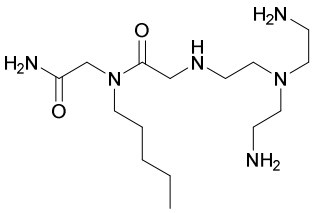
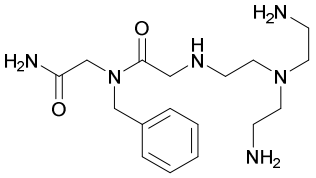
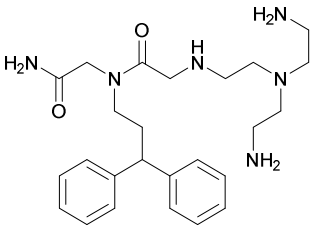
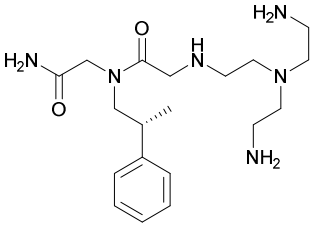
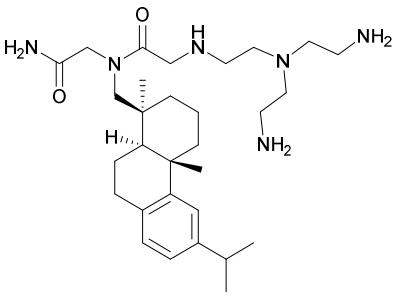
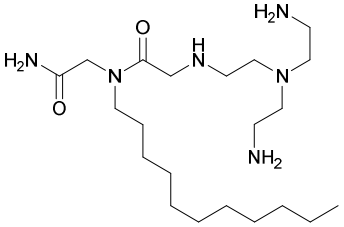
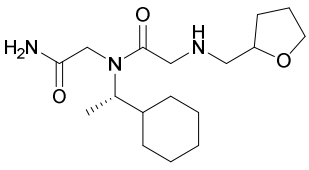
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
87		-	-	**289.4**	-	(–)	-
88		**17.2**	**71**	**309.4**	**310.1**	(–)	-
89		-	-	**413.5**	-	(–)	-
90		-	-	**337.4**	-	(–)	-
91		-	-	**487.7**	-	(+)	(–)
92		-	-	**373.5**	-	(–)	-

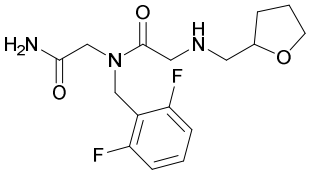
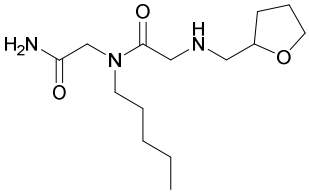
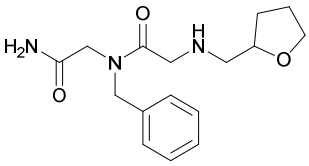
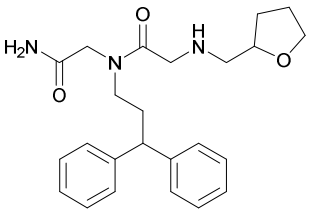
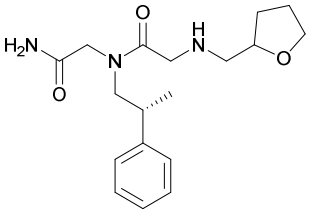
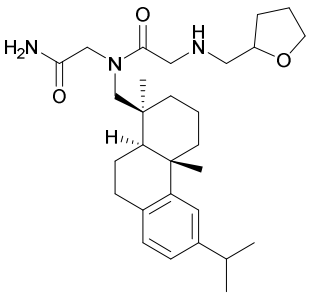
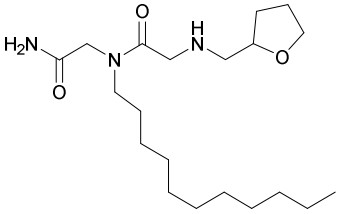
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 μM	Antibac. 25 μM
93		-	-	323.5	-	(–)	-
94		21.5	98	339.4	340.2	(–)	-
95		-	-	283.4	-	(–)	-
96		21.8	91	303.4	304.2	(–)	-
97		27.0	98	407.6	408.2	(–)	-
98		-	-	331.5	-	(–)	-
99		-	-	481.7	-	(+)	(+)

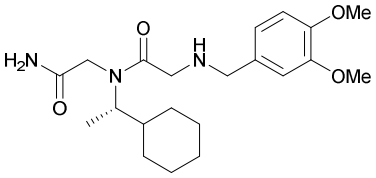
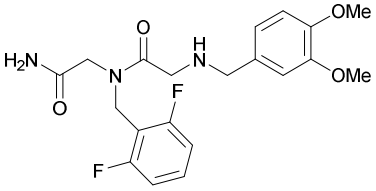
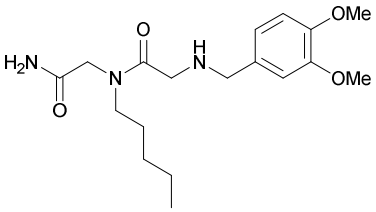
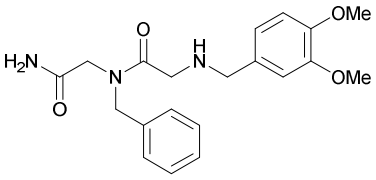
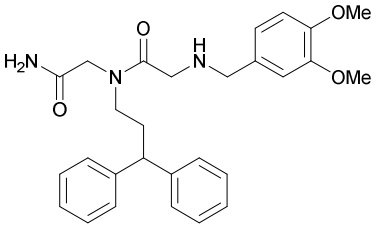
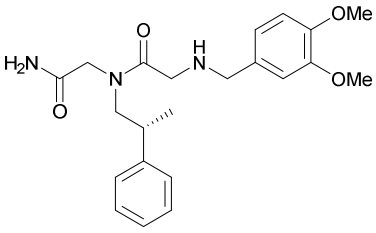
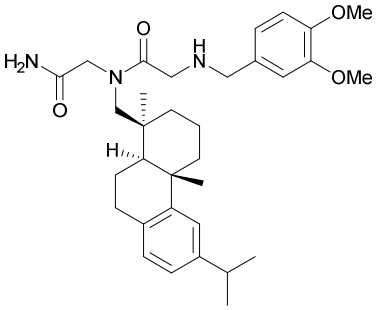
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 μM	Antibac. 25 μM
100		-	-	367.6	-	(+)	(+)
101		-	-	423.6	-	(–)	-
102		-	-	439.5	-	(–)	-
103		-	-	383.5	-	(–)	-
104		-	-	403.5	-	(–)	-
105		24.0	93	507.6	508.2	(–)	-

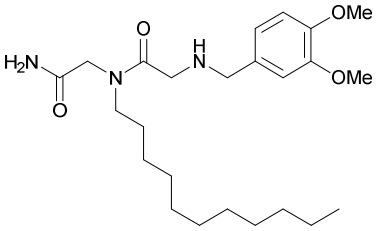
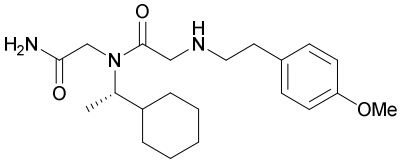
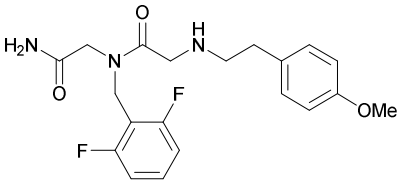
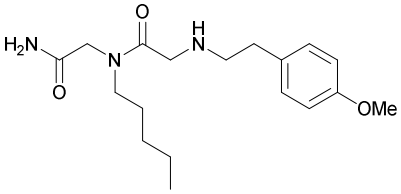
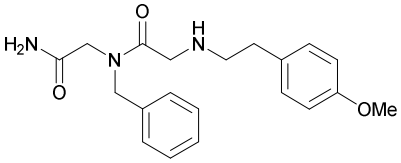
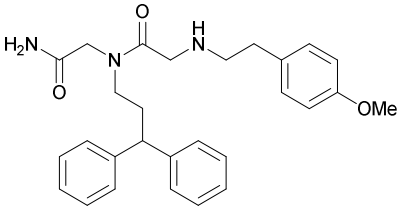
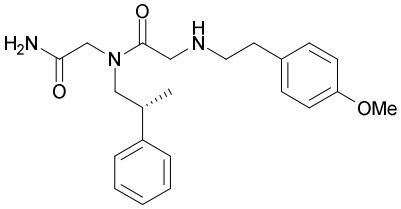
Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
106		-	-	**431.5**	-	(–)	-
107		**29.7**	**82**	**581.8**	**582.2**	(+)	(+)
108		-	-	**467.7**	-	(+)	(+)
109		-	-	**321.4**	-	(–)	-
110		-	-	**337.3**	-	(–)	-
111		**21.0**	**36 + 41**[1)]	**281.3**	**282.1**	(–)	-

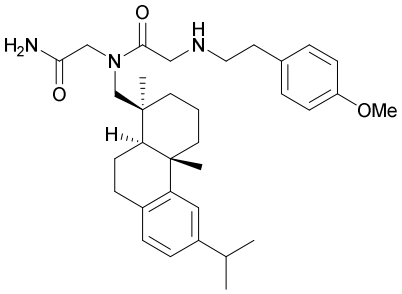
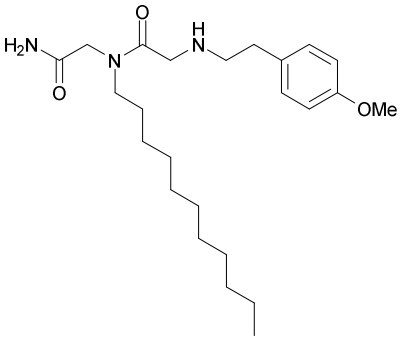
Dipeptoid	Structure	Ret.-time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
112		-	-	301.3	-	(–)	-
113		-	-	405.5	-	(–)	-
114		27.0	26	329.4	330.1	(–)	-
115		-	-	479.7	-	(+)	(+)
116		30.5	72	365.5	366.2	(–)	-
117		19.8	47 + 27[1)]	370.5	-[2)]	(–)	-
118		15.2	36 + 19 + 24[1)]	386.4	-[2)]	(–)	-

Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
119		-	-	**330.5**	-	(–)	-
120		**16.0**	**32 + 18 + 24**[1)]	**350.5**	-[2)]	(–)	-
121		-	-	**454.6**	-	(–)	-
122		-	-	**378.5**	-	(–)	-
123		-	-	**528.8**	-	(+)	(+)
124		-	-	**414.6**	-	(+)	(+)
125		-	-	**325.5**	-	(–)	-

Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
126		19.8	54 + 39	341.4	342.1[3)]	(–)	-
127		-	-	285.4	-	(–)	-
128		19.7	52 + 38	305.4	306.2[3)]	(–)	-
129		-	-	409.5	-	(–)	-
130		-	-	333.4	-	(–)	-
131		-	-	483.7	-	(+)	(+)
132		-	-	369.5	-	(–)	-

Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
133		-	-	391.5	-	(–)	-
134		-	-	407.4	-	(–)	-
135		26.5	77	475.6	476.0	(–)	-
136		-	-	371.4	-	(–)	-
137		32.3	67	549.7	550.1	(–)	-
138		-	-	399.5	-	(–)	-
139		23.9	67	335.4	336.3	(+)	(–)

Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 μM	Antibac. 25 μM
140		-	-	435.6	-	(–)	-
141		-	-	375.5	-	(–)	-
142		-	-	391.4	-	(–)	-
143		-	-	335.4	-	(–)	-
144		-	-	355.4	-	(–)	-
145		-	-	459.6	-	(–)	-
146		24.9	78	383.5	384.2	(–)	-

Dipeptoid	Structure	Ret. -time [min]	Purity [%]	Calc. mass [g/mol]	Found mass $[M+H]^+$	Antibac. 50 µM	Antibac. 25 µM
147		-	-	**533.7**	-	(+)	(+)
148		**32.2**	**75**	**419.6**	**420.3**	(+)	(+)

[1)] Fractions were combined; [2)] fractions were not collected; [3)] mass was not found in both fractions.

Experimental Procedures

General Methods

NMR spectra were recorded at 25 °C on a *Bruker* AC 250 [250 MHz (^{1}H)], *Bruker* AC+ 300 [300 MHz (^{1}H) and 75 MHz (^{13}C)] and a Bruker AM 400 [100 MHz (^{13}C)] spectrometer. Chemical shifts are reported in parts per million (ppm) on the δ scale from an internal standard (TMS). Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra of the peptoids were recorded on a *Bruker* Biflex IV or *Bruker* REXLEX II spectrometer with a pulsed ultraviolet nitrogen laser (200 µJ at 337 nm) and a time-of-flight mass analyzer with a 125 cm linear flight path. For every spectrum the samples were shot between 100 and 300 times with a repetition rate of 1 - 3 Hz. The software used for recording and processing the spectra was XACQ Version 4.0.4 and XMASS_TOF Version 5.1.0. The samples were spotted on a *Bruker* Standard stainless steel target with 386 spots. As matrix 2,5-dihydroxybenzoic acid (DHB) as saturated solutions in 50% acetonitrile in water with 0.1% trifluoroacetic acid (TFA) was used. The crystallisation was effected on air at room temperature. The abbreviation used for the protonated molecule ion is $[M+H]^+$. Reverse-phase HPLC was performed in a *Shimadzu* HPLC system (LC-10AT vp pump, SCL-10A vp controller, FCV-10ALvp low pressure gradient unit, SPD-M10A vp UV/Vis diode array detector (DAD)). For analytical measurements, a *Restek* Premier C_{18} column (5 µm, 4.6 mm × 250 mm) with a flow rate of 1 mL/min was used. Preparative purifications were performed with a *Vydac* protein & peptide C_{18}-column (10 µm, 22 mm × 250 mm) with a flow rate of 9 mL/min. An acetonitrile gradient in water with 0.1% TFA was used as eluent. The purity was calculated by integration of the signals at 218 nm. Furthermore, preparative reverse-phase HPLC was performed via a *Shimadzu* HPLC system with two LC-8A pumps, a CBM-10A system controller, a SIL-10A autoinjector, a FRC10A fraction collector and a SPD-10A vp UV/Vis detector using a *Vydac* protein & peptide C_{18} column (10 µm, 22 mm × 250 mm). The flow rates varied between 10 - 24 mL/min. The method (gradient, temperature and flow rate) was adjusted to each sample. Microwave reactions of dipeptoids on cellulose supports were carried out in a *Milestone* MicroSYNTH Labstation multimodal microwave synthesis reactor equipped with a continuous power source (1000 W max.). For reaction control, the instrument was interfaced with an *Ethos* MicroSYNTH Lab PC running EasyWave reaction monitoring software. Microwave reaction of dipeptoids on resins in fritted plastic syringes (Multisyntech GmbH) were carried out in a single mode *CEM* Discover LabMate microwave operated with *CEM*'s Synergy™ software. This instrument works with a constantly focused power source (0–300 W). Irradiation can be adjusted *via* Power- or Temperature Control. The temperature was monitored with an optical fiber at atmospheric pressure. Solid phase synthesis of the comparison dipeptoid was performed with Rink Amide aminomethyl polystyrene resin (from *Merck-Novabiochem*) as solid support. Resin loading was given as 0.61 mmol/g. All materials were purchased from

commercial sources and used without further purification.

Functionalization of Cellulose

For the synthesis of the dipeptoid library, Whatman 1 CHR chromatography paper (20 × 20 cm) was used. The spots were marked with a pencil.

First, the paper **1** was shaken in 20% trifluoroacetic acid (TFA) in dichloromethane for 20 min in a covered glass bowl. Subsequently, the cellulose was washed.[27] In this washing procedure, the sheet of cellulose was placed in a glass dish and covered with solvent. The filled bowl was gently swirled for 5 min. The solvent was decanted and the glass bowl with the cellulose was filled directly with next solvent. The solvents used for washing were as follows: DMF, ethanol/methanol (2 ×), dichloromethane. The support was then dried under an air stream.

For tosylation, the sheet was shaken in 2 M p-toluenesulfonyl chloride solution in pyridine for 20 min. The tosylated cellulose **2** was washed again as described before. The tosylated cellulose **2** was placed in a glass dish pre-heated to 80 °C with 75 mL of spacer **3** and covered. After 40 minutes at 80 °C in the oven, the sheet was washed as mentioned before with following solvents: DMF, ethanol, 2 M sodium hydroxide solution, water, ethanol (2 ×) and dichloromethane. The support was then dried under an air stream.[27] This washing step was used during the submonomer method.

A solution of 243 mg (450 µmol) of Fmoc-Rink linker, 52 mg (0.450 mmol) of HO-Su and 70 µL (57 mg of 0.450 mmol) of DIC in 1.0 mL of DMF was vortexed briefly and then allowed to stand for 30 min. The precipitated white solid was filtered off, and 6 µL of the filtrate was spotted onto the cellulose. The support was then placed in the oven at 80 °C for 30 min, after which it was washed as described above. To protect the unreacted amines, the support was then shaken in a glass dish with a solution of 20 mL of acetic anhydride and 10 mL of pyridine in 70 mL of DMF for 20 min at room temperature and washed as described above.

The loading of support **5** was determined via standard Fmoc quantification. Two spots of **5** were punched out and placed into separate vial. A 1 mL aliquot of 4% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was then added in DMF to each vial and vortexed for 30 s. After 15 min, the vials were vortexed again for 30 s. From these solutions, 100 µL were removed and diluted with 2 mL of DMF. The absorbance at 296 nm of these dilutions was determined, and the loading of the support **5** was calculated.

Optimized SPOT-Synthesis of Dipeptoids

Step 1: Deprotection: Support **5** was shaken in a solution of 20% piperidine in DMF in an uncovered glass bowl and then washed as mentioned before.

Step 2: Acylation: A 2 M solution of bromoacetic acid (**11**) in NMP was added with the equivalent amount of DIC. After vortexing, the precipitated solid was filtered off with a syringe filter. This solution (6 µL) was spotted on the support (in spatially localized positions to generate an 18 by 12 cm 96-spot grid), and the sheet was heated at 500 W in the microwave oven for 30 s. Spotting and heating in the microwave oven were repeated once. Subsequently, the acylated support was washed as described above.

Step 3: Amination: A 1 M solution of amine in NMP was prepared. From this solution, 3 µL were spotted onto the support (using a different amine at each localized spot). The support was heated at 550 W in the microwave oven for 90 s. Spotting and heating by microwave were repeated once. Subsequently, the aminated supported was washed as described above.

Steps 2) and 3) were repeated again for the coupling of the second amine, to generate each dipeptoid.

Thereafter, the support was dried under a stream of air. The individual spots were punched out and placed into separate glass vials. These vials were loosely capped and placed in a desiccator that also contained an open flask containing about 10 mL of TFA. The desiccator was evacuated for 10 min to generate a TFA vapor atmosphere, and then allowed to stand in the TFA atmosphere for a further 50 min. The desiccator was then aerated for 15 min, and 1 mL of acetonitrile was then added to each of the vials. The mixture was shaken for 15 min. The cellulose spots were removed from the solution with a clean pair of tweezers, and the solvent was removed *in vacuo*. The resulting residue was dissolved in acetonitrile for HPLC tests and dissolved in DMSO for antibacterial tests.

Microwave-Assisted Solid-Phase Synthesis *via* the Sumonomer Method with Bromoacetic Acid.

1.00 equiv. of dry Rink-Amide aminomethyl resin was swollen in a plastic fritted-syringe with twice its volume of DMF for 2 h. Then the solvent is removed. For Fmoc deprotection the resin is treated with a solution of 20% piperidine in DMF (approx. 0.5 – 1 mL/100 mg resin) for 5 min at room temperature. The solvent is filtered off and the procedure is repeated two more times. Afterwards the resin is washed with DMF (× 3) and *peptide synthesis grade* DMF (× 1).

Step 1: Coupling of Bromoacetic Acid: The solvent is removed from the resin. A 1.2 M solution of bromoacetic acid (8.00 equiv.) and DIC (8.00 equiv.) in peptide synthesis grade DMF is added to the resin. The mixture is stirred at 35 ºC under microwave irradiation (max. power: 40 W, open vessel, optical fiber temperature control) for 1 min. Then the resin is washed with DMF (× 4) and peptide synthesis grade DMF (× 1).

Step 2: Coupling of Primary Amine as Submonomer: Next, a 1.0 M solution of the desired amine (9.00 equiv.) in peptide synthesis grade DMF is added to the resin. The mixture is stirred at 60 ºC under microwave irradiation (max. power: 40 W, open vessel, optical fiber temperature control) for 30 or 45 min. Finally the resin is washed with DMF (× 4) and peptide synthesis grade DMF (× 1).

Step 1 and 2 were repeated until the desired chain length. After the final displacement is done, the resin is washed with dichloromethane (× 3). A 95% TFA solution

in dichloromethane is added to the resin (approx. 1 mL/100 mg resin) and shaken at room temperature for 15 min. The solution is collected, new cleavage mixture is added to the resin and shaken for another 15 min at room temperature. After filtering out the solution, the resin is washed with the same volume of dichloromethane (× 3) and methanol (× 3). The cleavage solutions and washes are combined and the solvent is removed under reduced pressure. The residue is taken up in approx. 5 mL of acetonitrile/water 1:1 (v/v) for HPLC. Lyophilization yielded of a fluffy white powder as pure product (>95% purity).

Antibacterial Tests of the Dipeptoids against MRSA

An aliquot of DMSO (~20 µL depending on the yield of the dipeptoid) was added to the crude product of the SPOT-synthesis, which had been cleaved and dried, to make 5 mM DMSO stock solutions of each dipeptoid. A methicillin-resistant *S. aureus* (ATCC 33591) overnight culture was diluted 1:250 into LB medium to obtain a ca. 10^7 CFU/mL culture, as verified by counting CFUs on LB/agar plates. A 2.0 µL aliquot of each DMSO stock solution was diluted with 198 µL of the culture in a polystyrene 96-well microtiter plate, resulting in 50 µM solutions. A positive control contained 2.0 µL DMSO and 198 µL culture; a negative control contained 198 µL LB medium only. Samples and controls were prepared in quadruplicate. The microtiter plates were incubated for 18 h at 37 °C with orbital shaking of 200 RPM. Absorbance at 595 nm was recorded with a BioTek Synergy2 plate reader. The compounds exhibiting complete growth inhibition had the same absorbance values as the negative controls (~ 0.05). Compounds that did not exhibit growth inhibition had absorbance values similar to the absorbance of the positive controls (about 0.7–1.5). The assays were repeated with TSB substituted for LB, with triplicates obtained on separate days for each concentration tested.

To validate the library, a subset of six dipeptoids was chosen for screening at lower concentrations. Authentic samples of the selected dipeptoids were synthesized and highly purified using RP-HPLC, lyophilized, and diluted to make 5 mM DMSO stock solutions. From the DMSO stocks, dilutions were prepared and the compounds were screened at concentrations of 50, 25, 20, 15, 12.5, 10, 5.0, and 2.5 µM as described above in LB medium as well as TSB (tryptic soy broth, Sigma) medium. No compound showed growth inhibition of MRSA strain ATCC 33591 at concentrations lower than 5.0 µM.

Conclusions

In summary, we have developed an efficient synthetic strategy for the synthesis of dipeptoids facilitated via microwave irradiation. A set of 29 dipeptoids with large unpolar residues, such as undecylamine and dehydroabiethylamine, were identified as active compounds against MRSA. For more detailed information on the activity and the mechanism of these antibacterial dipeptoids, the active agents must be investigated more closely. This optimized microwave-assisted SPOT-synthesis opens completely new possibilities for the generation of peptoid libraries, which can be tested directly on the carrier for biological activity.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge via the Internet on the ACS Publications website at DOI: XXXX.

Synthesis and characterization data (MALDI-TOF-MS) of comparison peptoid **7**.

Absorption curve for the dilution series of the dipeptoid.

Synthesis and characterization data (^{1}H NMR) of compound **17**.

Antibacterial tests (MIC) of the re-synthesized dipeptoids in LB and TSB medium.

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All authors have given approval to the final version of the manuscript.

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