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ARTICLE

Synthesis of Full Length and Truncated Microcin B17 Analogues as DNA Gyrase Poisons

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Microcin B17 (MccB17) is a post-translationally modified peptide containing thiazole and oxazole heterocycles that interrupt the peptide backbone. MccB17 is capable of poisoning DNA gyrase through stabilization of the gyrase-DNA cleavage complex and has therefore attracted significant attention. Using a combination of Fmoc-strategy solid-phase peptide synthesis and solution-phase fragment assembly we have prepared a library of full-length and truncated MccB17 analogues to investigate key structural requirements for gyrase-poisoning activity. Synthetic peptides lacking the glycine-rich N-terminal portion of the full-length sequence showed strong stabilization of the gyrase-DNA cleavage complex with increased potency relative to the full-length sequences. This truncation, however, led to a decrease in antibacterial activity of these analogues relative to their full-length counterparts indicating a potential role of the N-terminal region of the natural product for cellular uptake.

Introduction

The microcins are a family of small peptides produced by *Enterobacteriaceae* bacteria that are capable of inhibiting the growth of phylogenetically-related bacterial species.[1] Microcin B17 (MccB17, **1**) is a modified 43 amino acid antibacterial peptide produced and secreted by a variety of *Escherichia coli* strains containing plasmid pMccB17 that carries the *mcb* operon (Figure 1).[1-2] Similar toxins, that target gyrase, have also recently been found to be produced by *Pseudomonas syringae*.[3] The mature structure of MccB17 possesses extensive post-translational modifications in the form of aromatic oxazole and thiazole units throughout the linear peptide backbone. This structure arises from the initial production of a 69-amino acid precursor peptide (McbA) that is subsequently modified by the *mcbB*, *mcbC* and *mcbD* gene products. The enzymes encoded by these genes are responsible for the dehydrative cyclisation and dehydrogenation of specific serine and cysteine residues within the linear sequence to afford four oxazole and four thiazole heterocycles throughout the peptide backbone (including two *bis*-heterocycles containing an oxazole and thiazole unit).[4] Following heterocycle formation, the 26 amino acid leader sequence is cleaved to generate mature MccB17.

The antibacterial activity of MccB17 is due to its interaction with DNA gyrase.[5] DNA gyrase is a member of the ATP-dependent type II topoisomerase family of enzymes and is responsible for the introduction of negative supercoils into closed-circular DNA, a process that is essential for DNA replication and other processes in prokaryotes.[6] A number of antibacterial compounds are known to target DNA gyrase. These include gyrase inhibitors such as the aminocoumarins, e.g. novobiocin, which are competitive for ATP binding, and simocyclinone D8, which competes for DNA binding to the enzyme.[3] In contrast, the clinically-successful fluoroquinolone family of antibacterials operates through the stabilization of a transient gyrase-DNA covalent complex. The resulting 'cleavage complex,' in which both DNA strands have been cut and covalently linked to the enzyme, leads to the release of broken DNA, which is lethal to the organism. Because the mechanism of action of the fluoroquinolones induces DNA cleavage by the enzyme, these compounds are often called DNA gyrase poisons. Although the exact mode of action of MccB17 is unknown, it is thought to stabilize the cleavage complex with this action further enhanced by the presence of ATP. MccB17 is therefore also classified as a DNA gyrase poison.

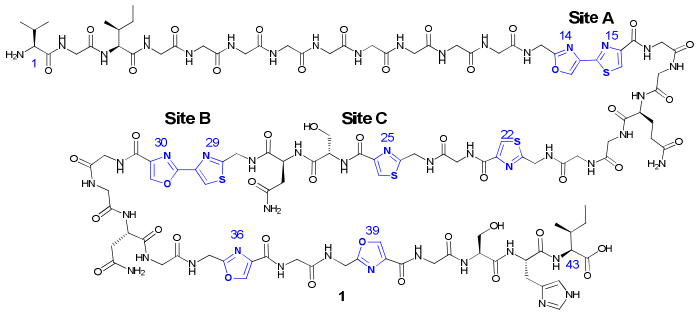
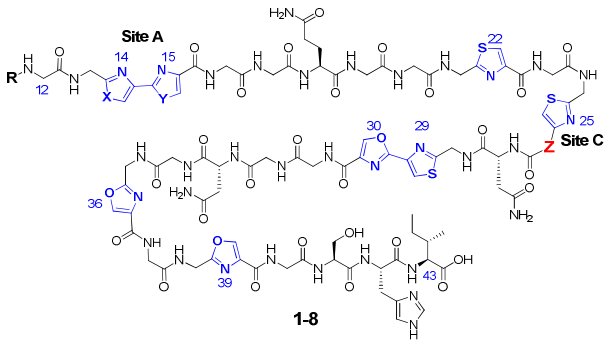


Figure 1. Microcin B17 (MccB17) **1**.



MccB17 Analogue	R =	Site A (X,Y =)	Site C (Z =)
1 (MccB17)	R^1	X = O,Y = S	
2	R^1	X = O,Y = S	
3	R^1	X = S,Y = O	
4	R^1	X = S,Y = O	
5	R^2	X = O,Y = S	
6	R^2	X = O,Y = S	
7	R^2	X = S,Y = O	
8	R^2	X = S,Y = O	

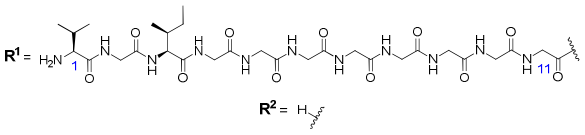


Figure 2. Target full length and truncated MccB17 analogues **1(MccB17)-8.**

With the rapid rise of resistance toward antibiotics, including the fluoroquinolones, new antibacterials that target DNA gyrase and other validated targets are urgently needed. Although MccB17 represents a unique and effective DNA gyrase poison, the poor physicochemical properties of MccB17 (a 3 kDa peptide natural product) prevent it from being considered as a lead candidate for antibacterial drug discovery. We have become interested in accessing full length and truncated analogues of MccB17 to enable structure-activity information to be elucidated with a view to developing simplified DNA gyrase poisons based on structural features present in MccB17. In seminal work by Walsh and co-workers a number of MccB17 analogues have been prepared *via* over-expression in *E. coli* using the biosynthetic machinery.[7] This demonstrated that the presence and positioning of heterocycles along the peptide backbone is important for activity. We have recently reported an efficient method for the convergent total synthesis of MccB17.[8] The synthetic strategy involves the use of a ligation-based assembly of heterocycle and *bis*-heterocycle-containing peptide and peptide thioester fragments that were synthesized *via* Fmoc-strategy solid-phase peptide synthesis (SPPS). Importantly, this synthetic strategy provides a potential means to access full-length and truncated analogues that cannot be prepared through use of the biosynthetic machinery of *E. coli* (McbB, McbC and McbD) or by a linear solid-phase strategy which has been used to assemble MccB17 previously.[9] In addition, we have recently shown that trypsin-digested MccB17 and synthetic peptide fragments (used to assemble the natural products) are capable of stabilizing the cleavage complex of DNA gyrase. This has provided confidence that synthetic analogues of MccB17 can serve as novel DNA gyrase poisons.[10]

Results and Discussion

Design

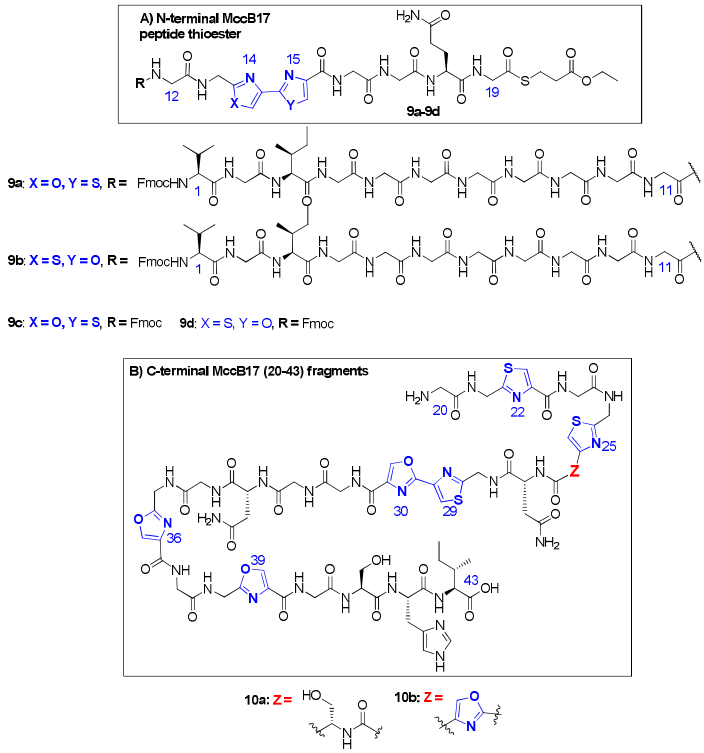


Figure 3. A) target *N*-terminal peptide thioester fragments **9a-d**; B) target *C*-terminal peptide fragments **10a** and **10b**

Herein, we describe the use of our synthetic methodology to access both full-length and truncated analogues of MccB17 to facilitate the systematic dissection of the structural components necessary for poisoning of DNA gyrase. We were particularly interested in accessing full length and truncated analogues that cannot be prepared through use of the McbBCD enzymatic machinery.[7] Modifications that we wished to explore included introduction of the alternate *bis*-heterocyclic isomer at site A and the introduction of a *bis*-heterocycle in place of the single thiazole unit at site C (Figure 2). We were also interested in investigating the importance of the *N*-terminal region on DNA gyrase inhibitory activity through the synthesis of truncated MccB17(11-43) analogues possessing a variety of heterocycles interrupting the peptide sequence. Our synthetic targets therefore included native MccB17 (**1**), three full length analogues (**2-4**) and four truncated analogues (**5-8**) (Figure 2). The synthesis of each of these analogues was proposed *via* a convergent fragment-based assembly through a single peptide ligation event. It was envisaged that this could take place between C-terminal fragments **10a** and **10b** (Figure 3B) with peptide thioesters corresponding to *N*-terminal residues 1-19 (**9a** and **9b**) for full length

(**1-4**) and 12-19 (**9c** and **9d**) for truncated (**5-8**) analogues (Figure 3A).

Synthesis

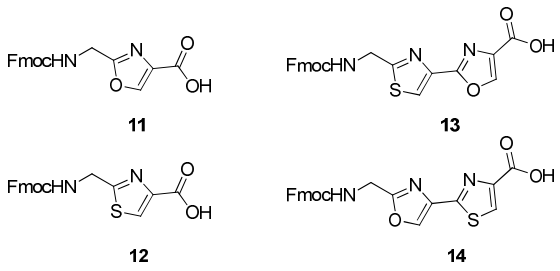
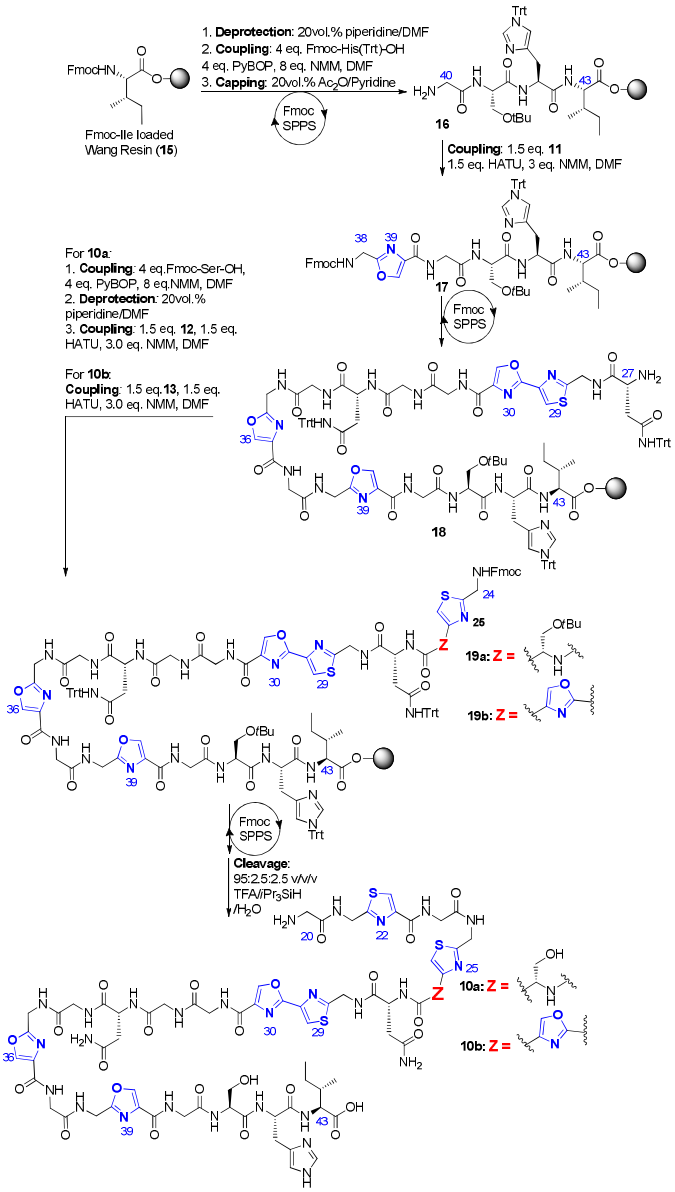
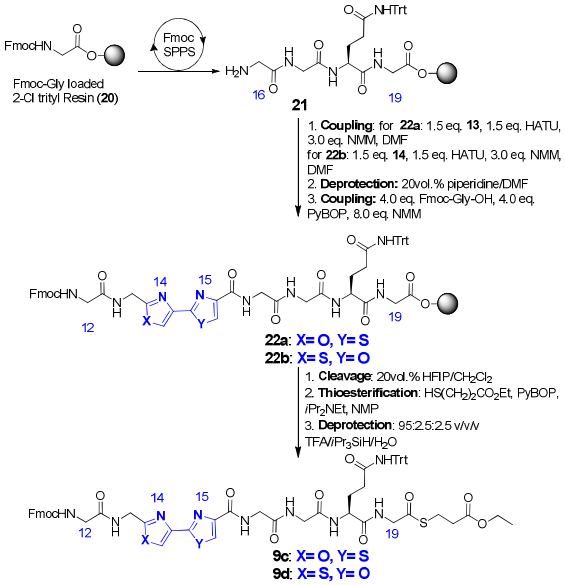


Figure 4. Heterocyclic building blocks **11-14** for direct incorporation into Fmoc-strategy SPPS of peptide fragments.

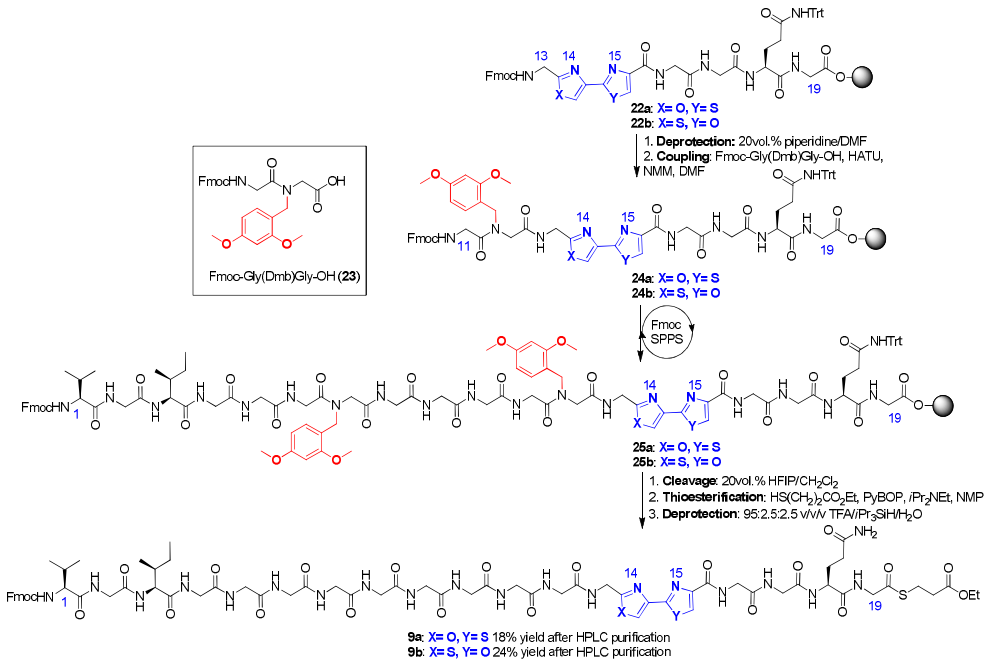


Scheme 1. Fmoc-SPPS of *C*-terminal MccB17(20-43) fragments **10a** and **10b**.

The synthesis of MccB17 analogues **1-8** began with the preparation of Fmoc-protected heterocyclic amino acids **11-14,** required for incorporation into peptide and peptide thioester fragments **10a,b** and **9a-d**, respectively, *via* Fmoc-strategy SPPS (Figure 4). These four building blocks were obtained using modified procedures to those reported previously for the synthesis of protected oxazole and thiazoles.[9, 11] The synthesis of *C*-terminal peptide fragments **10a** and **10b** began from Fmoc-isoleucine (Ile) preloaded Wang Resin **15** (Scheme 1). The *N*-terminal Fmoc-protecting group was first removed using piperidine in dimethylformamide (DMF). The resin-bound free amine was then coupled to Fmoc-His(Trt)-OH using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as a coupling reagent and *N*-methylmorpholine (NMM) as a base. The resin was then treated with 10% acetic anhydride in pyridine to cap any unreacted amine residues. This three-step procedure was iterated three times to afford resin-bound tripeptide **16**. At this stage heterocyclic amino acid **11** was coupled in slight excess (1.5 equivalents) using 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as the coupling reagent and NMM as the base to provide resin-bound **17**. Iterative Fmoc-strategy SPPS was continued and included insertion of another oxazole unit through coupling of **11** and an oxazole-thiazole *bis*-heterocyclic unit through coupling of preformed building block **13**. Following insertion of **13**, Fmoc-Asn(Trt)-OH (corresponding to Asn-27 in MccB17) was coupled to afford resin bound peptide **18**. At this point the resin was split into two equal batches. Both Fmoc-Ser(O*t*Bu)-OH and thiazole building block **12** were coupled to one batch of resin to provide resin-bound **19a** *en route* to peptide **10a**, while *bis*-heterocyclic building block **13** was coupled to the other half of the resin providing **19b** bearing an extra oxazole moiety. Further elongation of **19a** and **19b** with Fmoc-Gly-OH, thiazole building block **12**, and a final Fmoc-deprotection step provided the fully elongated resin bound MccB17(20-43) analogue fragments. Cleavage of the peptides from the resin with concomitant deprotection of the side chain protecting groups was achieved by treatment with an acidic cocktail consisting of 95% trifluoroacetic acid (TFA) containing 2.5vol.% tri*iso*propylsilane and 2.5vol.% water. Following reverse phase HPLC purification, native MccB17(20-43) peptide **10a** and MccB17(20-43) **10b**, bearing an additional oxazole moiety, were isolated in 45% and 38% yields respectively.



Scheme 2. Fmoc-SPPS of truncated MccB17(12-19) peptide thioester fragments **9c** and **9d**

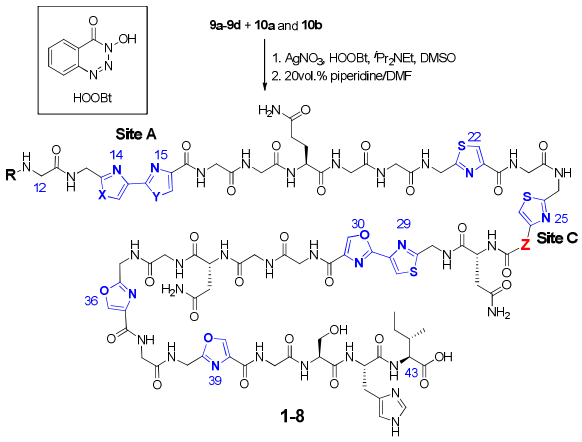


Scheme 3. Synthesis of MccB17(1-19) peptide thioesters **9a** and **9b** *via* Fmoc-SPPS.

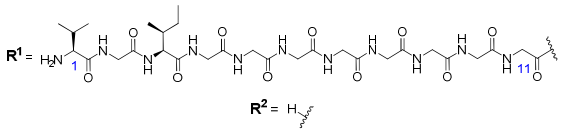
With the two *C*-terminal peptides in hand, our attention turned to the synthesis of peptide thioesters **9c** and **9d**, representing the *N*-terminal portion of truncated MccB17 analogues **5-8** (Figure 2). The synthesis began from Fmoc-Gly loaded 2-chlorotrityl resin **20** which was elongated using iterative Fmoc-strategy SPPS to afford resin bound peptide **21** (Scheme 2). At this point, the resin was split into two equal batches and *bis*-heterocyclic building blocks **13** and **14** were coupled to the two different batches of resin. This was followed by Fmoc-deprotection and elongation with Fmoc-Gly-OH to provide resin bound peptides **22a** and **22b**. Cleavage of the peptides from the resin, while keeping the side chain Trt protecting group of Asn-27 intact, was achieved using mildly acidic 20vol.% hexafluoroisopropanol (HFIP) in dichloromethane. The crude peptides were immediately subjected to thioesterification in solution by treating with ethyl 3-mercaptopropionate, PyBOP, and *N,N*-diisopropylethylamine in *N*-methylpyrrolidinone (NMP). Removal of the N-Trt protecting group by treatment with 95:2.5:2.5 v/v/v TFA/iPr$_3$SiH/H$_2$O then provided crude target peptide thioesters **9c** and **9d**. Finally, purification by reverse-phase HPLC provided **9c** and **9d** in 74% and 68% yields respectively, based on the original resin loading.

Synthesis of the peptide thioesters **9a** and **9b**, corresponding to MccB17(1-19), for use in the synthesis of the full length MccB17 analogues was achieved starting from resin bound peptides **22a** and **22b** (Scheme 3). Assembly of the nine consecutive Gly residues in the target peptide thioesters **9a** and **9b** represented a significant synthetic challenge due to the propensity of poly-Gly sequences to aggregate.[12] Initial attempts to synthesize the target sequences through stepwise coupling of single Fmoc-Gly-OH residues resulted in significant truncation. We therefore chose to employ acid labile *N*-2,4-dimethoxybenzyl (Dmb) moieties along the amide backbone. Backbone Dmb amide groups are known to suppress aspartimide formation as well as aggregation *en bloc* and therefore aid in the synthesis of difficult sequences.[13] To this end we first inserted a commercially available Fmoc-Gly(Dmb)Gly-OH (**23**) dipeptide unit into Fmoc-deprotected **22a** and **22b** to afford resin-bound peptides **24a** and **24b**. The sequence was further elaborated with three single Fmoc-Gly-OH residues *via* iterative Fmoc-SPPS before incorporating a further Fmoc-Gly(Dmb)Gly-OH dipeptide (**23**). Standard Fmoc-strategy SPPS was then employed to afford the fully assembled resin-bound MccB17(1-19) peptides **25a** and **25b**. It is important to note that incorporation of the bulky Dmb moieties into the peptide backbone significantly improved the quality of the peptide produced, with both **25a** and **25b** produced in higher purity (as judged by HPLC-MS analysis) than was achievable through stepwise coupling of single Gly residues without backbone modification. The fully assembled peptides were cleaved from the resin using 20vol.% HFIP in dichloromethane and the resulting crude peptides then subjected to solution phase thioesterification using ethyl 3-mercaptopropionate, PyBOP and di*iso*propylethylamine in NMP.[14] The acid labile protecting groups were removed using a TFA-based acidic cocktail to afford target thioesters **9a** and **9b** in 18% and 24% yields respectively after HPLC purification.

Having synthesized all the proposed target peptide (**10a** and **10b**) and peptide thioester (**9a-d**) fragments, we now turned our attention to the combinatorial ligation-based assembly of these units to afford our suite of full length (**1-4**) and truncated (**5-8**) MccB17 analogues, which included MccB17 (**1**) itself (Scheme 4). For the synthesis of the full length MccB17 analogues, *C*-terminal peptides **10a** or **10b** were each treated with a solution of peptide thioester **9a** or **9b** in the presence of silver(I) nitrate, 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HOOBt) and *N,N*-di*iso*propylethylamine in dimethylsulfoxide (DMSO).[15] After 18 hours each of the reactions were deemed to be complete, as indicated by complete consumption of peptides **10a** or **10b** by HPLC-MS analysis. At this point the reactions were treated with piperidine to effect *in situ* *N*-Fmoc deprotection. After HPLC purification, MccB17 (**1**) and the full length MccB17 analogues **2-4** were isolated in moderate to good yields (35-52%). It should be noted that the isolated yields for **1-4** were significantly lower than the analytical yields approximated from analytical HPLC analysis and was likely the result of poor recovery of the aggregation-prone peptides during reverse-phase purification. Notably, the presence of the extra oxazole heterocycle in peptides **2** and **4** rendered these analogues even more hydrophobic than their serine-containing counterparts **1** and **3**. The truncated MccB17 analogues **5-8** were synthesized by reacting C-terminal peptides **10a** and **10b** with truncated peptide thioesters **9c** and **9d** under identical Ag(I)-assisted ligation conditions to those described for **1-4** (Scheme 4). Following purification by reverse-phase HPLC the desired analogues **5-8** were isolated in good yields (54-72%). The improved isolated yields were the result of the improved solubility of these compounds due to the lack of the aggregation-prone poly-Gly N-terminus.



MccB17 Analogue	Thioester	Peptide	R =	Site A (X,Y =)	Site C (Z =)	Yield
1 (MccB17)	9a	10a	R^1	X = O,Y = S		46%
2	9a	10b	R^1	X = O,Y = S		38%
3	9b	10a	R^1	X = S,Y = O		52%
4	9b	10b	R^1	X = S,Y = O		35%
5	9c	10a	R^2	X = O,Y = S		63%
6	9c	10b	R^2	X = O,Y = S		54%
7	9d	10a	R^2	X = S,Y = O		72%
8	9d	10b	R^2	X = S,Y = O		60%



Scheme 4. Synthesis of MccB17 (**1**) and MccB17 analogues **2-7** *via* Ag(I)-assisted peptide ligation chemistry

Stabilisation of DNA gyrase cleavage complex by 1-8, 10a and 10b

Having prepared MccB17 and a range of full-length and truncated analogues, we next screened the synthetic analogues for their ability to inhibit *E. coli* gyrase supercoiling and to stabilize the gyrase-DNA cleavage complex. The activity of synthetic MccB17 (**1**), MccB17 analogues **2-8** and synthetic precursors **10a** and **10b** were measured in a gel electrophoresis-based assay.[10] DNA cleavage was measured at a range of concentrations and data are quoted relative to the potent DNA gyrase poison ciprofloxacin (CFX) (see Supporting Information for additional data). Table 1 shows the DNA cleavage activity of **1-8** and **10a** and **10b** at a concentration of 100 μM. The synthetic MccB17 (**1**) showed similar poisoning activity compared to the recombinant material (32% and 25%, respectively). Interestingly, modification of MccB17 through the addition of an extra heterocyclic unit in **2** led to a decrease in the amount of DNA cleavage (11%). It should be noted that this result is in contrast to the activity of a recombinant variant that possessed an additional heterocycle that was shown to be a more potent inhibitor of bacterial growth than MccB17.[7a] Further, alternating the *bis*-heterocyclic unit at site A from Thz14-Oxz-15 in MccB17 (**1**) to Oxz14-Thz15 in **3** also led to a decrease in the poisoning activity (25% for **1** vs 16% for **3**). The addition of an extra heterocycle in **4** did not lead to any further change in DNA cleaving activity. Interestingly, the truncated MccB17 analogue **5** (corresponding to Mcc12-43) missing the poly-Gly tail was the most potent DNA gyrase poison, exhibiting 78% of the cleavage activity of CFX (three times that of native MccB17). However truncated analogues **6** and **7**, bearing an extra heterocycle and an alternating *bis*-hereocyclic unit, were similarly potent to native MccB17. It is interesting to note that switching of the oxazole and thiazole rings at Site A results in an approximate 50% drop in cleavage activity, a result consistent with both full length and truncated analogues (compare compounds **1** and **3** with **5** and **7**, Table 1). Truncated compound **8** was identified as producing DNA cleavage both in the presence and absense of gyrase. This gyrase independent activity prompted us to cease further investigation into the gyrase-poisoning activity of this analogue, however, studies into the DNA cleavage mechanism of **8** will be the subject of future investigation. Compound **10a** (MccB17 20-43) and **10b** (containing an extra oxazole) that represent fragments of the natural product that are even shorter than truncated analogues **5-8** retained activity comparable to the natural toxin. It should be noted that in this case the truncated fragment with the native Ser-26 (**10a**) was equipotent to the analogue where an additional oxazole unit had been incorporated at this site (**10b**). Taken together these results suggest that synthetic peptides comprising only a fraction of MccB17 sequence can effectively poison gyrase. This is demonstrated by peptides **10a, 10b** and **5** being still able to stabilise the cleavage complex, the latter being significantly more potent than native MccB17. Other alterations of the full-length structure of the natural product, like the addition of an oxazole ring or the permutation of oxazole and thiazole in the first *bis*-heterocycle, appear to be moderately detrimental to the stabilisation of the cleavage complex in our assays.

Table 1. Stabilisation of the gyrase-DNA gyrase cleavage complex by MccB17 (**1**) and MccB17 analogues (**2-8**) measured by DNA cleavage relative to CFX.

MccB17 analogue[a]	Percentage DNA cleavage relative to CFX
Ciprofloxacin (CFX)	100%
Recombinant MccB17 (**1**)	25 ± 11%
Synthetic MccB17 (**1**)	32 ± 17%
2	11 ± 4%
3	16 ± 9%
4	12 ± 17%
5	78 ± 3%
6	34 ± 3%
7	28 ± 1%
8	41 %[b]
10a	39 ± 9%
10b	32± 5%

[a] Compounds tested at a concentration of 100 μM. [b] cleavage observed for **8** is independent of the presence of gyrase.

Antibacterial activity of 1-8, 10a and 10b.

Synthetic compounds (**1-8**, **10a** and **10b**) were also tested in halo assays against two strains of *E. coli*: a standard strain sensitive to MccB17 (MG1655), and a strain with an increased permeability to compounds due to a deficiency of the outer membrane (NR698). The results of the halo assays are summarised in Table 2 (see Supporting Information for halo images).[16] Synthetic MccB17 (**1**) exhibited inhibition of growth similar to the recombinant toxin. As expected, the permeable strain NR698 proved to be more susceptible to MccB17 and MccB17 analogues than the less susceptible MG1655 strain. Regardless of the strain of *E. coli* tested, native MccB17 showed the most potent bactericidal activity compared to the synthetic analogues. In MG1655 compound **3** exhibited the most potent bactericidal activity of the analogues, demonstrating that the substitution of the oxazole-thiazole by a thiazole-oxazole is slightly detrimental to antibacterial activity (20% loss). Compounds **2** and, **4** exhibited a decrease in bactericidal activity of ~50% compared to MccB17, suggesting that the addition of the oxazole at position 26 is detrimental to the antibacterial activity. Analogue **5**, which was the most potent stabiliser of the gyrase-DNA cleavage complex, also displayed bactericidal activity of ~80% relative to MccB17. This result reflects that, whilst truncation of the N-terminus does not have a negative effect on anti-gyrase activity, this modification leads to a significant loss in antibacterial activity, suggesting a possible role for this domain in internalisation of the gyrase poisons. Compounds **6** and **7** that constitute truncated variants of peptides **3** and **4** displayed negligible bactericidal activity on MG1655. Interestingly, **6** and **7** show a significant degree of antibacterial activity for the NR698 *E. coli* strain, supporting the dependence of the N-terminus for efficient internalisation of the gyrase poisons through the bacterial membrane. Compound **8**, which exhibited gyrase-independent DNA cleavage activity, was found to have no antibacterial activity with either strain. Finally, the smallest MccB17 fragments **10a** and **10b** possessed no antibacterial activity regardless of the strain, despite a stabilisation of the cleavage complex similar to MccB17 in the gyrase assays. This adds further evidence to the necessity of some structural features present within MccB17 for uptake into sensitive *E. coli* cells. Overall, the loss of the N-terminal poly-Gly sequence would appear to significantly reduce the bactericidal activity of the MccB17 analogues.

Table 2. Antibacterial activity of MccB17 (**1**) and MccB17 analogues (**2-8**) against MG1655 and NR698 strains of *E. coli* in halo assays (see Supporting Information for images).

MccB17 analogue[a]	MG1655 halo[b]	NR698 halo[b]
Ciprofloxacin (CFX)[c]	100% (17.6)	100% (25.4)
Recombinant MccB17 (**1**)	83 ± 6%	62 ± 4%
Synthetic MccB17 (**1**)	75 ± 6%	50 ± 4%
2	44 ± 6%	37 ± 4%
3	57 ± 6%	43 ± 4%
4	35 ± 6%	30 ± 4%
5	37 ± 6%	39 ± 4%
6	partial	25 ± 4%
7	partial	28 ± 4%
8	partial	partial
10a	0	0
10b	0	0

[a] 2 μL of a 1.5 mM stock solution added to the plate. [b] percent normalised to CFX. [c] 2 μL of a 120 μM stock solution added to the plate.'Partial' indicates that there was some antibacterial effect, however, complete clearance was not observed.

Experimental Section

Peptide Synthesis

Loading 2-chloro-trityl chloride resin: 2-Chloro-trityl chloride resin (1.22 mmol/g loading) was swollen in dry CH_2Cl_2 for 30 min then washed with CH_2Cl_2 (5 × 3 mL). A solution of Fmoc-AA-OH (2.0 equiv. relative to resin functionalization) and iPr$_2$NEt (4.0 eq.

relative to resin functionalization) in CH_2Cl_2 (final concentration 0.1 M of amino acid) was added and the resin shaken at rt for 16 h. The resin was washed with DMF (5 × 3 mL) and CH_2Cl_2 (5 × 3 mL). The resin was treated with a solution of CH_2Cl_2/CH_3OH/*i*Pr_2NEt (17:2:1 v/v/v, 3 × 3 mL × 5 min) and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL). The resin was subsequently submitted to iterative peptide assembly (Fmoc-SPPS).

Loading estimation of amino acid loading: The resin was treated with 20% piperidine/DMF (3 mL, 3 × 3 min) and the combined deprotection solution made up to 10 mL with DMF. The solution was diluted 200-fold with DMF and the UV absorbance of the resulting piperidine-fulvene adduct measured (λ = 301 nm, ε = 7800 M^{-1} cm^{-1}) to estimate the amount of amino acid loaded onto the resin.

General iterative peptide assembly (Fmoc-SPPS):

Deprotection: The resin was treated with 20vol.% piperidine/DMF (3 mL, 3 × 3 min) and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL) and DMF (5 × 3 mL).

General amino acid coupling: A solution of protected amino acid (4 eq.), PyBOP (4 eq.) and NMM (8 eq.) in DMF (final concentration 0.1 M) was added to the resin. After 1 h, the resin was washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL) and DMF (5 × 3 mL).

Capping: Acetic anhydride/pyridine (1:9 v/v) was added to the resin (3 mL). After 3 min the resin was washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL) and DMF (5 × 3 mL).

Coupling conditions for preformed Fmoc protected amino acids (**11-14**) *and Fmoc-Gly(Dmb)Gly-OH* (**23**): A solution of amino acid (**11-14, 23**) (1.5 eq.), HATU (1.5 eq.), and NMM (3.0 eq.) in either DMF (**11-13**, **23**; final concentration 0.1 M) or 1:1 v/v DMF/DMSO (**14**, final concentration 0.1 M) was then added to the resin (1.0 eq.) and shaken at rt for 16 h. The resin was then washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), DMF (5 × 3 mL).

Cleavage from Wang resin: The resin was washed with CH_2Cl_2 (10 × 3 mL) and dried under vacuum. A mixture of TFA, tri*iso*propylsilane and water (95:2.5:2.5 v/v/v) was then added to the resin. After 2 h, the resin was washed with TFA (3 × 2 mL). The combined cleavage solution was concentrated under a stream of nitrogen. The residue was dissolved in DMSO and purified by preparative HPLC and analyzed by LC-MS and ESI mass spectrometry.

Cleavage from 2-chloro-trityl chloride resin: The resin was incubated in a solution of 20% hexafluoro-2-propanol in CH_2Cl_2 (3 × 3 mL × 20 min) and then washed with CH_2Cl_2 (3 × 3 mL). The combined cleavage solution and washings were concentrated under reduced pressure to give the cleaved protected peptide.

Solution-Phase Thioesterification

Crude protected peptide (1 equiv.) was dissolved in *N*-methylpyrolidinone at 0 °C. Ethyl 3-mercaptopropionate (30 equiv.) was added, followed by PyBOP (5 equiv.) and *i*Pr_2NEt (5 eq.). The solution was warmed to ambient temperature with stirring over 16 h. A solution of TFA/*i*Pr_3SiH/H_2O (95: 2.5: 2.5 v/v/v, 10 mL) was added at 0 °C and the reaction mixture was warmed to room temperature over 2 h. The TFA was removed under N_2(g) and the crude residue was purified by preparative RP-HPLC

Silver-Promoted Fragment Condensation General Procedure

A solution of peptide **10a** or **10b** (1 equiv.), $AgNO_3$ (5 eq.) and hydroxy-1,2,3-benzotriazin-4(3H)-one (HOOBt, 30 eq.) in dry DMSO (10 mM concentration of **10a** or **10b**) was added to thioester **9a-d** (1.3 eq.). *i*Pr_2NEt (20 eq.) was added and the resulting yellow suspension was gently agitated, followed by incubation at 37 °C for 18 h. The mixture was then further diluted with DMSO followed by piperidine, giving a 1:2 (v/v) solution of piperidine in DMSO. After 0.5 h the reaction was quenched with 2:1 v/v TFA/H_2O at 0 °C and directly purified by preparative RP-HPLC.

Gyrase cleavage assays: Cleavage assays were carried out as described previously.[17] Reaction mixture (30 µL) contained 45 mM Tris.HCl (pH 7.5), 44 mM KCl, 4 mM $MgCl_2$, 4 mM DTT, 0.2 mM EDTA, 1.8 mM spermidine, 1 mM ATP, 8.5% v/w glycerol, 0.1 g/L BSA), 0.5 µg relaxed pBR322, and 240 nM gyrase, 3.3% DMSO. The inhibitors were added as 1 µL of a solution in DMSO. Reaction mixtures were incubated for 30 min at 37 °C, then SDS (0.2% w/v) and proteinase K (2 µg/µL) were added and the reaction was incubated for another 30 min at 37 °C. Chloroform/isoamyl alcohol (30 µL, 24:1) and 40% sucrose (30 µL), 100 mM Tris·HCl (pH 8.0), 100 mM EDTA, 0.5 mg/mL bromophenol blue were added to stop the reactions. The mixtures were vortexed for 10 s and centrifuged for 2 min at 13000 rpm. Aqueous layers were loaded on a 1% w/v agarose gel and run either overnight at 18 V or 3 h at 60 V, and stained with ethidium bromide (1 mg/L). Gel bands corresponding to the different DNA topoisomers were quantified with Genetools (Syngene).

Halo assays: MccB17 and derivatives were tested against two different strains in halo assays: MG1655 a standard *E. coli* strain and NR698, an *E. coli* drug-permeable strain.[16] The halo assays were performed using the following method: 200 µL of an overnight culture in 5 mL LB of either MG1655 or NR698 were added to 3 mL of molten LSS agar that was poured onto a LB agar plate. 2 µL of DMSO containing MccB17 or MccB17 analogue was applied onto the surface of the plate. The plate was incubated overnight at 37 °C before measuring the diameter of the halo created by each compound.

Conclusions

In summary, we have developed a convergent synthetic route to both MccB17 itself, as well as structural analogues not accessible through biosynthetic means. Interestingly, analogue **5**, which lacks the poly-Gly *N*-terminus, showed a 2-fold enhancement in gyrase cleavage complex stabilizing ability *in vitro* relative to the native sequence. This trend was observed for all of the truncated analogues relative to their full-length partners. This strongly suggests that the glycine-rich *N*-terminus is unlikely to provide interaction with the gyrase-DNA complex. The native arrangement of oxazole and thiazole heterocycles within **1** and **5** appears optimal for gyrase-DNA stabilization, with modifications including a serine-to-oxazole mutation and *bis*-heterocyclic isomerization leading to significant decreases in activity. *In vivo* bactericidal activity was, however, greatly diminished for the truncated analogues **5-8** relative to the full-length peptides **1-4**. This indicates that the antibacterial activity of the gyrase-poisoning peptides necessitates recognition-based uptake into the target cells based on the full-length sequence of MccB17. Overall, the promising activity of the truncated sequences now enables investigations into the fundamental gyrase-DNA stabilizing activity imbedded within the MccB17 sequence. Furthermore, the information from this study may allow for the generation of structurally simplified DNA gyrase poisons as potential antibacterial leads in the future.

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