Microcins are small (<10-kDa) ribosomally synthesized peptide antibiotics produced by Enterobacteriaceae (17). Three microcins, B, C, and J, form a subgroup of posttranslationally modified microcins. Members of this subgroup have highly unusual structures and inhibit cellular enzymes that are validated targets for antibacterial drug development (25). Posttranslationally modified microcins are attractive as drug candidates because of their strong antibacterial action and because virtually limitless numbers of their derivatives can be generated by means of mutation, chemical synthesis, or both. Microcin B (McB), a 43-residue peptide with thiazole and indole rings (13), inhibits DNA gyrase (21). Microcin J, a 21-amino-acid peptide, assumes an unusual threaded lasso structure (2, 23, 27) and inhibits bacterial RNA polymerase (1, 18). The structure of the subject of this study, McC (compound 1) is shown in Fig. 1a. McC is a heptapeptide with a formylated O-propylamine group (9).

The passage of McC through the inner layer of the Escherichia coli cell wall is carried out by the YejABEF transporter (19). Once inside the cell, McC is specifically processed by one of the several broad-specificity E. coli cytoplasmic aminopeptidases (12). The product of processing, modified aspartyl-adenylate (compound 2) (15), closely resembles Asp-AMP (compound 3) (Fig. 1c), the natural reaction intermediate of the tRNA<sup>Asp</sup> aminoacylation reaction catalyzed by AspRS. However, because the bond between the α-carboxyl of C-terminal aspartate and the phosphoramidate nitrogen is nonhydrolyzable, compound 2 inhibits AspRS. Unprocessed McC has no effect on tRNA<sup>Asp</sup> aminoacylation, while processed McC has no effect on McC-sensitive cells at concentrations at which intact McC strongly inhibits cell growth. Thus, McC is a Trojan horse inhibitor (22): the peptide part allows McC to enter sensitive cells, where it gets processed, liberating the inhibitory part of the drug.

Aminoacyl-tRNA synthetases (aaRSs) carry out the condensation of genetically encoded amino acids with cognate tRNAs. When 1 of the 20 aaRSs present in the cell is inhibited, the corresponding tRNA is not charged. This leads to protein synthesis inhibition and cell growth arrest. In principle, variation of the last amino acid of the McC peptide, the product of the mccA gene, should allow investigators to obtain McC derivatives targeting aaRSs other than AspRS. Unfortunately, the results of systematic structure-activity analyses of the McC peptide revealed that substitutions in the seventh codon of mccA invariably prevented McC production, presumably by interfering with posttranslational modifications of the MccA peptide by the McC maturation enzymes (11). Indeed, in vitro analysis showed that the C-terminal asparagine of MccA is required for the addition of the adenosine moiety by the MccB protein (24).

Aminoacyl-sulfamoyl adenosines are well-known nanomolar inhibitors of their corresponding aaRSs (5, 20, 26). However,
these compounds show low in vivo activities due to limited membrane permeability and the absence of a transporter for these compounds. Here, we show that through chemical attachment of aminoacyl-sulfamoyl adenosines to the first 6 amino acids of the MccA peptide, potent antibacterial agents can be generated. The new compounds share the Trojan horse mechanism of action with McC but target aaRSs specified by the last amino acid of the peptide moiety.

**MATERIALS AND METHODS**

**General chemistry.** Reagents and solvents were from commercial suppliers (Acros, Sigma-Aldrich, Buchem, and Novabiochem) and used as provided, unless indicated otherwise. Dimethylformamide (DMF) and tetrahydrofuran were analytical grade and were stored over 4Å molecular sieves. For reactions involving 9-fluoroenylmethoxy carbonyl (Fmoc)-protected amino acids and peptides, DMF for peptide synthesis (low amine content) was used. All other solvents used for reactions were analytical grade and used as provided. Reactions were carried out in oven-dried glassware under a nitrogen atmosphere and stirred at room temperature, unless indicated otherwise.

\[ \text{Asp-AMP (3)} \]

\[ 4, 7, 11 (D): R = -\text{CH}_2\text{-COO}^- \]

\[ 5, 8, 12 (E): R = -(\text{CH}_2)_2\text{-COO}^- \]

\[ 6, 9, 13 (L): R = -(\text{CH}_2)_3\text{-CH(\text{CH}_3)2} \]

\[ 4, 5, 6 \]

\[ (i) \text{Fmoc-MRTGNA-OH, HOBt, DIC, DIPEA; (ii) Et}_3\text{N/DMF (1:1 [vol/vol])} \]

\[ \text{MRTGNAD-SA (7), MRT GNAE-SA (8), MRTGNAL-SA (9) DSA (4), ESA (5), LSA (6)} \]

\[ \text{Asp-AMP (3)} \]

\[ (i) \text{Fmoc-MRTGNA-OH, HOBt, DIC, DIPEA; (ii) Et}_3\text{N/DMF (1:1 [vol/vol])} \]

\[ 4, 5, 6 \]

\[ (i) \text{Fmoc-MRTGNA-OH, HOBt, DIC, DIPEA; (ii) Et}_3\text{N/DMF (1:1 [vol/vol])} \]

\[ 4, 5, 6 \]

\[ (i) \text{Fmoc-MRTGNA-OH, HOBt, DIC, DIPEA; (ii) Et}_3\text{N/DMF (1:1 [vol/vol])} \]

\[ 4, 5, 6 \]

\[ (i) \text{Fmoc-MRTGNA-OH, HOBt, DIC, DIPEA; (ii) Et}_3\text{N/DMF (1:1 [vol/vol])} \]

\[ 4, 5, 6 \]

\[ (i) \text{Fmoc-MRTGNA-OH, HOBt, DIC, DIPEA; (ii) Et}_3\text{N/DMF (1:1 [vol/vol])} \]
acetyl)sulfamoyladenosines (compounds 4 to 6) and their intermediates are not described here.

**Synthesis of the hexapeptide Fmoc-MRTGNA-OH (compound 10).** The synthesis of hexapeptide 10 was done by standard-phase peptide synthesis using Fmoc-protected amino acids and Wang resin (680 μmol of reactive groups per gram) as the solid support. The amino acid building blocks were coupled using activation mixtures consisting of appropriately protected amino acid (4.0 eq.), N-hydroxysuccinimidyl carbonate (HOBt; 0.4 eq.), diisopropylcarbodiimide (DIC; 4.0 eq.), and N,N-diisopropylethylamine (DIPEA; 2.0 eq) relative to the active support (1.0 eq). For Asn, Arg, and Met, building blocks with acetyl-labile side-chain protecting groups were used: Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pmc)-OH, and Fmoc-Thr(OrtBu)-OH. Cleavage from the solid support and side chain deprotection were done by treatment with 5% thioanisole and 5% H2O in trifluoroacetic acid. The resulting Fmoc-hexapeptide 10 was purified by reverse-phase HPLC (RP-HPLC) (solvent A, 5% CH3CN, 0.1% HCOOH, H2O; solvent B, 5% CH3CN, 0.1% HCOOH, H2O; solvent B, 5% CH3CN, 0.1% HCOOH, H2O, CH3CN). The peak corresponding to compound 7 was identified by ESI-MS and lyophilized. HRMS for C55H76N17O17S2 [M-H]− calculated, 1310.50468; found, 1310.50428.

Design and synthesis of McC analogs. Given our failure to generate McC derivatives with targets other than AspRS by means of mutation (9), we decided to obtain McC analogs by total chemical synthesis. In all, three different compounds were prepared. Compound 7 was designed to target AspRS, which is also the target of McC. Two other compounds (8 and 9) were expected to target GluRS and LeuRS, respectively. To simplify synthesis, synthetic compounds differed from natural McC in several ways. First, the N-acetyl-O-aminopropylphosphoramidate linker of the aspartyl-adenylate moiety of processed McC (compound 2) was replaced by its close analog sulfamate, giving 5′-O-(N-L-aspartyl)-sulfamoyladenosine [DSA; compound 4]; XSA abbreviates representation 5′-O-(N-t-aminosulfamoyl)-sulfamoyladenosine, where X can be replaced by any 1-letter amino acid code to indicate the corresponding amino acid derivative] as the expected processing product of the compound expected to retain the target specificity of McC and ESA (compound 5) and LSA (compound 6) as the expected processing products of the other two compounds. DSA inhibits AspRS with an inhibition constant of 8.0 nM (3). ESA and LSA inhibit, respectively, GluRS and LeuRS with inhibition constants of 2.8 nM (4) and 8.4 nM (8). Compounds 7, 8, and 9 lack the O-amino propyl group present in intact McC, as well as the N-formyl group. While both groups contribute to the antibacterial activity of McC, a natural derivative lacking both groups is still active (12).

As nothing was known in advance about the stability of compounds 7 to 9, the use of strong acid, strong base, and strong nucleophiles during their synthesis was avoided, to limit possible decomposition. We chose to use a convergent ap-
proach of coupling of Fmoc-protected hexapeptide 10 (Fmoc-MRTGNA-OH), corresponding to the first 6 amino acids of MccA prepared by standard solid-phase peptide synthesis, with XSAs 4 to 6. This was followed by deprotection of the coupling product to yield MRTGNA-SAs 7 to 9. The existing synthesis scheme for XSAs was modified to allow for the preparation of larger amounts of the compounds. The main difference between our method and previous syntheses (3, 4, 6) was the use of 2′,3′-O-t-butyl-dimethylsilyl protection instead of 2′,3′-O-isopropylidene. This modification was developed by Ferreras et al. (7) for the preparation of 5′-O-(N-salicyl)-sulfamoyladenosine. We found that this method is preferential, as intermediates are far less prone to undergo intramolecular cyclization to N3′-5′-cy cloadenosine (18).

Triethylamine salts of XSAs 4 to 6 were coupled to Fmoc-MRTGNA-OH (compound 10) by in situ preactivation of hexapeptide 10 with HOBT in the presence of DIC and subsequent reaction of the activated hexapeptide with XSA·Et3N in the presence of DIPEA (Fig. 1d). Prolonged incubation with Et3N-DMF at room temperature led to successful deprotection of the presence of DIPEA (Fig. 1d). Prolonged incubation with Et3N-DMF at room temperature led to successful deprotection allowing us, following HPLC purification, to obtain synthetic MccA analogs in amounts sufficient for biological and biochemical analyses. The identity of the 5′-O-(N-salic yl)-sulfamoyladenosine intermediates 4 to 6 was confirmed by HR-MS and nuclear magnetic resonance. The identities of the Fmoc-MRTGNA-OH hexapeptide 10 and the synthetic Mcc analogs 7 to 9 were confirmed by HR-MS.

In vivo activity of Mcc analogs. The antibacterial activities of MRTGNA-SAs 7 to 9 and corresponding XSAs 4 to 6 were determined by monitoring the appearance of growth inhibition zones (anabiosys halos) on lawns of Mcc-sensitive E. coli K-12 BW28357 cells (10). As controls, Mcc and its derivative, without the N-terminal formyl and the aminopropyl groups were used; as we have described elsewhere (16), such a derivative is produced by E. coli cells lacking aminopeptidases A, B, and N and harboring an Mcc-producing plasmid with a disrupted mcdc and/or mccE gene). The results, presented in Fig. 2a, can be summarized as follows. First, all MRTGNA-SAs possessed antibacterial activity. Clear zones of growth inhibition were observed with a 10 µM solution of MRTGNAD-SA, the most active of the synthetic compounds. Growth inhibition zones of comparable size were detected with a 25 µM solution of MRTGNAL-SA and a 50 µM solution of MRTGNAE-SA. MRTGNAD-SA was less active than intact Mcc but considerably more active than the Mcc derivative without the N-terminal formyl and the aminopropyl groups. Second, detectable growth inhibition zones were observed with 100 µM and 250 µM solutions of LSA and DSA, respectively. No antibacterial activity of ESA was detected at concentrations of up to 1,000 µM. We therefore conclude that the addition of the MRTGNA heptapeptide increases the antibacterial activity of XSAs by at least an order of magnitude. Moreover, a comparison of MRTGNAD-SA and the Mcc derivative lacking the formyl and propylamine groups indicates that the sulfamoyl linkage increases antibacterial activity. Additional modifications such as the presence of an N-terminal formyl group or the presence of an aminopropyl group may increase the antibacterial activities of MRTGNAD-SA and other MRTGNA-SAs even further.

Cells carrying mutations in the yej genes coding for the YejABEF inner membrane ABC transporter are resistant to Mcc because they are unable to internalize the drug (19). BW28357 cells harboring a deletion in the yejA gene (19) were resistant to up to 100 µM MRTGNA-SAs (Fig. 2b). In contrast, the sensitivities of these cells to XSAs were indistinguishable from those of the wild-type control cells (compare Fig. 2a and b). We therefore conclude that the YejABEF transporter is responsible for the uptake of MRTGNA-SAs. We further conclude that YejABEF is not involved in XSAs transport.
Once inside the cell, McC is deformylated and then processed by the action of one of the three broad-specificity amino-peptidases, A, B, or N (12). BW28357 cells harboring a triple deletion of the pepA, pepB, and pepN genes coding, respectively, for peptidases A, B, and N, are resistant to the drug because they cannot process it (12). These cells were also resistant to up to 100 $\mu$M MRTGNAX-SAs, while sensitivity to XSAs was indistinguishable from that of the wild-type control cells (Fig. 2c). The results therefore suggest that peptidases A, B, and N are required for processing of MRTGNAX-SAs, while sensitivity to XSAs was indistinguishable from that of the wild-type control cells (Fig. 2c). The results therefore suggest that peptidases A, B, and N are required for processing of MRTGNAX-SAs (see below, also). Additional analysis involving McC-sensitive double (pepA pepB, pepA pepN, and pepB pepN) and single (pepA, pepB, and pepN) mutants revealed that they were all sensitive to MRTGNAX-SAs (data not shown). Thus, any one of the three peptidases is sufficient to impart sensitivity to MRTGNAX-SAs.

**In vitro activity of McC analogs.** Our next step was to determine the intracellular targets of MRTGNAX-SAs 7 to 9. To this end, in vitro tRNA aminoacylation reactions in *E. coli* extracts were carried out using radioactively labeled aspartate, glutamate, and leucine. As expected, each XSA 4 to 6 inhibited aminoacylation of cognate tRNA but had no effect on noncognate tRNA aminoacylation (i.e., LSA abolished aminoacylation of tRNA$^{\text{Asp}}$ but not of tRNA$^{\text{Glu}}$) (Fig. 3a). The addition of MRTGNAX-SAs also inhibited cognate (as determined by the last amino acid of the peptide moiety) tRNA aminoacylation (Fig. 3a). XSAs also inhibited tRNA aminoacylation in extracts prepared from cells lacking pepti-
dases A, B, and N (Fig. 3b). In contrast, MRTGNAX-SAs had no effect on tRNA aminoacylation in mutant cell extracts (Fig. 3b). We therefore conclude that synthetic McC-like compounds are processed by aminopeptidases and, upon processing, inhibit AaRSs specified by the last amino acid of the peptide moiety.

In vivo targets of McC analogs. The results presented so far suggest that synthetic McC analogs enter the cell through the YejABEF transporter, are processed by cytoplasmic aminopeptidases, and then target AaRSs specified by their last amino acids. To prove this conjecture, we determined whether *E. coli* BL21(DE3) cells overproducing AspRS from *Deinococcus radiodurans*, GluRS from *Acidithiobacillus ferrooxidans*, or LeuRS *Methanothermobacter thermautotrophicus* become resistant to compounds 7, 8, and 9. As control, cells overproducing *D. radiodurans* ProRS were used. Previously, we showed that overproduction of *D. radiodurans* AspRS but not ProRS makes *E. coli* resistant to McC (15). Because the initial plasmid overproducing LeuRS from thermophilic *A. ferrooxidans* did not lead to changes in sensitivity to any of the compounds tested (data not shown), a plasmid overproducing *E. coli* LeuRS was created. As can be seen in Fig. 4, only overproduction of “cognate” mesophilic AaRSs afforded protection from synthetic McC analogs and McC. Likewise, AspRS and LeuRS overproduction led to increased resistance to DSA and LSA. No protection...
from ESA could be observed (data not shown), as this compound lacks antibacterial activity (see above). Based on these results we conclude that, in vivo, synthetic McC analogs target AaRSs whose identities are determined by the nature of the last amino acid of an McC analog.

**DISCUSSION**

The principal result of our work is the demonstration that McC can be used as a platform to prepare synthetic compounds that target AaRSs other than AspRS, the target of wild-type McC. This is a significant advance, since neither site-specific mutagenesis of the mcca gene coding for the peptide moiety of McC (25) nor bioinformatics searches for mcc gene homologs (25) have led to compounds with altered target specificities.

Our experiments reveal that synthetic McC-like compounds retain the essential Trojan horse features of the original compound. First, the McC-like compounds are at least 10 times more active than the corresponding XSSAs, due to the contribution of the MccA hexapeptide MTRGNA. The facilitated transport of McC-like compounds is due to the action of the YejABEF transporter, which is also responsible for McC uptake (19). Finally, the McC-like compounds are processed inside the cytoplasm of sensitive cells by the same broad-specificity aminopeptidases that process McC (12).

The current and previous results (11) show that changes in the McC peptide moiety, including substitutions altering the enzyme-inhibiting part of McC and changes in the linker between the peptide and nucleotide parts of McC, are tolerated with limited effects on activity and may even increase the whole-cell antibacterial activity of McC-like compounds. This modularity of McC is interesting from a drug development point of view. An important question that remains is how much the structure of the toxic part may deviate from the native processed McC (compound 2) structure to retain the uptake advantage. The antibacterial activity of MRTGNAL-SA (compound 9) suggests that these differences can be quite extensive, as this compound differs from compound 2 both at the linker moiety and the aminocycl side chain (an uncharged isobutyl side chain versus an anionic carboxymethyl). Thus, total chemical synthesis should allow investigators, in principle, to generate McC-like compounds targeting each of the 20 AaRSs in the cell. It would be interesting to see if amides or esters consisting of the MRTGNA peptide and inhibitors of essential bacterial cell components other than AaRSs also lead to antibacterials with improved potencies.

By bypassing the need for specific maturation enzymes acting on the MccA heptapeptide, our results open several avenues for preparation of novel McC-like compounds that act on bacteria other than those targeted by McC. For example, compounds containing peptide moieties shorter than the MccA heptapeptide can be readily prepared and tested. Conceivably, such compounds may enter sensitive cells through transporters other than YejABEF. Since McC-like compounds are processed by ubiquitous broadly specific aminopeptidases and since the ultimate target, an aaRS, is evolutionarily conserved, such compounds may inhibit the growth of bacteria other than E. coli. An alternative strategy is to couple a nonhydrolyzable XSA to a peptide known to specifically enter a particular group of bacteria and thus create a narrow-spectrum inhibitor. Finally, peptide library-based approaches can be used for the generation and screening of McC-like compounds with desired properties. This work is currently under way in our laboratories.

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