Abstract: The increasing emergence of resistances against established antibiotics is a substantial threat to human health. The discovery of new compounds with potent antibiotic activity is thus of utmost importance. Within this work, we identify strong antibiotic activity of the natural product myxocoumarin B from *Stigmatella aurantiaca* MYX-030 against a range of clinically relevant bacterial pathogens, including clinical isolates of MRSA. A focused library of structural analogs was synthesized to explore initial structure-activity relationships and to identify equipotent myxocoumarin derivatives devoid of the natural nitro substituent to significantly streamline synthetic access. The cytotoxicity of the myxocoumarins as well as their potential to cure bacterial infections *in vivo* was established using a zebrafish model system. Our results reveal the exceptional antibiotic activity of the myxocoumarin scaffold and hence its potential for the development of novel antibiotics.

Myxobacteria are talented producers of complex, biomedically interesting specialized metabolites from most diverse natural product classes. Prominent examples include epothilone A (1) and analogs as anti-cancer chemotherapeutics,[2] the antifungal soraphen A$_{16}$ (2),[3] or the antibacterial cystobactamids,[4] such as 3 (Figure 1). These organisms have thus proven to be a promising resource for the discovery of new small molecules with potent biological functions, particularly for applications with increasing demand due to emerging resistance against currently known agents, both in the agrochemical and medical sectors. In 2013, we have described the discovery of two natural products from *Stigmatella aurantiaca* MYX-030, myxocoumarins A (4) and B (5), both of which being equipped with a long alkyl side chain and a rare aromatic nitro group.[5] Myxocoumarin A (4) displayed potent inhibitory effects against a range of agrochemically relevant pathogenic fungi, while 5 could not be biologically assessed due to the low production titers and the loss of a viable producing strain. To allow for antifungal evaluation of 5, a concise total synthesis of this compound was developed, revealing a complete lack of the anticipated antifungal properties.[6] A possible natural function as well as...
prospective applications of myxocoumarin B (5) thus remained elusive. This raised our interest in exploring potential other biological functions of 5. We therefore set out to more broadly explore potential antimicrobial properties of myxocoumarin-type scaffolds within this work.

Initial screening of synthetic 5 against *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* using classical disk-diffusion assays revealed a strong activity of 5 against the latter two, Gram-positive bacteria, comparable to the employed standard kanamycin. Owing to these promising preliminary results, a closer inspection of the antibiotic potential of the myxocoumarins was targeted. Given the straightforward and flexible synthetic access developed for coumarins was targeted. Given the straightforward and flexible synthetic access developed for coumarins was targeted. Given the straightforward and flexible synthetic access developed for coumarins was targeted.

![Scheme 1. Synthesis of hydroxy-substituted myxocoumarin analogs 10a–k and 11a–k by Pechmann condensation with β-keto esters 6a–k with different side-chains R'.](image)

**Table 1.** Substitution pattern and synthetic yields of analogs 10 and 11.

| Compound | R'   | Yield [%] of 10 (R'=H) | Yield [%] of 11 (R'=OH) |
|----------|------|------------------------|-------------------------|
| a        | H    | 69                     | 73                      |
| b        | n-C8 | 42                     | 30                      |
| c        | n-C8 | 42                     | 33                      |
| d        | n-C8 | 26                     | 27                      |
| e        | n-C8 | 36                     | 32                      |
| f        | n-C8 | 35                     | 25                      |
| g        | n-C8 | 33                     | 42                      |
| h        | n-C8 | 30                     | 25                      |
| i        | n-C8 | 30                     | 26                      |
| j        | n-C8 | 25                     | 28                      |
| k        | iso-C8 | 37               | 19                     |

alkyl substitution with phenol building blocks in a TFA-mediated Pechmann condensation.[6] Ethyl 2-methylacetooacetate (6a) was deprotonated with n-BuLi/DIPA and selectively acetylated with iodoalkanes 7b–k to deliver the desired β-keto esters 6b–k (Scheme 1). The analogs bearing linear side chains from C4 to C13 (6b–6i) and C17 (6j) were obtained in yields ranging from 43% to 62%. The terminally branched 6k gave a lower yield of 38%. The β-keto esters were subsequently condensed with resorcinol (8) and phloroglucinol (9) to give the corresponding mono- (10a–k) and dihydroxy (11a–k) myxocoumarin analogs (for yields, see Table 1).

To test the role of the methyl group at the position neighboring the ester functionality, derivatives bearing a proton (12) or an ethyl group (13) at R' were prepared by alkylation of...
ethyl acetoacetate or ethyl 2-ethyldiacetoacetate with 7g followed by Pechmann condensation with phloroglucinol (9).

To further increase the structural diversity of the myxocoumarin chemical library beyond hydroxyl analogs of 5, a selection of O-methylated (14–17) and O-acetylated (18–21) derivatives was additionally prepared (Figure 2), either bearing a proton at position R1 or an \( n \)-C5 alkyl chain as in the original myxocoumarin scaffold. While compounds 14 and 18–21 were obtained by either O-methylation or O-acetylation of the corresponding phenol analogs 10a and 10a, 11a, 10g, 11g using MeI or Ac2O, respectively, 15–17 were generated by Pechmann condensation of 3,5-dimethoxyphenol or 3-methoxyphenol with either 6a or 6g. Furthermore, four myxocoumarin derivatives 22–25 bearing the natural nitro function were prepared. Compounds 22–24 were accessed by triflation and a subsequent nitration reaction of the corresponding hydroxy coumarins,\(^{[8]}\) while 25 was synthesized by O-acetylation of myxocoumarin B (5). In addition, a dimethylamino- (26), amino- (27), chloro- (28) and iodo- (29) analog were accessed by Pechmann condensation of the correspondingly functionalized phenols with 6g (see Supporting Information for experimental details on the syntheses of 12–29).

Having this myxocoumarin library composed of the natural product 5 and 40 synthetic structural analogs in hands, we set out to evaluate their antimicrobial potential. All compounds were inactive against the opportunistic pathogenic yeast \( C. albicans \), the gram-negative bacterium \( Pseudomonas aeruginosa \), as well as the gram-positive \( Micrococcus luteus \) and \( Listeria monocytogenes \) at the maximum tested concentrations (250 \( \mu \)g/mL). However, strong antibacterial potential against the gram-positive bacteria \( Bacillus subtilis \) NCTC5398 and \( S. aureus \) NCT6571 was observed for a number of analogs (Table 2, see Table S1). The natural product 5 exhibited very strong anti-bacterial activity against these test strains, with MIC values as low as 0.3 \( \mu \)g/mL against \( S. aureus \) NCT6571 combined with an up to 400-fold higher LC50 value in a zebrafish model (119.6 \( \mu \)g/mL). For the mono-hydroxylated 10 and dihydroxylated 11 compound series, a clear structure-activity relationship became obvious, with the activity correlating with the length of the alkyl side chain at R1. Considering the activity of analogs 10 and \( S. aureus \) NCT6571, strong effects were only observed for 10e–g with \( C_7 \) to \( C_9 \) alkyl substitution, with lowest MICs for the natural \( C_7 \) substituent. Inhibition of \( B. subtilis \) followed a similar trend, but activity extended to \( C_8 \) and to a smaller extent to \( C_9 \) substitution. The series of dihydroxylated analogs generally had a broader activity profile in terms of alkyl chain length, with significant inhibition already starting from \( C_4 \) up to \( C_9 \) substitution.

Alkyl chain lengths beyond the natural \( C_7 \) substituent lead to a fast drop in antibacterial activity for both compound series, 10 and 11. When correlating antibacterial structure-activity relationships with toxicity in the zebrafish model, it became evident that longer side chains also lead to drastically increased LC50 values and thus less toxicity. Overall, besides the natural product 5, 10f (\( C_6 \)), 10g (\( C_8 \)) and 11e–11g (\( C_4–C_8 \)) exhibited the most promising activity profiles.

When considering R1 substitution, analog 12 bearing a proton at this position had dramatically reduced activity when compared to its methylated congener 11g, while activity in ethyl derivative 13 was retained. Among all other tested compounds 14–29, the O-acetylated analogs 20 and 21 and to an even lesser extent O-methylated derivative 17 and chlorinated 28 all bearing the natural \( C_7 \) alkyl substitution, retained weak antibacterial effects. Interestingly, the antibacterial activity of the O-acetylated analog 25 of myxocoumarin B (5) was approx. 25-fold higher against \( B. subtilis \) NCTC5398 and 2–4-fold higher against \( S. aureus \) when compared to the natural product. However, this was accompanied with an approx. 4-fold increase in toxicity.
in toxicity in the zebrafish model, overall leading to similar activity versus toxicity ratios.

Overall, these generated structure-activity data indicated that substitution at R₁ is required for strong antibiotic activity, preferably with OH or OAc, that analogs with nitro substitution at R₁ generally show higher activity (roughly 10-fold when compared to the respective phenolic derivatives), and that alkyl side-chain lengths from C₇ to C₁₀ seem to be optimal for activity, with increased side-chain lengths correlating with decreased toxicity in the zebrafish model. This structure-activity correlation was corroborated by synthesis of the C₇ analogs of 5, without (30) and with (31) O-acetylation of the phenol at R₁. Both compounds indeed showed activity comparable to that of 5, yet with approx. 6-fold increased toxicity. Most importantly, the antibiotic potential of all active myxocoumarin analogs was also retained against S. aureus MRSA, showing the high potential of these compounds to combat this clinically relevant, highly resistant bacterial pathogen.

We next evaluated the cytotoxicity of all myxocoumarin analogs with significant antimicrobial activity (at least one MIC value at or below 15.6 μg/mL) by screening against healthy human MRC-5 fibroblast cells (see Table S2). This revealed low selectivity indices (SI) for some derivatives (e.g., below 1 for 11g and 17), with up to an SI of 87.5 for the natural product 5. For further in-depth studies, we selected three analogs with high antibacterial activity combined with a range of selectivity indices, namely myxocoumarin B (5) (SI = 87.5), its O-acetyl analog 25 (SI = 25), and the most potent dihydroxylated congeners 11e equipped with a C₁₂ alkyl chain (SI = 3.8). These compounds were evaluated against a larger panel of S. aureus strains, including clinical isolates (Table 3). The strains were collected from veterinary specimens (dog urine, ear swab and mouth swab) and showed resistance to one or more commonly used antibiotics in veterinary practice. Enterococcus faecium, as another Gram-positive opportunistic pathogen, was also included in the assessment. To our delight, all compounds showed pronounced antibacterial effects against all tested strains, with 25 being the most active antibacterial across all evaluated organisms (Table 3).

| Table 3. Antibiotic activity profile of 5, 25 and 11e against a selection of S. aureus strains and Enterococcus sp. (top), including clinical isolates (bottom). |
|------------------|------------------|------------------|
| Compound        | S. aureus MRSA ATCC43300 (μg/mL) | S. aureus ATCC9144 (μg/mL) | Enterococcus faecium ATCC6057 (μg/mL) |
| 5                | 0.6              | 0.3              | 2 |
| 25               | 0.15             | 0.15             | 0.6 |
| 11e              | 4                | 2.5              | 2.5 |
| Compound        | Staphylococcus sp. 80103770 | Staphylococcus sp. 80100861 | Staphylococcus sp. 80100865 |
| 5                | 0.3              | 0.3              | 2 |
| 25               | 0.15             | 0.15             | 0.6 |
| 11e              | 2.5              | 5                | 2.5 |

The likelihood of resistance development against 5, 25 and 11e was assessed by multistep resistance selection of S. aureus ATCC9144 by propagating cultures with subinhibitory concentrations of the respective individual compound for 20 rounds, followed by one round of growth without selective pressure.[9] Over the course of this treatment, S. aureus developed a 5-fold increase in MIC against 5, a 4-fold increase against 25 and only a 2-fold increase for 11e. Therefore, high antibiotic potential was retained for all compounds. This indicates a low likelihood of S. aureus for developing rapid resistance to the tested myxocoumarins.

Based on above resistance development assays and SI values, 5 and 25 were selected for evaluation of the antibiotic potential in vivo in a S. aureus- zebrafish infection model (Figure 3). The zebrafish infection model is a well-recognized platform for studying host-pathogen interactions, as well as for the development of therapeutic strategies.[10] In this experiment, wild type zebrafish embryos were challenged with the lethal systemic infection with S. aureus MRSA 43300, and followed for the survival and bacterial burden after 3-days treatments with 5 and 25. Antibacterial efficacy of the selected myxocoumarins was evaluated in relation to the efficacy of vancomycin and linezolid, two antibiotics of clinical relevance. Analyses of zebrafish embryo survival coupled with bacterial proliferation confirmed that both compounds are highly efficient in rescuing zebrafish from infection, with the effect being dose dependent (Figure 3 A, B). Remarkably, both 5 and 25 proved approx. 10-fold more efficient at 0.5×MIC, with even approx. 35-fold increased activity at 1×MIC when compared to vancomycin, and approx. 2-fold higher activity at these concentrations when compared against linezolid.

Using 2×MIC of either 5 or 25 completely eliminated bacterial infection in vivo, as reflected in the efficiency of reduction of the bacterial burden (Figure 3C). Most importantly, treatment of infected zebrafish with 5 restored survival rates at all tested concentrations to the level of uninfected specimen (Figure 3A), thus proving both, high antibiotic efficiency and low toxicity of this compound upon application in vivo.

Overall, our work provides first structure-activity relationship data on a new class of antibiotics based on the myxocoumarin scaffold. It is interesting to note that the natural nitro substitution seems to be crucial for strong antibiotic activity. The most potent congeners provide outstanding sub-nanomolar antibacterial potency, even against drug-resistant S. aureus strains accompanied with low toxicity, both, in vitro and in vivo. Particularly encouraging are the obtained results on eliminating bacterial S. aureus infections in zebrafish by 5, clearly outcompeting the established antibiotics vancomycin and linezolid, not only in terms of clearing infections, but most importantly also in terms of restoring survival of infected zebrafish to levels of healthy specimen. The myxocoumarins thus represent a valuable scaffold for the development of new antibiotics against clinically relevant bacterial pathogens, particularly for desperately needed new treatment option against MRSA.[11]
Figure 3. Myxocoumarin B (5) and derivative 25 rescued zebrafish embryos of S. aureus MRSA 43300 infection (A–B) and significantly decreased the bacterial burden (C). The Kaplan-Meier curves of the infected embryos survival upon different doses of vancomycin (Van), linezolid (Lin) and myxocoumarins 5 and 25 are shown. Survival of the treated infected fish was compared to those in the group without treatment (infected control) and the uninfected group (injected with 5% PVP [polyvinylpyrrolidone], which was used as a vehicle for the S. aureus suspension for infection). Embryos were monitored daily for survival. Data are compilations of two independent experiments using two replicates (n = 20 embryos/replicates) for each group. Bacterial burden was determined at 4 dpi by plating of the crushed embryos for colony forming units (CFUs). Data are compilations of two independent experiments using ten embryos for each group. Each dot represents an individual fish (square – untreated embryos, circle – treated embryos).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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