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Synthesis and initial biological evaluation of myxocoumarin B†

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The myxocoumarins A and B from *Stigmatella aurantiaca* MYX-030 are natural products featuring unusual nitro- and long-chain alkyl substitution. While myxocoumarin A was shown to exhibit strong antifungal properties, the antifungal potential of myxocoumarin B was not yet assessed due to low production titers during initial isolation. We therefore developed a total synthesis of myxocoumarin B that involves a late-stage Pd-catalyzed nitration of the coumarin core. The availability of synthetic material facilitated the initial evaluation of the bioactivity of myxocoumarin B, which revealed a lack of activity against medically relevant *Candida* sp. and low cytotoxicity *in vitro* against human fibroblasts (MRC-5) and *in vivo* (zebrafish).

Introduction

Natural products have a huge impact as leads and drugs in medicine and agrochemical applications. Owing to the continued emergence of new and untreatable maladies and the evolution of pathogens exhibiting resistance against existing treatment options, the continued discovery of novel scaffolds with potent biological activities is of utmost importance. A rich source of such natural products with truly novel molecular architectures and activity profiles are myxobacteria. These mostly soil-dwelling δ-proteobacteria are particularly talented producers of unusual ribosomal and non-ribosomal peptides and polyketides with often unprecedented modes of actions.[1–5] It is thus not surprising that many myxobacterial natural products have intensely been studied in recent years. Intriguing examples include the antineoplastic pretubulusin (**1**),[6] the antifilarial corallopyronin A (**2**),[7,8] the antimalarial chlorotonil A (**3**),[9,10] the cystobactamids, *e.g.* **4**, as potent antibiotics against Gram-negative bacteria,[11] and the antifungal soraphen A1α (**5**) (Fig. 1).[12] In 2013, we reported the discovery of the myxocoumarins A (**6**) and B (**7**) from liquid cultures of *Stigmatella aurantiaca* MYX-030.[13] Compound **6** was shown to exhibit promising inhibitory activities against a wide range of fungal pathogens, including *Botrytis cinerea*, *Magnaporthe grisea*, *Phaeospaeria nodorum*, *Blumeria graminis*, and *Fusarium culmorum*. Due to the initially low production titers of myxocoumarin B (**7**), combined with the unfortunate inability to regrow the producing strain from all existing stock cultures, the antifungal potential of **7** could not be evaluated during this study. Given the significant activity of **6**, we thus decided to develop a short synthetic access towards **7** to facilitate its biological evaluation.

Results and discussion

A broad range of synthetic approaches for the construction of coumarin core structures can be found in the literature, including Perkin,[14] Reformatsky,[15] Wittig[16] and Knoevenagel[17] reactions. The most widely used method is the Pechmann condensation[18] by which phenolic substrates are fused to β-keto esters catalysed by a (Lewis) acid. Disconnecting the respective building blocks accordingly in myxocoumarin B (**7**) leads to resorcinol **8** with substitution at *C*-5 and β-keto ester **9**, which in turn can readily be prepared from **10** by regio-selective alkylation with iodo-octane (Scheme 1A). Owing to the activation of the *ortho*/*para* aromatic positions by the phenolic functions in **8**, the introduction of suitable substituents at *C*-5 within this substrate is not directly possible. An elegant access to the corresponding nitro-substituted resorcinol **13** is provided by Pd-catalyzed nitration of aryl chlorides as developed by the Buchwald laboratory.[19] Following this approach, the commercially available substrate **11** was smoothly converted to **12** in 75% yield (Scheme 1B). BCl_3-promoted *O*-demethylation gave

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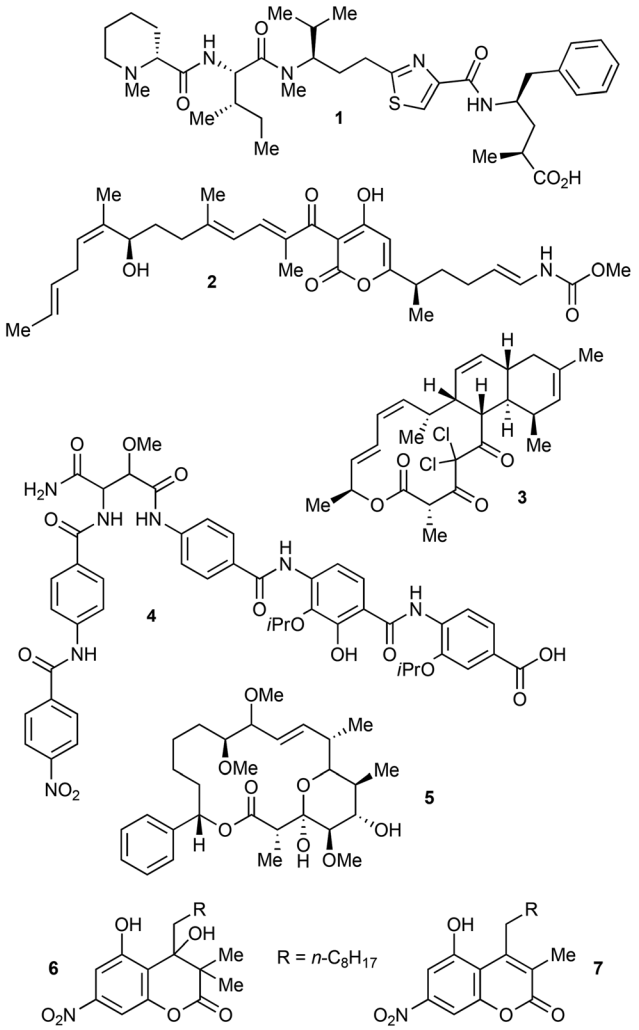
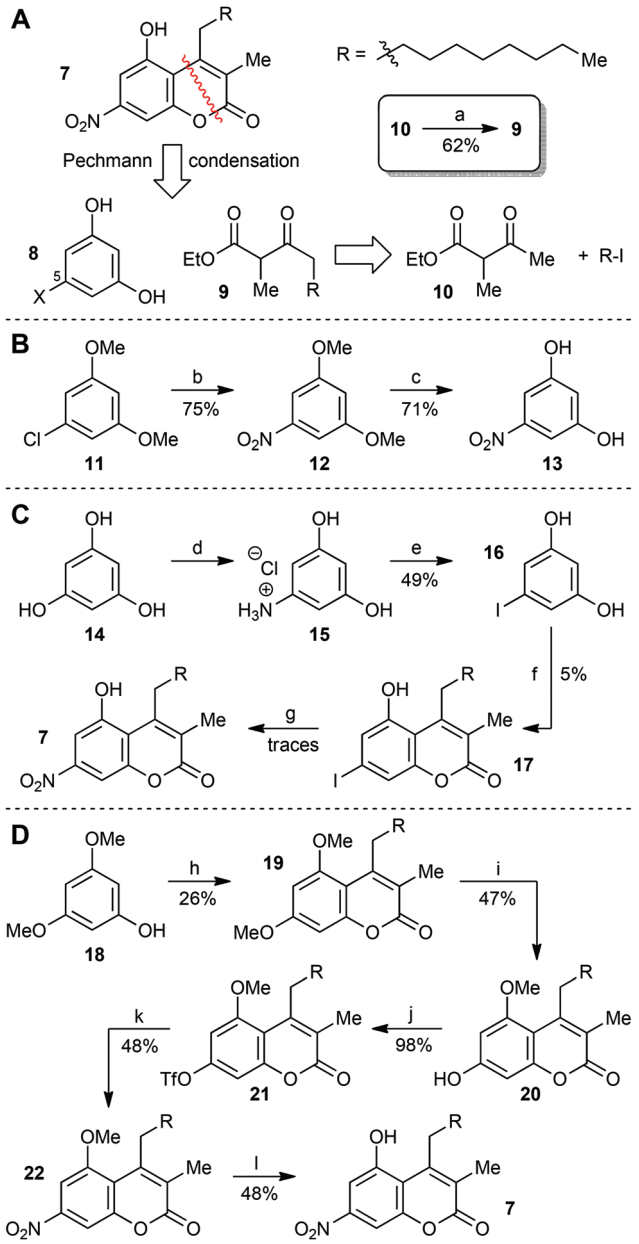


Fig. 1 Structures of myxobacterial compounds **1**–**7**.

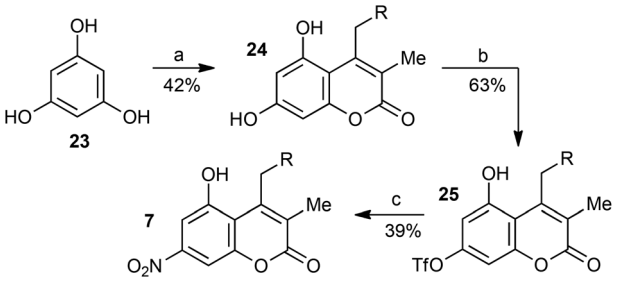


Scheme 1 A. Retro-synthetic analysis (A), unsuccessful synthetic routes (B, C) and first generation synthesis (D) of myxocoumarin B (**7**). Reagents and conditions: (a) 1. *n*BuLi (2.5 eq.), DIPA (2.5 eq.), THF, −78 °C, 1 h. 2. **10** (1.0 eq.), 0 °C, 1 h. 3. R–I (1.2 eq.), −78 °C to rt, 16 h. (b) $Pd_2(dba)_3$ (0.5 mol%), *t*BuBrettPhos (1.2 mol%), TDA (5.0 mol%), $NaNO_2$ (2 eq.), *t*BuOH, 130 °C, 24 h. (c) BCl_3 (2.0 eq.), toluene, 130 °C, 40 h. (d) 1. NH_4OH, RT, 18 h. 2. HCl (e) 1. H_2SO_4 (3.6 eq.), $NaNO_2$ (2.8 eq.), H_2O, 0 °C, 15 min. 2. KI (3.6 eq.), H_2O, rt, 5.5 h (49% over two steps d, e). (f) **9** (1.0 eq.), SSA (9.6 eq.), $CHCl_3$, 65 °C, 20 h. (g) CuI (0.2 eq.), KNO_2 (1.1 eq.), 18-crown-6 (1.0 eq.), *N*,*N*′-diethylethylenediamine (0.4 eq.), DMF, 100 °C, 26 h. (h) **9** (1.0 eq.), TFA (6.2 eq.), MW, 100 °C, 30 min. (i) BCl_3 (2.1 eq.), toluene, 110 °C, 18 h. (j) Tf_2O (1.3 eq.), pyridine (2.0 eq.), DCM, 0 °C to rt, 30 min. (k) $Pd_2(dba)_3$ (0.5 mol%), *t*BuBrettPhos (1.2 mol%), TDA (5.0 mol%), $NaNO_2$ (2 eq.), *t*BuOH, 130 °C, 24 h. (l) LiCl (6.0 eq.), DMF, 155 °C, 18 h. All yields are un-optimized.

13 in 71% yield. Despite literature precedence for the utilization of nitro-substituted phenols in Pechmann reactions to yield the corresponding nitro coumarins,[20] our attempts to convert **13** to **7** under various condensation conditions failed to provide the desired product (data not shown). As this likely can be rationalized by the deactivation of resorcinol **13** due to the nitro substitution, we decided to use an alternative strategy that involves a later-stage nitration at the fully elaborated coumarin core (Scheme 1C). Following a protocol by Thorn *et al.* one of the hydroxy functions in phloroglucinol (**14**) was replaced by an amino group by stirring of this substrate in ammonium hydroxide.[21] The resulting crude hydrochloride **15** was directly used in a Sandmeyer reaction to generate 5-iodoresorcinol (**16**) in 49% yield over two steps. The latter was utilized in a Pechmann condensation reaction with **9** to deliver iodo coumarin **17**, although only in unsatisfactory 5% yield. **17** was subjected to an Ullmann-type copper-catalyzed nitration utilizing a protocol by Saito *et al.*[22] However, only traces of myxocoumarin B (**7**) were detectable in the reaction mixture by MS analysis.

Given the power of the Pd-catalyzed nitration utilized in route B for the synthesis of **12**, we decided to evaluate this reaction for the introduction of the desired nitro functionality later in the synthetic route. Coumarin **19** was constructed by a Pechmann condensation of 3,5-dimethoxyphenol (**18**) with **9** in 26% yield. Likely owing to a shielding effect of the long-alkyl chain R located at the coumarin core, a regioselective *O*-demethylation of the desired methoxy functionality using BCl_3 was possible in 47% yield. The resulting phenol **20** was converted into **21** by triflation in 98% yield. Triflate **21** served as the substrate in a Pd-catalyzed nitration again following the Buchwald protocol.[19] This indeed delivered the fully functionalized coumarin core **22** in 48% yield. The latter was *O*-deprotected in 48% yield to conclude the first total synthesis of **7**. Taken together, this sequence gave access to myxocoumarin B (**7**) in five steps from **18**, with an overall yield of approx. 3%.

To improve the unsatisfyingly low yield of the overall sequence, we set out to further streamline the synthetic route. As the yields of Pechmann condensation generally improve with the electron-richness of the employed phenols, we decided to first construct the corresponding dihydroxy derivative of **19**, coumarin **24**. This compound is available by condensation of phloroglucinol **23** with **9** in 42% yield. Given the apparent protective effect of the long-chain *n*-octyl group as observed in the selective *O*-demethylation of **19** to **20** shown above, we hypothesized that the desired activation by regioselective mono *O*-triflation of **24** should be possible, as well as the following Pd-catalyzed nitration reaction in the presence of the free phenol. Indeed, selective triflation proceeded smoothly to deliver **25** in 51% yield, along with >5% of the bis-triflated analog and 18% of re-isolated starting material **24**, thus leading to 63% of **25** brsm (Scheme 2). Compound **25** was subjected to Pd-catalyzed nitration with slightly modified conditions (5-fold increase of Pd-catalyst and ligand), furnishing 39% of the natural product **7**. Overall, this route thus reduced the number of required steps from five (Scheme 1D) to only three, accompanied by a more than 3-fold increase in overall yield.



Scheme 2 Alternative synthetic route to myxocoumarin B (**7**) *via* triflate **25**. Reagents and conditions: (a) **9** (1.0 eq.), TFA (6.2 eq.), MW, 110 °C, 75 min. (b) Tf_2O (1.2 eq.), pyridine (3.0 eq.), DCM, 0 °C, 4 h. (c) $Pd_2(dba)_3$ (2.5 mol%), *t*BuBrettPhos (6.0 mol%), TDA (5.0 mol%), $NaNO_2$ (2 eq.), *t*BuOH, 130 °C, 24 h.

Table 1 MIC values, IC_{50} and LC_{50} for myxocoumarin B (**7**)

Test	*C. albicans* MIC[a] [μg mL^{-1}]	*C. krusei* MIC[a] [μg mL^{-1}]	MRC-5 IC_{50} [b] [μg mL^{-1}]	Zebrafish LC_{50} [c] [μg mL^{-1}]
Compound **7**	>500	>500	35	120
μM			100	344

[a] Minimum inhibitory concentration. [b] Calculated IC_{50} value corresponds to the concentration required to inhibit 50% of cell growth. [c] Calculated LC_{50} value corresponds to the concentration required to kill 50% of the embryos.

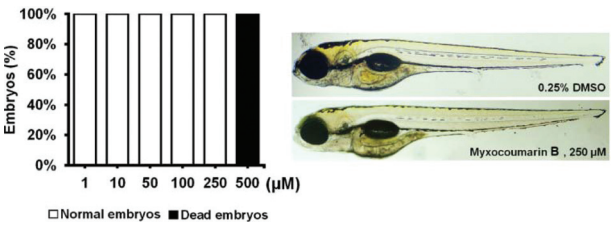


Fig. 2 Toxicity assessment of myxocoumarin B (**7**) in zebrafish embryos exposed to different concentrations and representative images of zebrafish embryos at 114 hpf upon treatment with 250 μM of myxocoumarin B (**7**) compared to a DMSO control.

Having sufficient amounts of synthetic myxocoumarin B (**7**) in hands we next aimed at the evaluation of its antifungal effects against fungal pathogens, *i.e.*, *Candida albicans* and *C. krusei* (see ESI† for details). Unfortunately, no effect on the planktonic growth of these strains was observed up to concentrations of 500 μg mL^{-1} (Table 1).

Cytotoxicity of **7** *in vitro* was assessed against healthy human lung fibroblasts cell line MRC-5 and was found to be moderate (IC_{50} at 100 μM). Toxicity of **7** *in vivo* was determined using zebrafish embryos and found to be at least 3-fold lower (IC_{50} at 344 μM). Importantly, myxocoumarin B (**7**) elicited no toxic response (lethal nor teratogenic) in zebrafish embryos upon treatment with doses up to 250 μM, with all embryos dying upon exposure to 500 μM (Fig. 2).

Conclusions

In conclusion we developed a short total synthetic access to **7** with a late-stage Pd-catalyzed nitration reaction as the key step. Our work nicely showcases that the Pd-catalyzed nitration reaction developed by Buchwald *et al.*[19] can readily be applied to elaborate substrates such as the employed triflated coumarin derivatives **21** and **25** and therefore constitutes a valuable transformation for late-stage natural product functionalization. The obtained synthetic material facilitated initial assessment of the biological properties of **7**, which, in contrast to myxocoumarin A (**6**), despite only small structural differences, revealed a complete lack of activity against fungal pathogens and low toxicity both *in vitro* and *in vivo*. Access to **7** now permits investigations into the likely ecological functions of

the myxocoumarin class of natural products in their natural producer. This work is currently ongoing in our laboratory.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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All experiments involving zebrafish embryos were performed in compliance with the European directive 2010/63/EU (stating that ethical approval is not needed for zebrafish embryos providing that the experiments are done until 120 hpf) and the ethical guidelines of the Guide for Care and Use of the Laboratory Animals of Institute of Molecular Genetics and Genetic Engineering, University of Belgrade.

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