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COMMUNICATION

Photochemical oxazole–nitrile conversion downstream of rhizoxin biosynthesis and its impact on antimitotic activity†‡

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Through metabolic profiling of mutants and wild type of the endofungal bacterium *Burkholderia rhizoxinica* two novel rhizoxin derivatives with unusual nitrile substitutions were discovered. The nitrile groups result from a photochemical oxidative cleavage of the oxazolyl moiety. *In vitro* studies revealed that the photooxidation by singlet oxygen also takes place in the absence of a photosensitizer, and that also a thiazolyl-substituted rhizoxin analogue undergoes the same transformation. The resulting nitriles have antimitotic properties but are significantly less active than the parent compounds. These results highlight the impact of photoreactions onto the antiproliferative agent and encourage the introduction of bioisosteric groups that render the compound less susceptible towards photooxidation.

Introduction

Rhizoxin (**1**, Fig. 1) is a potent antimitotic macrolide that plays a key role in rice seedling blight, a plant disease accounting for severe losses in agriculture.[1] It exerts its function by binding to the β-tubulin of eukaryotic cells, thus preventing the polymerization of tubulin subunits into microtubules, and consequently, the formation of the mitotic spindle.[2] Besides its infamous role as a toxin, rhizoxin has been thoroughly studied as a potential antitumor drug due to its approved mode of action. The mitotic spindle represents one of the prime targets of currently used cytostatic drugs.[3,4] Originally, rhizoxin and several derivatives had been isolated from cultures of the plant pathogen *Rhizopus microsporus* but ongoing studies have shown that the rhizoxin complex is in fact produced by endofungal bacteria of the genus *Burkholderia* residing in the cytosol of the fungus.[5] Isolation and cultivation of the endosymbionts enabled the characterization of several rhizoxin analogues, some of which possess significantly increased cytostatic properties.[6] Moreover, insights into the molecular basis of rhizoxin biosynthesis could be gained by cloning, sequencing and molecular analysis of the biosynthetic gene cluster coding for a giant polyketide assembly line.[7] Through pathway dissection and mutational analyses, various unusual enzymatic tailoring processes have been elucidated that are critical for the biological activity of rhizoxin, such as double bond shifts and introduction of the C-5 side chain.[8,9]

Here, we report the unexpected photochemical processing of the rhizoxin oxazole group into a nitrile moiety and the impact of the functional group conversion on the biological activity of rhizoxin.

Results and discussion

Cultivation of the endosymbiotic bacteria in the absence of the fungal host represents an easy and sustainable method for the generation of cytostatic rhizoxin derivatives. Additionally, a number of more potent analogues were discovered from the axenic cultures.[6] During the metabolic profiling of these

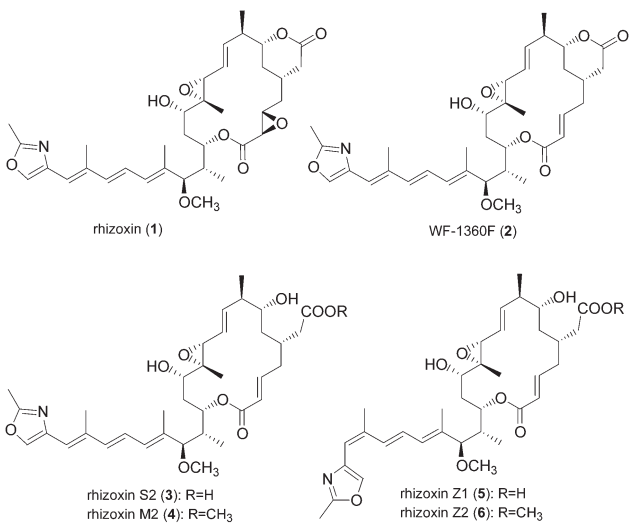

Fig. 1 Structures of rhizoxin derivatives.

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endosymbiont cultures, we detected traces of previously undescribed metabolites. The compounds seemed to be related to rhizoxin, as was deduced from HR-MS and MS^n analysis, but showed a different UV spectrum compared to the known macrolides. Instead of the typical polyene spectrum of rhizoxin with a UV maximum at 311 nm, these minor metabolites displayed a UV maximum at 304 nm and were lacking the distinct tetraene chromophore. This difference as well as the MS^n fragmentation pattern pointed to a modified polyketide backbone with an altered side chain. Interestingly, we detected the same compounds as the only representatives of the rhizoxin complex in an extract of a mutant strain (Δ*rhiG-AT1*) that had been created in the course of studying rhizoxin biosynthesis (Fig. 2).

We have shown previously that the tandem acyltransferase (AT), RhiG is in charge of loading the multimodular polyketide synthase with malonyl building blocks in *trans*, which enables the stepwise assembly of the macrolide toxin.[7] To clarify the role of the AT domains encoded by *rhiG*, we had created various mutants deficient in both or individual AT domains. HPLC-MS analysis of the mutant culture extracts revealed that Δ*rhiG-AT1* solely produced rhizoxin analogues with a UV maximum at 304 nm, and this indicated that these new compounds result from a deficient pathway. To elucidate the structures of these unknown derivatives, we isolated both metabolites from a 14 L culture of the endosymbiotic bacteria finally yielding ~1 mg of each compound allowing for a full structural characterization by 1D and 2D NMR experiments as well as by HRESI-MS and IR measurements.

For compound **7** (*m/z* 594 $[M + Na]^+$) a molecular formula of $C_{32}H_{45}NO_8$ was deduced from HRESI-MS. ^1H and ^{13}C NMR data revealed the same 16-membered macrolide skeleton as known from rhizoxin derivatives. However, instead of the conjugated tetraene system of the side chain, only three double bonds and the lack of the oxazole ring were noticeable. An upfield shift of H-23 (δ_H = 5.42 ppm in **7** *vs.* 6.23 ppm in rhizoxin S2 (**3**)) and its HMBC coupling with the quaternary carbon at 118.8 ppm disclosed the presence and connectivity of a nitrile substituent. This conclusion was fully supported by the occurrence of the characteristic band at $\tilde{\nu}$ = 2207 cm^{-1} in the IR spectrum. The ESI-MS of compound **8** exhibited a quasimolecular ion at *m/z* 608 $[M + Na]^+$. HRESI-MS data established the molecular formula of $C_{33}H_{47}NO_8$, suggesting that **7** and **8** differ by one methyl group. This was corroborated by additional signals in the ^{13}C and ^1H NMR spectra (52.0 ppm and 3.67 ppm, respectively) of **8**. HMBC long-range correlation of the methyl protons and C-5b determined the partial structure of a methyl ester. The ester moiety is derived from methanolysis of the corresponding δ-lactone during the isolation process. The relative configuration of the chiral centres was elucidated by NOESY experiments (Fig. 3).

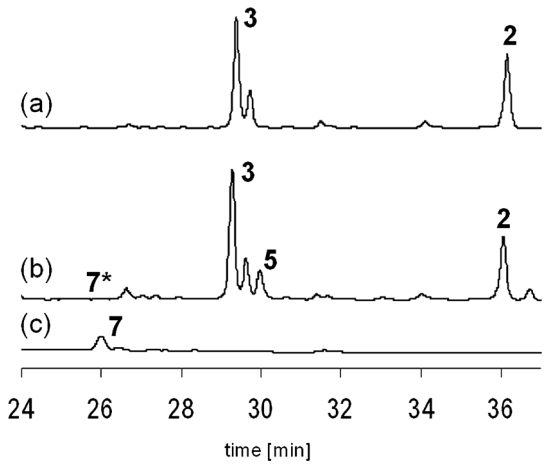

Fig. 2 HPLC profiles of extracts from cultures of (a) wild-type *B. rhizoxinica* grown in the dark, (b) ditto, exposed to daylight, (c) *B. rhizoxinica* Δ*rhiG-AT1* mutant exposed to daylight; * compound present in traces.

Although nitrile-containing natural products have been described from diverse organisms, their occurrence in nature is relatively rare. The most abundant class are the cyanogenic glycosides that are produced by several plant species. In microorganisms however, the formation of nitriles is much less common.[10]

Prominent examples include borrelidin from *Streptomyces parvulus*,[11] the sponge metabolites calyculin A and hemi-phorboxazole A,[12] and the cyanosporasides from *Salinispora pacifica*.[13] Interestingly, all of these metabolites possess potent pharmacological properties, and in some cases the nitrile moiety even seems to confer the biological activity (*e.g.* saframycin A).[14]

However, there are only few reports about the biosynthetic origin of the cyano function. The plant cyanogenic glycosides, the most thoroughly studied examples, result from the conversion of an amino acid into a nitrile. First, the amine is oxidized to yield an aldoxime, which then undergoes enzymatic dehydration.[10] In bacteria, different mechanisms of nitrile formation have been described. Some species seem to be able to sequester cyanide from their environment.[15] The nitrile moiety of the macrolide borrelidin is derived from the methyl group of a methylmalonyl-CoA PKS extender unit, which is oxidized by a cytochrome P450, and transamination and dehydration finally lead to the cyano function.[16] An amide to nitrile conversion has been proposed for bezerramycins,[17] and the hydrolysis of closthioamide thioamide bonds represents one of the most exotic sources of nitriles in nature.[18] These scenarios, however, are unlikely for rhizoxin-derived nitriles because of previous biosynthetic considerations regarding the PKS/NRPS-derived polyene side chain. As was shown by stable isotope labelling experiments[19] and prediction of the adenylation domain specificity, the methyloxazoline ring of rhizoxin originates from the incorporation of serine by a single NRPS module of the rhizoxin

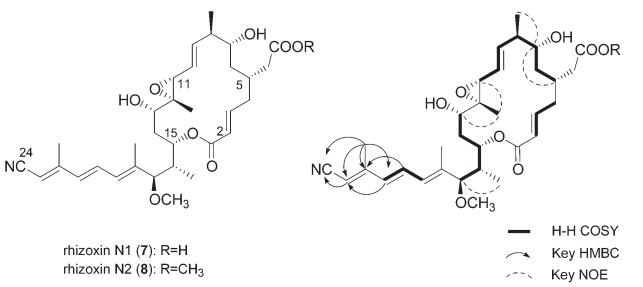

Fig. 3 Structures of nitrile-substituted rhizoxin derivatives and key 2D NMR correlations.

assembly line involving heterocyclization and oxidation.[7] In principle, the nitrile could originate from a misprocessed oxazolyl precursor or result from the oxidation of a glycine residue that was loaded in lieu of serine. To investigate whether the rhizoxin nitriles share the same biosynthetic origin we performed feeding experiments with ^{13}C–^{15}N-labelled amino acids. LC-MS analysis unequivocally showed that both amino acids, serine and glycine, are incorporated into the rhizoxin nitrile backbone. However, since conversion of serine to glycine by serine hydroxymethyltransferase is a common biotransformation, these results were not unambiguous. Thus, we also investigated the timing of nitrile formation by monitoring the fate of rhizoxin derivatives in culture, and indeed we noted that nitriles **7** and **8** form after completion of rhizoxin biosynthesis. This observation could be rationalized by enzymatic degradation or photooxidation of the oxazole moiety. A similar scenario has only been described for two natural products, hemicalyculin A and hemiphorboxazole A.[20,21] According to a plausible model cycloaddition of singlet oxygen to an oxazole ring would yield a reactive peroxide, which could further react and rearrange to a nitrile (Fig. 4A).[22]

To test this hypothesis, we irradiated a solution of rhizoxin derivatives under a commercial 40 W lamp using methylene blue as a photosensitizer and bubbling oxygen through the reaction mixture. At −78 °C we observed a complete conversion of oxazoles into nitriles by HPLC, MS and comparison with authentic standards. To test whether this photooxidation may also take place during the cultivation, extraction or isolation procedure, we repeated the same reaction without addition of a photosensitizer and at room temperature. Thus, we could show that the reaction also proceeds in the absence of a photosensitizing dye, albeit to a much lower extent (Fig. 4B). At room temperature, only trace amounts of nitriles could be detected, which is in accordance with the natural abundance of nitriles in the bacterial cultures. However, these experiments disclose that rhizoxin itself, obviously the oxazolyl-substituted polyene, acts as a photosensitizer. We reason that this is sufficient to quantitatively convert the small amounts of rhizoxins produced by the *B. rhizoxinica* Δ*rhiG-AT1* mutant strain into the corresponding nitriles. It should be noted that also the *E*/*Z* isomerization of the C-22/C-23 double bond is induced by light as was shown by comparative profiling of the culture extracts of bacteria grown in the dark and exposed to light, respectively (Fig. 2).

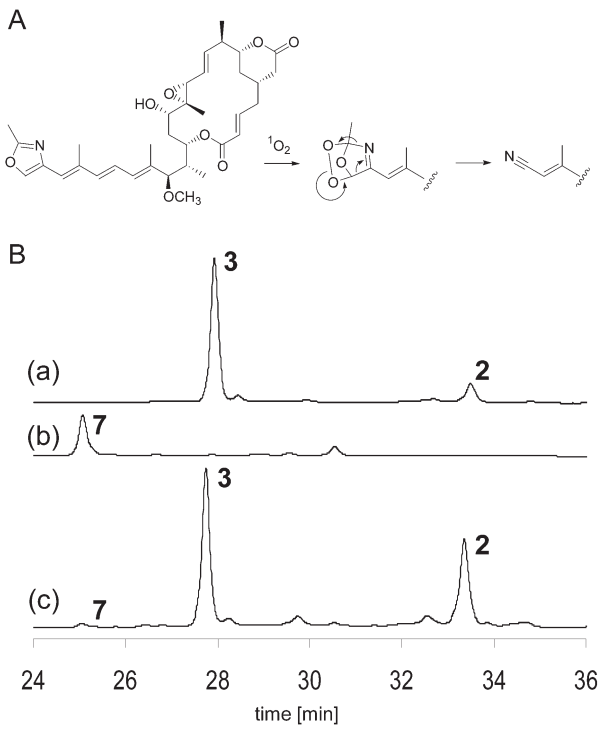

Fig. 4 Photochemical conversion of oxazole rhizoxins into nitrile derivatives: A. Proposed mechanism of photochemical conversion; B. HPLC profiles of (a) fraction containing rhizoxin S2 and WF-1360F, (b) same fraction irradiated with a 40 W lamp in the presence of methylene blue, and (c) ditto without photosensitizer.§

Does this photochemical conversion have an impact on the biological activity? Rhizoxin derivatives exhibit potent antitumoral properties due to their interference with microtubule dynamics during mitosis. Previous structure–activity-relationship studies revealed that beside the pharmacophoric C-5b carbonyl function, an intact macrocycle with either one or two epoxides (C-2/3 and C-11/12, respectively) that might be replaced by double bonds, and a free hydroxyl function at C-13 confers biological activity. Additionally, a side chain of at least six carbon atoms is required to function as a cytostatic. The distal end of the polyene side chain, however, was found to be less important for the interaction with tubulin.[23] To investigate the impact of the oxazole–nitrile conversion on bioactivity, we tested selected derivatives in antiproliferative assays using L-929 and K-562 cell lines and a cytotoxicity assay using the HeLa cell line. In this primary activity profiling, we noted that although the nitrile derivatives possess cytostatic activity, they are substantially less potent than their oxazole counterparts (Table 1). For this purpose, we investigated the stability of the thiazolyl-substituted rhizoxin analogue that was generated by mutasynthesis.[24] Yet again, in the presence of methylene blue we noted the transformation of the thiazole into the nitrile and various other, unidentified compounds. These results indicate that the substitution of oxazole and thiazole moieties in natural products with bioisosteric groups would render the compounds less susceptible towards photooxidation. To this end, our findings further encourage semisynthesis and mutasynthesis approaches.

Conclusions

In summary, by metabolic profiling of mutants and wild type of the endofungal bacterium *Burkholderia rhizoxinica*, we detected two novel rhizoxin derivatives. Isolation and structure

Table 1 Antiproliferative and cytotoxic activity of rhizoxin derivatives

Compound	Antiproliferative activity L-929 GI_{50} [μg mL^{-1}]	Antiproliferative activity K-562 GI_{50} [μg mL^{-1}]	Cytotoxicity HeLa CC_{50} [μg mL^{-1}]
Rhizoxin N1 (**7**)	>50	0.5	3.1
Rhizoxin N2 (**8**)	4×10^{-2}	1×10^{-3}	0.17
Rhizoxin S2 (**3**)	8×10^{-2}	1×10^{-6}	0.15
Rhizoxin M2 (**4**)	0.5	5×10^{-7}	2×10^{-3}

elucidation revealed that these compounds feature an unusual nitrile substitution. Stable isotope labelling experiments and time-course monitoring showed that the nitrile moiety derives from glycine or serine, and that oxazole assembly precedes nitrile formation. Through *in vitro* assays with and without a photosensitizer, we could show that both oxazolyl- and thiazolyl-substituted rhizoxins decompose to yield the corresponding nitriles. Notably, the photooxidation products display significantly lower antimitotic activities than their precursors. Thus, our findings not only disclosed a rare non-enzymatic transformation of rhizoxin, but also have implications for the future development of related bioisosteric compounds into more stable antitumoral agents. Additionally, our studies further highlight the impact of photoreactions on natural product diversity[25] and provide an alternative option for the semisynthesis of new chemical entities with nitrile moieties.

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Notes and references

§ For HPLC analysis, compounds were dissolved in acetonitrile, therefore compound **8** is not formed.

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