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A new bactericidal lead structure for the protection of materials

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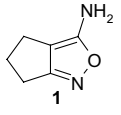
Abstract—Our search for a new broad spectrum bactericide for preserving materials lead to the discovery of a highly active bicyclic amine (**1**) and a number of derivatives. The synthesis and biological evaluation as well as a first toxicological assessment of these compounds are described. Compound **1** shows strong bactericidal activity down to levels of below 100 ppm but unfortunately increases the number of mutations in Ames tests.

Microbicides are chemicals, which, at small concentrations, provide effective protection against the microbiological spoilage of a wide variety of products, including foodstuffs, paints, polymer emulsions, carbonate slurries, metal working fluids, detergents, cosmetics, adhesives, or plastics. The relevant market for biocides/microbicides is projected to stand at approximately € 3 billion in 2005.[1] New chemical classes of such microbicides have become extremely rare.[2] The challenge with new microbicides lies in finding long lasting broad spectrum activity against a variety of *extremely* different organisms while toxicity toward mammals and persistency should be minimal. In addition, the biocide industry is also affected by regulatory issues and the increasing cost of registration. As existing biocides are relatively inexpensive chemicals, the cost of new actives must not be above approximately € 100 per kg.

Here we want to report on a new lead structure for a bactericide and the course of our discovery process. Our attention was first caught while microbiologically screening libraries of small organic molecules. In a modified sensor assay[3] and classical screening, a compound **1**, containing an isoxazole moiety, exhibited excellent in vitro activity against a variety of bacteria (Table 1).

Table 1. Minimum inhibitory concentrations (MICs) of **1**, determined in vitro (Agar)



1

Bacterium	MIC (ppm)
Bacillus subtilis	5
Pseudomonas aeruginosa ATCC 15442	5
P. aeruginosa NCIB 6749	<1
P. aeruginosa NCIB 12201	7.5
Alcaligenes faecalis	<1
Corynebacterium	<1

We subsequently initiated a program on testing other analogues of **1**. By focusing on the bicyclic ring structure we thought to track down essential parts in respect to activity. However, much to our surprise, the results at this stage were disappointing. For example, none of the analogues displayed in Figure 1 showed any significant activity against the bacteria tested. The aliphatic five-membered ring structure as well as the isoxazoline part both seemed indispensable.

That finding was also significant as being in perspicuous contrast to the series of isothiazolone biocides, established on the market (Fig. 2). Changes on the ring system are obviously tolerated here, without activity

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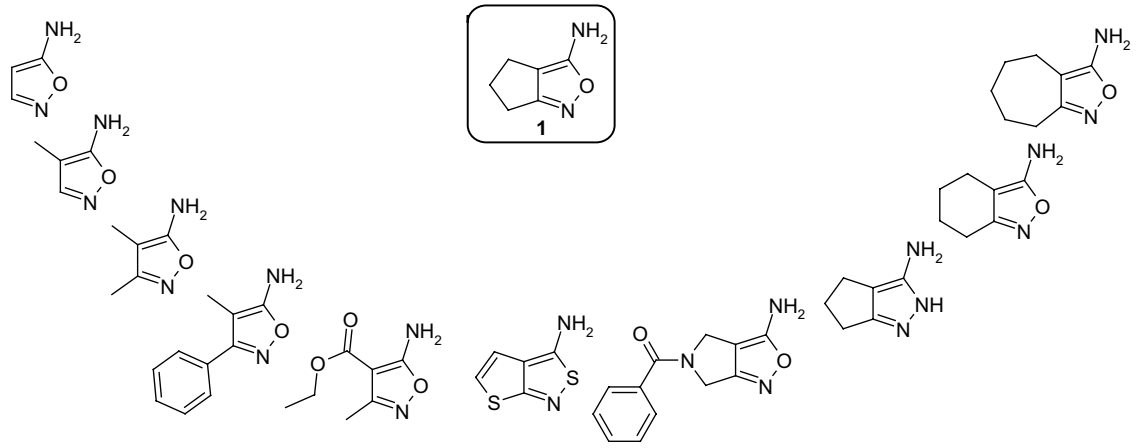


Figure 1. Some analogues of **1**. None of them showed significant activity, however.

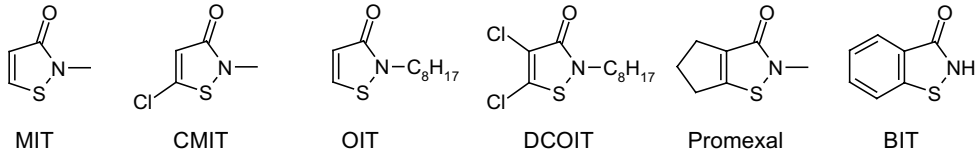
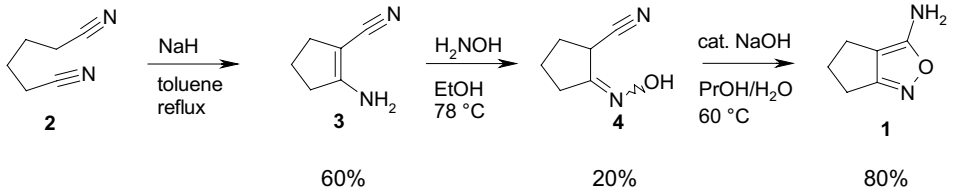


Figure 2. Commercial biocides of the isothiazolone class.

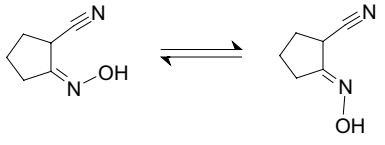


Scheme 1.

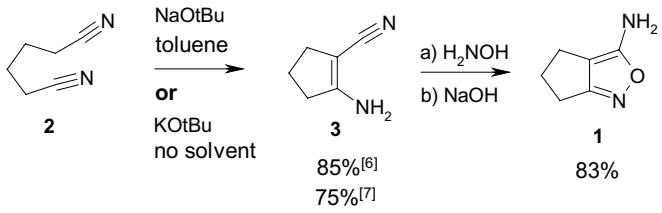
being abruptly lost. (However, distinct shifts in their spectrum of activity do occur, e.g., while MIT is only bactericidal, OIT is only fungicidal and algaecidal.) Ascribed to the activated nitrogen sulfur bond, a reactive mechanism of biocidal action has been widely accepted for this class of microbicides.

So for the next step we returned to **1** and focused on its synthesis. As the ^{1}H NMR spectrum of **1** seemed relatively unspecific, the structure of **1** was unequivocally confirmed by X-ray analysis.[4] Bond lengths are all in the expected range, the molecule is relatively flat with the methylene groups being disordered. To produce larger quantities of the compound required for tests of more practical relevance, we started from adiponitrile **2** (Scheme 1).

Compound **2** is available in bulk as it is an intermediate of Nylon-6,6. Reacting **2** with sodium hydride gave a cyclized product **3**, which after cooling of the reaction mixture could be filtered off (Thorpe reaction).[5] A number of bases has been suggested for this reaction. Treating **3** with hydroxyl amine affected a formal ammonia hydroxyl amine exchange. The resulting product mixture was distilled for purification. The cyclization to yield the final product was then initiated by catalytic amounts of NaOH.[6] However, when we tried to utilize this route to produce 100 g quantities of **1** we encountered a strongly exothermic decomposition during the distillation of the hydroxyl amine **4**, which previously had been carried out successfully on a smaller scale. Hydroxyl amines are not without danger as reactants in processes and during distillation.[7] We thought this distillation to be essential for the purification of compound **4** as we had old reference samples of **4**, which displayed perfect ^{1}H NMR purity, while freshly prepared samples showed additional signals. During our investigation we learned, however, that the initially formed 1:2-mixture of *cis–trans*-isomers (Scheme 2) will gradually shift to



Scheme 2. *cis–trans*-Isomers of **4**.



Scheme 3.

one side during drying and storage of the solid sample at room temperature, affecting the observed simplification of the NMR spectrum. The initial mixture of isomers with a proportion of ca. 1:2 can be regenerated by heating the single isomer to 100 °C for 1 h.

After this clarification and some additional experimentation to improve the synthesis employing a phase transfer-catalyst instead of NaH to affect the Thorpe cyclization we realized that PTC results mainly in the formation of a dimerized side product.[8] But while initially managing to purify **4** by column chromatography to avoid decomposition during distillation we found that crude **4** can favorably be converted to the final product **1** by in situ cyclization after treatment with base. In a recent study it was shown that **3** can even be produced under solvent-free conditions.[9] We could therefore arrive at a very short and economically feasible reaction sequence (Scheme 3).

Once we had larger quantities of **1** at hand we could continue with the biological study. As shown in Figure 3, isoxazole **1** protects metal working fluids perfectly against microbiological spoilage. At concentrations of 100 ppm the activity lasts over 10 weeks. In addition we observed that in a 'time-to-kill' test using *Pseudomonas fluorescens* the bactericidal effect of **1** was of course much more rapid than that of the standard bactericide BIT, both at 20 ppm. This means that compound **1** is among the strongest broad spectrum bactericides known, yet structurally simple.

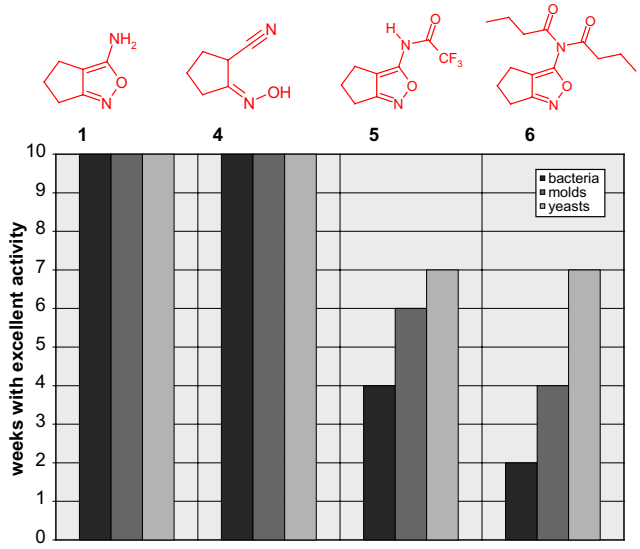
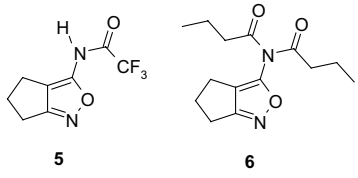


Figure 3. Preservation of a metal working fluid (mineral-oil based) at a concentration of 100 ppm active ingredient

As for the acute toxicity, the LD_{50} (oral, rat) was determined to be >100 < 300 mg/kg. Toward alkaline hydrolysis, **1** turned out to be relatively stable with $T_{1/2}$ = 97 h (pH 9, 50 °C). In addition **1** was only weakly reactive in enzyme assays with ADH[10] and coenzyme A[11] but reactive toward glutathione.[12] Unfortunately the Ames test[13] for mutagenicity was clearly positive and in addition, according to the Magnusson–Kligmann test,[14] compound **1** was a potential sensitizer. So we could not proceed with the development of this compound.

In our efforts to find other derivatives of comparable biocidal activity we then synthesized a large number of different derivatives substituted at the nitrogen atom. The in vitro screening gave promising MICs with two *N*-acylated compounds, **5** and **6**.



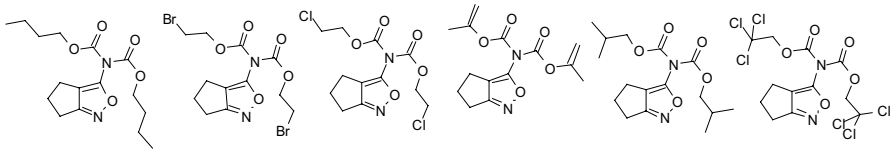
Even more relevant than the in vitro results, **5** and **6** kept metal working fluids free of microbes for a remarkable length of time. As shown in Figure 3, activity is particularly good against yeasts and molds. While both derivatives are certainly less potent than the parent compound **1**, they still do exhibit strong activity, with **5** being a touch better than **6**. After analyzing the rates of hydrolysis of **5** and **6** we found that a simple deprotection of **5** or **6** yielding **1** as the real underlying active compound could not be excluded nor be confirmed ($T_{1/2}$ = 105 h for **5** and $T_{1/2}$ = 6 h for **6**, pH 9, 50 °C. Note that the primary hydrolysis product in case of **6** is not **1**.)

However, our hopes of having identified a development candidate faded nevertheless when we looked at the Ames mutation rates: compounds **5** and **6** both clearly led to increased number of mutations in this test.

We nevertheless tried to find other active derivatives and started looking at the next generation of backup candidates, identified during in vitro screening. One promising class of derivatives were again modified at the nitrogen atom, resulting in derivatives of

Table 2. Representative in vitro data for imidodicarbonate derivatives of **1**

Bacterium/Agar	MIC (ppm)					
P. aeruginosa NCIB 6749 complex medium	10	5	5	10	5	5
P. aeruginosa NCIB 6749 chem. def. medium	10	10	10	10	10	10



imidodicarbonates. When trying to synthesize gram quantities, however, we noticed that these compounds were thermally not very stable, with strongly exothermic decompositions taking place around 80 °C. The compounds were perfectly stable at room temperature but for safety considerations and because the whole class (we synthesized over 20 derivatives) behaved similarly, they were abandoned as a whole even though the in vitro data had been very promising. Table 2 shows some representative in vitro results.

Finally, and as a last attempt to utilize lead structure **1**, we thought of using a pro-drug approach: when looking at intermediate **4** we noticed that, as just trace amounts of base or acid present in the environment or media to be protected would be sufficient to affect the cyclization to **1**, intermediate **4** itself might be added to materials as bactericide. The result would be a slow and steady supply of **1** in low concentrations, possibly sufficient to provide a bactericidal activity high enough to protect, but low enough to avoid undesired toxicity issues. The synthesis of larger amounts of **4** was not a trivial undertaking though, due to the aforementioned instability reasons and the need of purification by column chromatography. But we managed to isolate sufficient amounts of **4** and our pro-drug idea turned out to be thoroughly true: Figure 3 shows that only 100 ppm of **4** are sufficient to preserve a metal working fluid for over 10 weeks against bacteria, molds, and yeasts. We immediately proceeded with toxicity studies. Unfortunately even **4** had a positive Ames test result and in addition a sensitizing potential according to the Magnusson–Kligmann test. It is obviously impossible to distinguish at this point if these unfavorable toxicity data of **4** are due to a rapid transformation of **4** to **1** or due to inherent substance characteristics of **4** itself. It would be interesting (but also tedious) to clarify if **4** is transformed to **1** before entering the cell or after entering the cell or if **1** is a product of cell metabolism. But even then the current Ames test results would not be altered.

In summary, we discovered a remarkably active new class of biocides.[15] Looking at the compounds investigated so far, the activity seems to be confined to a small window of derivatives of **1**, substituted at the nitrogen atom, if not to **1** exclusively. This narrow confinement is surprising.

It might be the case that developing a new powerful *broad spectrum* bactericide for non-pharma applications without mutagenic or sensitizing potential is not feasible at all. None of the existing broad spectrum bactericides would comply with the stringent legal requirements, if they were developed again under current circumstances. The results of this work clearly underline that the development of bactericides being environmentally more compliant than existing ones is a very challenging goal.

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