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Antibiotics

Regio- and Stereoselective Epoxidation and Acidic Epoxide Opening of Antibacterial and Antiplasmodial Chlorotonils Yield Highly Potent Derivatives

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Abstract: The rise of antimicrobial resistance poses a severe threat to public health. The natural product chlorotonil was identified as a new antibiotic targeting multidrug resistant Gram-positive pathogens and *Plasmodium falciparum.* Although chlorotonil shows promising activities, the scaffold is highly lipophilic and displays potential biological instabilities. Therefore, we strived towards improving its pharmaceutical properties by semisynthesis. We demonstrated stereoselective epoxidation of chlorotonils and epoxide ring opening in moderate to good yields providing derivatives with significantly enhanced solubility. Furthermore, in vivo stability of the derivatives was improved while retaining their nanomolar activity against critical human pathogens (e.g. methicillin-resistant *Staphylococcus aureus* and *P. falciparum*). Intriguingly, we showed further superb activity for the frontrunner molecule in a mouse model of *S. aureus* infection.

Introduction

Antimicrobial resistance is increasing at an alarming rate and it poses a severe threat to public health. Increasing numbers of multidrug-resistant and even pan-resistant bacterial pathogens are reported worldwide and the development of resistance against last resort antibiotics such as colistin is of particular concern.[1–3] Likewise, resistance is also observed among other human pathogens such as the malaria parasites *Plasmodium falciparum* and *Plasmodium vivax.*[4,5] After the introduction of artemisinin-based combination therapies (ACTs) as standard treatment for malaria in 2006, the number of malaria-related fatalities decreased worldwide. However, the clinical efficacy of ACTs is

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hampered by the rise of mutated parasites being less susceptible to artemisinin treatment.[6,7] Thus, in order to circumvent known resistance mechanisms, there is an urgent need to discover potent antimicrobial compounds with novel molecular scaffolds for the development of new anti-infective drugs. Indeed, of the 11 approved antibiotics in the last five years, only two represented a new class, with a similar lack of innovation observed in the clinical pipeline.[8–10] Natural products are the predominant source of such new scaffolds that can overcome resistance by addressing novel targets.[11,12] Nevertheless, most of the new antibacterial drug candidates never reach the market as many hurdles have to be overcome prior to the approval of a new drug for human therapy. Despite the often cited "supply issue", natural products exhibit usually favorable biological properties whereas physico-chemical and pharmacological properties often need improvement to enable their therapeutic application.[13,14] Chlorotonil A (**1**, Figure 1) was first isolated from *Sorangium cellulosum* Soce1525 in 2007.[15] The natural product **1** is a highly lipophilic, tricyclic macrolide bearing an unusual *gem*-dichloro-1,3-dione moiety, a unique feature among natural polyketides. In addition to its high potency against blood-stage *Plasmodium falciparum* (half-inhibitory concentration, $IC_{50} = 9.1$ nM), **1** displays very pronounced activity against several Gram-positive pathogens, including *Staphylococcus aureus* (minimum inhibitory concentration, $MIC = 0.006\ \mu g\,mL^{-1}$) and *Streptococcus pneumoniae* ($MIC = 0.0125\ \mu g\,mL^{-1}$).[16,17] Although **1** was shown to efficiently reduce parasitemia in a murine model of *Plasmodium berghei* infection, its poor solubility is a major drawback and the compound could only be administered orally through mixing with peanut butter.[16]

Nevertheless, the outstanding biological activity of **1** prompted us to start a scaffold optimization program in order to make chlorotonils accessible for early pre-clinical development. A total synthesis was reported but it relies on 21 steps for the longest linear sequence with an overall yield of less than 1.2 %.[17] Thus, we excluded total synthesis as viable and economic route towards new and structurally diverse analogues. Encouragingly, we found that **1** can be produced by fermentation on >150 L scale of the natural host Soce1525. An efficient downstream process enables gram-scale isolation of **1** and it also allows purification of a minor component, the α-chloro-β-diketone derivative Chlorotonil B (**2** and **3**, Figure 1), setting the stage for subsequent scaffold optimization through semi-syntheses. In this work, we focused on improving the aqueous solubility and increasing in vivo stability of **1** while maintaining the potent biological activity.

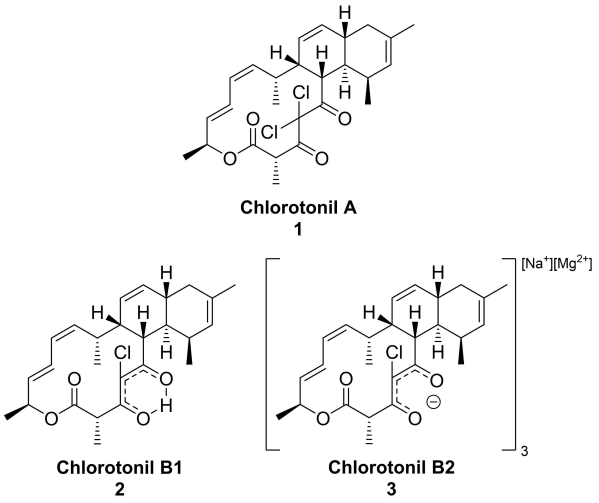


Figure 1. Chemical structures of naturally occurring chlorotonil A (**1**), chlorotonil B1 (**2**) and chlorotonil B2 (**3**).

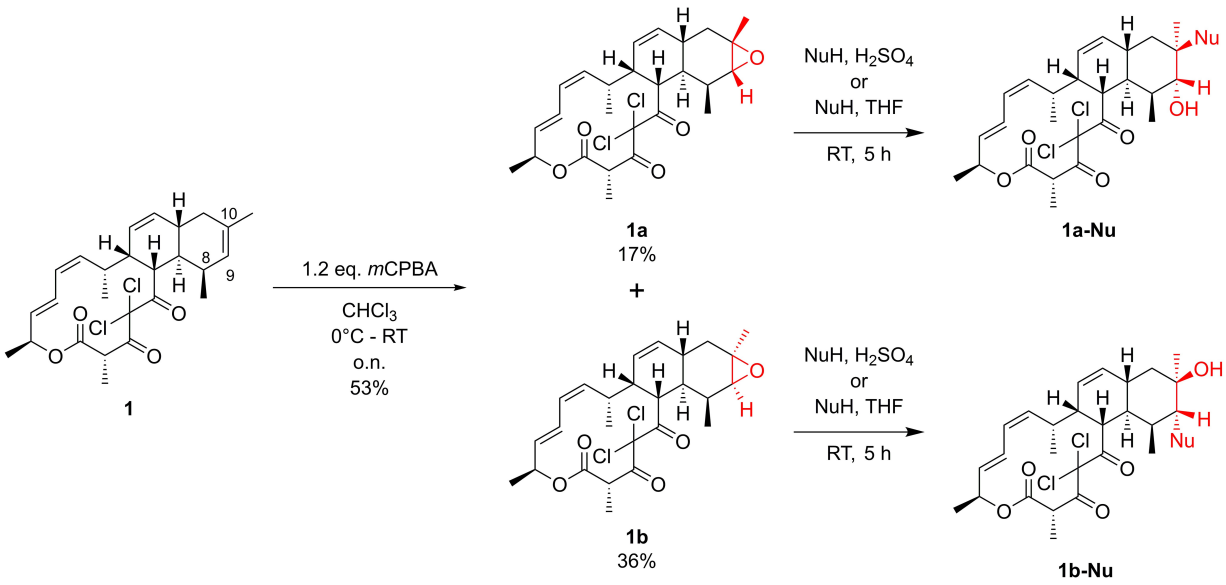
Results and Discussion

After establishing large scale fermentation and purification processes for the starting material **1** as described in the Supporting Information using the natural producer, we obtained sufficient material for the investigation of new derivatives of **1**.[15] Prileschajew epoxidation with *meta*-chloroperoxybenzoic acid (*m*CPBA) was chosen as the most suitable reaction to introduce new polar groups after screening numerous reactions (Scheme 1).[18] The epoxidation reaction afforded moderate yields of 53 % and could be even applied on gram-scale. The reaction was regioselective to position C9 and C10 when using only 1.2 equivalents of the peroxide reagent and it resulted in a diasteremoeric ratio of 1:2 for **1a** to **1b**. The resulting diastereomers could be separated by column chromatography. NMR and X-ray crystallography confirmed the structures (Figures S1 and S2). The stereoselectivity could be explained by the steric hindrance of the axial methyl group at position 8 hampering the epoxidation at the *re* face at position 9 and 10.

In order to further enhance solubility, ring opening of the epoxide was conducted. Basic conditions (triethylamine), nucleophiles (NaOMe, MeMgCl, *n*BuLi) or activation by Lewis acids (LiCl, CuCl, $AlCl_3$, $BF_3{\cdot}Et_2O$) resulted either in decomposition or starting material recovery. Only under harsh acidic conditions with excess of acid in tetrahydrofuran (THF) or excess of concentrated H_2SO_4 in the corresponding neat alcohol resulted in products with yields ranging from 40–95 % (Scheme 1). Unfortunately, amines did not react or were too reactive to afford desired products. The regioselectivity of the epoxide opening of **1a** and **1b** was distinct. In case of **1a**, the nucleophile attacked at C10 and for **1b**, the nucleophile attacked at C9. The structures of the opened epoxides **1a**-ONO_2 and **1b**-ONO_2 were determined by NMR and additionally, **1b**-ONO_2 was verified by X-ray diffraction (Figures S3 and S4).

Due to the more polar hydroxy groups at the C9-C10 double bond of **1**, we assumed that all newly generated derivatives display improved aqueous solubility. In order to establish an initial structure–activity relationship, the new derivatives were tested with a small panel of bacterial indicator strains.

Additionally, we assessed their activity against drug-sensitive and chloroquine-resistant *P. falciparum* (Table 1 and Table S1). Intriguingly, we found several derivatives with retained activity compared to the parent compound **1**.



1a-Nu =	-OMe	-OEt	-Cl[a]	-ONO$_2$
yield	70%	65%	88%	65%

1b-Nu =	-OMe	-OEt	-O*n*Bu	-O-isoamyl	-glycerol	-O-benzyl
yield	85%	82%	90%	80%	45%	50%
	-OH	-F	-Cl	-Br	-ONO$_2$	
	50%	40%	95%	70%	80%	

Scheme 1. Epoxidation of **1** followed by epoxide ring opening assisted by H_2SO_4 in the neat alcohol or with a saturated hydrogen halide solution in tetrahydrofuran (THF) and the corresponding yields. [a] Epoxide ring opening was performed in $CHCl_3$ instead of THF.

Table 1: Biological evaluation of new semi-synthetic derivatives of **1**.

	MIC [μg mL^{-1}][b]			IC$_{50}$ [μM][c]
Compound[a]	*S. aureus*	*B. subtilis*	*C. glutam.*	*P. falcip.* 3D7
1	0.0125	0.0125	0.0125	0.014 ± 0.005
1a	0.025	0.0125	0.5	0.101 ± 0.055
1b	0.0125	0.025	0.05	0.062 ± 0.019
1a-OMe	4–8	> 3.2	> 3.2	> 7.811
1a-OEt	2–4	> 3.2	> 3.2	n.d.
1a-Cl	0.05	0.1	0.4	0.102 ± 0.084
1b-OMe	1.6	6.4	> 6.4	> 11
1b-OEt	> 3.2	> 6.4	> 6.4	> 11
1b-O*n*Bu	1.6	0.8	> 3.2	> 11
1b-O-isoamyl	1.6	0.8	3.2	> 11
1b-O*n*BuOH	> 3.2	> 3.2	3.2	n.d.
1b-glycerol	4	> 3.2	> 3.2	> 1.9
1b-O-benzyl	0.4	0.4	3.2	n.d.
1b-OH	4–8	> 6.4	> 6.4	> 2.2
1b-F	0.2	0.2	0.4 - 0.8	0.135 ± 0.026
1b-Cl	0.2	0.1	0.2–0.4	0.021 ± 0.004
1b-Br	0.05	0.05	0.1	0.025 ± 0.006
1b-ONO$_2$	0.025	0.025	0.1	0.0009 ± 0.00015

[a] See structures in Scheme 1. Functional groups refer to Nu. [b] Minimum inhibitory concentrations (MICs) were determined in two independent experiments using *S. aureus* str. Newman, *Bacillus subtilis* DSM-10, and *Corynebacterium glutamicum* DSM-20300. [c] Half-inhibitory concentrations (IC$_{50}$s) are given as mean ± SD (n = 2) and were determined using drug-sensitive *P. falciparum* 3D7 (chloroquine IC$_{50}$ = 7.4 ± 2.6 nM) and chloroquine-resistant *P. falciparum* Dd2 (Table S1; chloroquine IC$_{50}$ = 262 ± 76 nM).

The epoxide diastereomers **1a** and **1b** showed similar potency as **1** against the Gram-positive bacterial strains with MICs in the ng mL^{-1} range. Equipotent activity is also observed with *P. falciparum* 3D7 where **1a** and **1b** showed IC$_{50}$s of 0.1 μM and 0.06 μM, respectively. In general, we did not observe significant cross-resistance with chloroquine, and IC$_{50}$s on *P. falciparum* Dd2 were on average only 2- to 3-fold higher than observed with the drug-sensitive strain 3D7 (Table S1). Among the substituted derivatives based on **1b**, the halogen residues (F, Cl, Br) and the ONO$_2$ residue were most favorable as the corresponding derivatives displayed low to mid nanomolar IC$_{50}$s with *P. falciparum* and sub-μg mL^{-1} MICs on Gram-positive bacteria. In contrast, the ether-derived substances e.g. **1b**-OMe, including the diol showed decreased activity. We were also able to demonstrate excellent activity of **1** and its semi-synthetic derivatives **1b**, and **1b**-Cl against a set of 180 clinical isolates of methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) exhibiting MIC$_{90}$ of 0.1 μg mL^{-1} for all three compounds (Table 2).

Furthermore, no apparent toxicity in a potential therapeutic window (at least 10× MIC) was found in HepG2 cells for the parent compound and its epoxidized derivatives (Table S1). Higher concentrations could not be analyzed due to low solubility of chlorotonils.

During the investigation of derivatization reactions, it turned out that the scaffold of **1** is relatively stable. However, under various conditions (Table S2), the *gem*-dichloro moiety could release a chlorine forming the

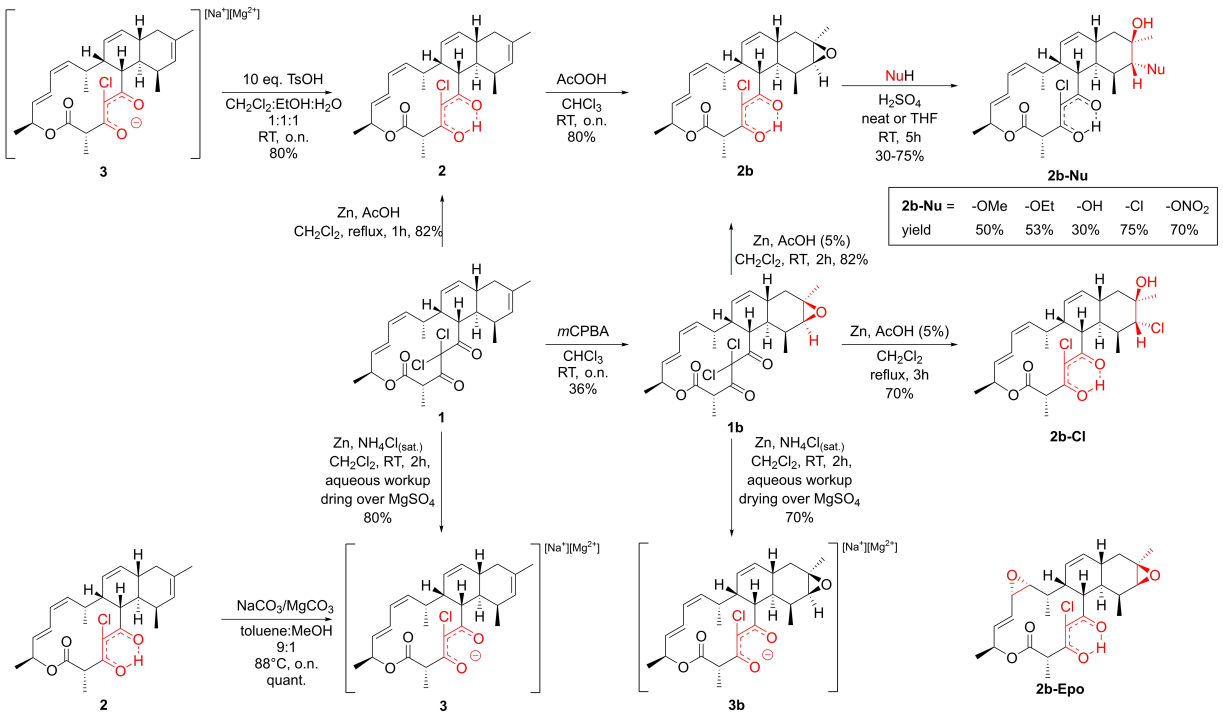
Table 2: MIC distribution in clinical isolates of *S. aureus* ($n=180$).

Compound	MIC range [μg mL^{-1}]	MIC_{50} [μg mL^{-1}][a]	MIC_{90} [μg mL^{-1}][b]
1	0.0125–0.4	0.05	0.1
1b	0.0125–0.2	0.05	0.1
1b-Cl	0.025–0.2	0.1	0.1

[a] Concentration inhibiting growth in ≥50% of all tested isolates.
[b] Concentration inhibiting growth in ≥90% of all tested isolates.

naturally occurring chlorotonil B1 (**2**) or its complex B2 (**3**). We assumed that this chemical liability may cause unwanted transformation also in vivo. Therefore, we first decided to study the conversion of chlorotonils ex vivo in blood. No transformation of **1** was observed in different blood fractions under the tested conditions (Table S3). Next, we studied in vivo pharmacokinetics (PK) of **1** in mice after oral administration. Unfortunately, it was not possible to establish blood PK because of the challenging analytical method development for the poorly ionizable compound and due to presumably low plasma levels. However, we were able to detect chlorotonils in pooled urine and feces of animals receiving a single dose of 50 mg kg^{-1} of **1** (Figure S5). In summary, we found **1** and **2** in pooled feces samples 24 h post-treatment whereas **1** degraded to **2** in a ratio of **2:1**= ca. 6:1. Interestingly, we also detected small amounts of **2** in pooled urine whereas **1** was not detectable. Based on these findings, we decided to continue further scaffold optimization using mono-chlorinated **2** instead of **1** as parent compound to avoid unwanted in vivo biotransformation caused by chlorine liberation.

In order to obtain mono-chlorinated chlorotonil, several reductions were investigated (Table S4). Compound **1** could be transformed into **2** using 5% acetic acid and zinc. Substance **3** could be obtained by treating **1** with saturated aqueous ammonium chloride solution with excess of zinc (Scheme 2 and Table S4). Furthermore, the transformation between **2** and **3** was scrutinized (Tables S5 and S6). Since the structural difference between **2** and **3** was not fully understood yet, X-ray diffraction analysis was used to decipher the exact differences between **2** and **3**. Indeed, **3** appeared to be a metal complex with two coordination centers although the resolution was not sufficient to determine the exact counter ions. By heating a mixture of **2**:Na_2CO_3:$MgCO_3$ in a 1:1:1 ratio in toluene, we established **3** as the Na/Mg salt complex of **2**. With this in hand, we attempted the direct epoxidation of **2** with *m*CPBA to **2b**. However, this led to an inseparable mixture. Another route to obtain the desired **2b** derivatives followed the same reaction conditions as previously described for the parent products **1a** and **2**. Compound **2b** can be easily synthesized from **1b** in the presence of zinc and 5% acetic acid (overall yield 30%). In addition, **3b** was obtained by using a saturated solution of ammonium chloride and zinc. Owing to the poor yield of obtaining **2b** from **1a**, a thorough epoxidation condition screening of **2** was performed (Table S7). Here, the replacement of *m*CPBA with peracetic acid (AcOOH) led to the stereoselective formation of **2b** in 80% yield (Table S8 and Figure S6). With **2b** readily available, selected ring opening reactions were conducted. The stereo- and regioselectivity for the ring opening reaction remained as for the **1a** and **1b** derivatives (Figure S7).



Scheme 2. Transformation of **1** into **2**, **3** and epoxidation of **2**, epoxide ringopening of **2b** including yields and the structure of **2b-Epo**. AcOOH: peracetic acid. TsOH: *p*-toluenesulfonic acid.

Table 3: Biological evaluation of new semi-synthetic derivatives of **2** and comparison to **1**.

Compound[a]	MIC [μg mL^{-1}][b]										IC_{50} [μM][c]
	S. aureus	MRSA	*E. faecalis*	*E. faecium*	VRE	*S. epid.*	*B. subt.*	*C. glut.*	*E.coli* WT	*E. coli* Δ*tolC*	*P. falcip.* 3D7
1	0.0125	0.05	0.025	0.0125	0.025	0.05	0.0125	0.0125	>3.2	>3.2	0.014 ± 0.005
2	0.2	0.8	0.2	0.1	0.1	0.4	0.4	0.1	>3.2	>3.2	0.04 ± 0.004
3	3.2	>3.2	1.6	0.4	0.8	3.2	0.8	0.2	>3.2	>3.2	0.091 ± 0.010
2a[d]	0.4	0.8	0.8	0.8	0.8	0.8	0.4	0.4	>3.2	>3.2	0.363 ± 0.005
2b	0.05	0.05	0.1	0.05	0.05	0.1	0.025	0.05	>3.2	>3.2	0.220 ± 0.029
3b	0.2	0.2	0.2	0.2	0.2	0.4	0.1	0.4	>3.2	>3.2	0.282 ± 0.187
2b-Epo	3.2	3.2	3.2	3.2	3.2	>3.2	3.2	3.2	>3.2	>3.2	>23
2b-OMe	3.2	3.2	3.2	1.6	3.2	>3.2	1.6	1.6	>3.2	>3.2	>23
2b-OEt	>3.2	>3.2	>3.2	>3.2	>3.2	>3.2	>3.2	>3.2	>3.2	>3.2	>23
2b-Cl	0.05	0.1	0.1	0.1	0.1	0.2	0.05	0.1	>3.2	>3.2	>2.3
2b-ONO_2	0.2	0.2	0.4	0.2	0.2	0.8	0.1	0.4	>3.2	>3.2	>2.2

[a] See structures in Scheme 2. **2b** residues (except epoxides) refer to Nu. [b] Minimum inhibitory concentrations (MIC) were determined in two independent experiments using *S. aureus* str. Newman, *S. aureus* str. N315 (methicillin-resistant, MRSA), *Enterococcus faecalis* DSM-20478, *Enterococcus faecium* DSM-20477, *E. faecium* DSM-17050 (vancomycin-resistant, VRE), *Staphylococcus epidermidis* DSM-28765, *B. subtilis* DSM-10, *C. glutamicum* DSM-20300, *E. coli* BW25113 (K12; WT), and efflux-deficient (Δ*tolC*) *E. coli* K12. [c] Half-inhibitory concentrations (IC_{50}) are given as mean ± SD ($n=2$) and were determined using drug-sensitive *P. falciparum* 3D7 (chloroquine $IC_{50}=7.4\pm2.6$ nM) and chloroquine-resistant *P. falciparum* Dd2 (Table S1; chloroquine $IC_{50}=226\pm76$ nM). [d] Structure can be found in the Supporting Information.

Furthermore, when **1b** was refluxed under reductive condition, the free chlorine could attack the epoxide, forming **2b**-Cl.

The newly generated derivatives of **2** were tested along with **1** as a reference compound in an extended panel of bacterial strains and their activity against *P. falciparum* was determined (Table 3 and Table S9). The parent compound **2** was on average by one order of magnitude less active than natural product **1**. However, **2b** was more potent than **2** with MICs in a similar range as **1**. For the opened epoxides of **2b**, we observed a similar trend as seen in the series for **1**, where the halogen (Cl) and the nitrate ester are well tolerated, whereas ether derivatives were much less active. However, it is important to note that **2b**-Cl and **2b**-ONO_2, although being potent antibacterials were not active against *P. falciparum.* In summary, **2b** was determined as the new frontrunner molecule of the semisynthetic chlorotonils. The new derivative did not only suppress growth of multidrug resistant Gram-positive bacteria and *P. falciparum* in the nanomolar range, but it also showed an increased aqueous kinetic solubility at pH 7.4 (16.2 μM) by more than 200-fold compared to **1** (0.073 μM).

Having achieved a significant improvement of the physico-chemical properties of derivatives of **1** retaining superior antimicrobial activity, we studied in vivo PK of our newly generated semi-synthetic derivative **2b**. For this, we developed a fit-for-purpose formulation based on soy oil that allows for administration of precise amounts—in contrast to the previously used mixture of **1** in peanut butter.[16] In summary, the new derivative **2b** was the first chlorotonil derivative for which we were able to quantify blood levels. We could also confirm our assumption that a mono-chlorinated derivative is stable in vivo. Indeed, we determined a very long half-time in blood with a single oral dose of 10 mg kg^{-1} ($t_{1/2z}$ 31.9 h). For the higher dose of 50 mg kg^{-1}, $t_{1/2}$ could not be calculated and blood levels remained for 24 h above MIC for this compound against Gram-positive test strains analyzed here with an $AUC_{0\text{-}tz}$ of 1409 ng h mL^{-1} and a peak concentration (C_{max}) of 147.7 ng mL^{-1} (Figure 2, Table 4; additional PK data of **2b** administered in peanut butter is given in Figure S5 and Table S10).

Strikingly, the new frontrunner **2b**, despite showing four-fold reduced in vitro activity against *S. aureus* compared to the parent molecule **1** (Table 3), was found highly effective in a mouse model of *S. aureus* thigh infection (Figure 3). An intravenous dose of 2×5 mg kg^{-1} of **1** led to

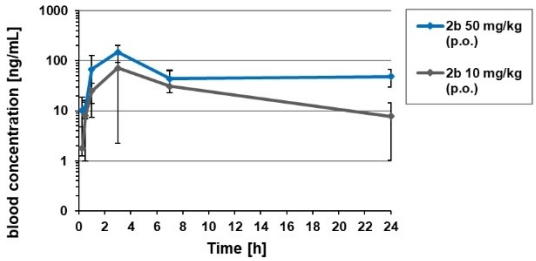


Figure 2. Pharmacokinetics of **2b** in mice ($n=3$) after p.o. administration in soy oil.

Table 4: PK parameters given as mean ($n=3$) of **2b** in mice after p.o. administration in soy oil.

Parameters[a]	10 mg kg^{-1}, p.o.[b]	50 mg kg^{-1}, p.o.[b]
C_{max} [ng mL^{-1}]	75.3	147.7
t_{max} [h]	3	3
t_z [h]	18	24
$t_{1/2z}$ [h]	31.9	n.c.[c]
$AUC_{0\text{-}tz}$ [ng h mL^{-1}]	603	1409

[a] C_{max}: maximum concentration; t_{max}: time at which C_{max} is reached; t_z: time of the last sample which has an analytical quantifiable concentration; $t_{1/2z}$: half-life of the terminal slope of a concentration-time curve between t_0 and t_z; $AUC_{0\text{-}tz}$: area under the concentration-time curve up to the time t_z. [b] **2b** was given orally in soy oil formulation. [c] Not calculable.

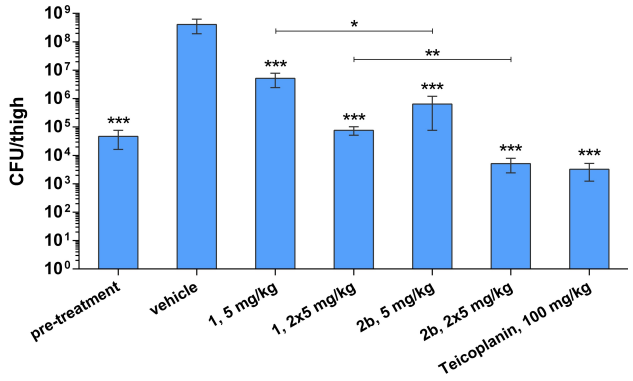


Figure 3. In vivo efficacy of **1** and **2b** in a neutropenic mouse model of *S. aureus* thigh infection. The infection was established using a bacterial inoculum of $\approx 10^6$ colony-forming units (CFU) per animal. Chlorotonils **1** and **2b** were administered i.v. with individual doses of 5 mg kg^{-1}. Teicoplanin (Targocid, SANOFI) was used as reference antibiotic. Values are displayed as mean ± SD ($n=3$). Treatment groups and the pre-treatment group were compared to the vehicle group (10% ethanol, 5% DMSO in 85% saline; *v/v*) by one-way ANOVA with Dunnett's post hoc test ($F=10.74$, $^{***}p<0.001$). Individual samples were compared using an unpaired t-test: $^{*}p<0.05$, $^{**}p<0.01$. Analysis was performed using GraphPad Prism.

an approximately 4-log reduction of bacterial burden in thigh. Importantly, the same dose of **2b** led to an approximately 5-log reduction and in turn, almost 1-log below stasis (p value = 0.0786).

As detailed above, balancing the in vitro potency and pharmacokinetic properties thus yielded an improved, stable and highly effective derivative of the chlorotonil class.

Intrigued by the outstanding biological activity of chlorotonils and their "resistance-breaking" properties in Gram-positive bacterial pathogens and *P. falciparum*, we set out to study potential acquired resistance towards **1** and **2b** in *S. aureus*. Strikingly, we were not able to generate any resistant mutant under standard conditions (i.e. selection of mutant colonies at 4×/8×MIC with an inoculum of $\approx 10^9$–10^{10} colony-forming units). Instead of applying standard conditions to determine frequency of resistance, we slowly adapted *S. aureus* to increasing concentrations of **1** starting with 0.5×MIC. After several passaging steps, we were able to increase the concentration of **1** to 1 μg mL^{-1}, while applying higher concentrations resulted again in cell death. Importantly, this approach was so far not successful for the new semi-synthetic frontrunner, and we thus lack any evidence for resistance development against **2b**. We then characterized the obtained step selected mutants and determined MICs for **1**, which were on average only 10-fold higher than the MIC of the wild type strain *S. aureus* Newman. This finding is not surprising as the initially used concentration for selection (1 μg mL^{-1}) led to significantly delayed growth (after approximately 5 days), while MIC is determined after 24 h incubation. Genome sequencing of these low-level resistant mutants was performed and revealed several mutations mostly in regulatory genes (Table S11). For example, the sensor histidine kinase of the two component system (TCS) SaeRS was found to be mutated in five out of nine analyzed individual resistant clones. Interestingly, SaeS has been linked to sensing of cell membrane alterations and the TCS was found to be dysregulated after exposure of *S. aureus* to e.g. daptomycin.[19,20] However, when we assessed transposon mutants (*saeR* and *saeS* disruption mutants) of *S. aureus* USA300 we did not observe a shift in MIC (Table S12), which further hints towards a complex mode of resistance, where e.g. dysregulation of *saeRS* possibly contributes to resistance in concert with other mutations. Thus, we currently lack a detailed understanding of chlorotonil resistance in *S. aureus* and underlying mechanisms appear to be different to common mechanisms such as increased efflux, impaired uptake or target modifications, which further underlines the potential of chlorotonils to escape pre-existing resistance in clinical isolates along with a low probability of resistance development. Similarly, we were not able to generate *P. falciparum* resistant mutants, which further underlines the superior properties of chlorotonils in combating antimicrobial resistance.

Conclusion

In summary, we established stereoselective epoxidation as well as epoxide ring opening reactions of the antimicrobial natural product chlorotonil A and its natural derivative chlorotonil B. By these chemical modifications, we were able to easily access the different derivatives and study the importance of different moieties and their impact on antimicrobial activity against multidrug resistant bacteria and *P. falciparum* including multidrug resistant laboratory strains and clinical isolates. Our current frontrunner molecule **2b** retained nanomolar activity while being stable in vivo as well as having a significantly improved aqueous solubility. The development of experimental formulations enabled us to study PK properties of chlorotonils for the first time, and we demonstrated in vivo safety for **2b** as no toxicity up to 50 mg kg^{-1} in a single per oral dose was found whereas blood concentrations in these experiments reached levels above inhibitory concentrations for Gram-positive bacteria and *P. falciparum*. Furthermore, we demonstrated superiority of **2b** against *S. aureus* when compared to the parent compound **1** in vivo. The newly generated derivative reduced the bacterial burden by up to 5-log compared to the vehicle control, and a 1-log reduction of *S. aureus* burden below stasis was achieved. Studying potential development of resistance, low-level resistant *S. aureus* mutants were obtained by slowly adapting the bacteria to **1**, and analyses of these mutants revealed a complex mode of resistance. Intriguingly, analogous experiments with the new frontrunner **2b** did not result in resistant *S. aureus* colonies. Although this most promising molecule with its α-chloro-β-diketone moiety as well as its epoxide is relatively inert, other modifications replacing possible reactive groups and studies aiming at enhancing its properties will be performed in the future. We thus aim to continue to explore the pharmaceutical development of the unique chlorotonil skeleton and identify its intriguing mode of action.

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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 Supporting Information of this article.

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