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Investigating the promiscuity of the chloramphenicol nitroreductase from *Haemophilus influenzae* towards the reduction of 4-nitrobenzene derivatives

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Abstract

Chloramphenicol nitroreductase (CNR), a drug-modifying enzyme from *Haemophilus influenzae*, has been shown to be responsible for the conversion of the nitro group into an amine in the antibiotic chloramphenicol (CAM). Since CAM structurally bears a 4-nitrobenzene moiety, we explored the substrate promiscuity of CNR by investigating its nitroreduction of 4-nitrobenzyl derivatives. We tested twenty compounds containing a nitrobenzene core, two nitropyridines, one compound with a vinylogous nitro group, and two aliphatic nitro compounds. In addition, we also synthesized twenty-eight 4-nitrobenzyl derivatives with ether, ester, and thioether substituents and assessed the relative activity of CNR in their presence. We found several of these compounds to be modified by CNR, with the enzyme activity ranging from 1-150% when compared to CAM. This data provides insights into two areas: (i) chemoenzymatic reduction of select compounds to avoid harsh chemicals and heavy metals routinely used in reductions of nitro groups and (ii) functional groups that would aid CAM in overcoming the activity of this enzyme.

Graphical Abstract

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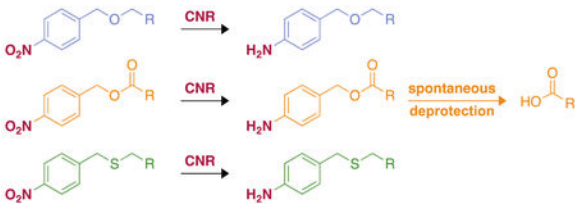
Author contributions

K.D.G., M.Y.F., and S.G.-T. design the study, analyzed the data, wrote the manuscript and supporting information, and made figures. M.Y.F. and A.S.M. synthesized all compounds used in this study. K.D.G. performed the biochemical assays and large-scale chemoenzymatic reactions. K.D.G., M.Y.F., A.S.M., and S.G.-T. reviewed the manuscript and supporting information. All authors approved the manuscript and supporting information.

Supporting information available: The Supporting Information is available free of charge. Copies of ^{1}H and ^{13}C NMR spectra for all compounds synthesized are provided in Figs. S1-S56. The graphs for optimization of pH (Fig. S57) and temperature (Fig. S58), chemical structures of the synthesized nitro-containing molecules tested (Fig. S59), mass spectra of crude extract of large-scale CNR reduction reaction (Fig. S60), as well as HPLC traces for time course of large-scale reduction of compound **26h** with CNR (Fig. S61) are also provided. A Table (Table S1) of the numerical values used to generate Fig. 3 is also presented.

The authors declare no conflict of interest.



Keywords

Antibiotic; Bacterial enzyme; Chemoenzymatic deprotection; Drug-modifying enzyme; Nitroreduction

Chloramphenicol (CAM) (Scheme 1) is an antibiotic with a wide spectrum of activity against Gram-positive and Gram-negative cocci and bacilli.[1] It is primarily bacteriostatic and works by binding to specific residues of the 23S rRNA on the 50S subunit of the bacterial ribosome,[2, 3] disrupting the action of the peptidyltransferase enzyme and leading to the inhibition of important biological functions such as peptide bond formation,[4] termination of translation,[5] and translational accuracy.[6] However, treatment with CAM may be accompanied by deleterious side effects notably neurotoxicity,[7] bone marrow depletion and aplastic anemia,[8] and as a result, its use is often limited to topical ophthalmic infections and other serious infections when other suitable drugs are unavailable, such as meningitis caused by *Haemophilus influenzae, Streptococcus pneumoniae*, and *Neisseria meningitides*.[9] The clinical use of CAM has also been hampered by the rapid emergence of resistant bacterial strains. Mechanisms of resistance include reduced bacterial membrane permeability towards CAM uptake,[10, 11] efflux pumps that decrease CAM concentration in the bacterial cell,[12] and mutations/modifications of the ribosomal target.[13-17] Another common mechanism of resistance to CAM is its enzymatic modification by CAM acetyltransferases (CAT), CAM phosphotransferases (CPT),[18-20] CAM hydrolase,[21] and CAM nitroreductase (CNR).[22, 23]

Antibiotic resistance by drug inactivation/modification is not specific to CAM; it is also present in other families of antibacterials. For instance, aminoglycosides (AGs), another well-known class of antibiotics, have suffered from the emergence of aminoglycoside-modifying enzymes (AMEs).[24] Indeed, more than 100 AMEs have been identified,[25] threatening the long-term use of AGs. These AMEs include AG acetyltransferases (AACs), nucleotidyltransferases (ANTs), and phosphotransferases (APHs). AACs catalyze the *N*-acetylation of AGs, while ANTs and APHs catalyze the transfer of phosphate and adenosine phosphate moieties, respectively, to hydroxyl groups of AGs.[26] We have previously reported a methodology that couples AACs with analogues of acetyl coenzyme A cosubstrate. Specifically, acyl derivatives of coenzyme A (CoA) were chemically synthesized and served as AAC cosubstrates in the chemoenzymatic generation of *N*-acylated AG analogues.[27] We also expanded the substrate promiscuity of two AACs by exploring their structural modification of a variety of AGs.[28, 29] Substrate promiscuity is documented with multiple drug-modifying enzymes including β-lactamases,[30, 31] kinases,[32, 33] dethiobiotin synthetase,[34] acetyltransferases,[35-37] and *O*-nucleotidyltransferases.[38, 39]

Our group has been engaged in the investigation of CAM resistance enzymes. We have determined the crystal structures of CAT_I in its unbound and CAM-bound forms, enabling us to further understand the broad substrate specificity of one of the most prevalent types of CATs.[40] We have also developed homo- and heterodimers of CAM and examined their susceptibility to enzymatic modifications by CAT and CPT. Although CAT and CPT have been extensively studied, CNR remains underexplored. Because of our long-standing interest in CAM resistance enzymes, we decided to investigate the CNR from *H. influenzae*,[41] recombinantly expressed in *Escherichia coli*. Here, we investigate its properties at different pHs, at different temperatures, and determine the kinetic parameters of CNR with CAM. Structurally, CAM contains a 4-nitrobenzene moiety, and a recent study has shown that CNR was able to reduce the nitro group of CAM to an amine (Scheme 1). To that end, we (i) examined some commercially available nitro-containing molecules to assess the versatility of this resistance enzyme and (ii) synthesized a series of 4-nitrobenzyl derivatives to explore the promiscuity of the CNR enzyme. A better understanding of the CNR substrate promiscuity is important to (a) give indications as to what modifications of CAM would help alleviate the action of this enzyme, and (b) potentially develop CNR as a chemoenzymatic tool.

Prior to testing any compounds with CNR, we determined the pH and temperature at which the CNR enzyme is most active. The reaction of CAM with CNR was first tested at various pHs ranging from pH 3.0 to pH 9.0 using either citrate, phosphate, or Tris buffer in their appropriate pH ranges (Fig. S57). We established that the CNR enzyme is most active at pH 8.0. From this point on, we used pH 8.0 with 50 mM Tris-HCl as this was one of the reactions with the highest rate of reaction, and Tris-HCl had less variability in the pH range tested (pH 6.8-9.0) than phosphate in the similar pH range (6.0-8.0). With an optimum pH established, we next investigated the optimal temperature for CNR activity (Fig. S58). Again, the reaction rates of CAM with CNR at 21, 25, 30, 37, 42, 50, and 60 °C were determined. From these data we observed that, surprisingly, the optimal temperature for the enzyme is 50 °C, despite the fact that *H. influenzae* grows optimally at 37 °C. To reconcile this discrepancy, we tested the CNR enzyme at these two temperatures in the next set of experiments.

To establish the effect of temperature on the kinetic parameters of CNR, we next determined the K_m and k_{cat} values for CAM with the enzyme at both 37 and 50 °C, the optimal temperature for growth of *H. influenzae* and that for CNR activity, respectively (Fig. 1). The binding constants (K_m) of CAM to CNR at both temperatures were on the same order of magnitude (273 ± 87 μM at 37 °C and 191 ± 48 μM at 50 °C). The catalytic turnover (k_{cat}) values were nearly identical at both temperatures (61 ± 6 s^{-1} at 37 °C and 65 ± 5 s^{-1} at 50 °C). As the K_m and k_{cat} values were similar at both temperatures, so were the catalytic efficiencies (k_{cat}/K_m) (0.22 ± 0.07 $s^{-1}\mu M^{-1}$ at 37 °C and 0.34 ± 0.09 $s^{\pm 1}\mu M^{-1}$ at 50 °C). Based on these data we decided there was no disadvantage to using 37 °C over 50 °C and therefore performed the rest of the experiments at 37 °C.

With the optimized conditions for CNR activity, we determined the initial rate (first 5 min) of nitroreduction of twenty-five commercially available nitro containing compounds (**1-25**, Fig. 2) by UV-visible assays (Fig. 3A). The compounds consisted of two aliphatic nitro-

containing compounds, one vinylogous nitro-containing molecule, twenty nitrobenzene analogues, and two nitropyridine compounds. Most of the aromatic purchased compounds contained the nitro group in *para*-position to better compare with CAM, which also has its nitro group in that position. A few compounds were selected to be aliphatic or aromatic with their nitro group located in *ortho*- or *meta*- of other functionalities in order to preliminarily establish if *para*-substitution on aromatic compounds was required for CNR activity. The initial rate of CNR was set to 100% for the known substrate CAM for comparison purposes. From these 25 molecules, we found that 8 compounds (*n*-nitrohexane (**1**), nitrocyclohexane (**2**), nitrobenzene (**4**), 3,4-dichloronitrobenzene (**5**), 4-nitrobenzyl bromide (**12**), 4-nitroaniline (**18**), 4-fluoro-2-nitroaniline (**19**), and (3,5-dimethyl-4-nitro-2-pyridyl)-1-methanol (**25**)) were not modified at all by CNR. More promisingly, we also observed that 10 compounds (nitrofurantoin (**3**), 4-nitrobenzenesulfonamide (**7**), 4-nitrophenol (**8**), 2-nitrobenzaldehyde (**13**), 4-nitrobenzaldehyde (**14**), 5,5'-dithiobis(2-nitrobenzoic acid) (**17**), 2-nitro-4,5-difluoroaniline (**20**), 1-(4-nitrophenyl)piperidine (**22**), 5-nitroindole (**23**), and 4-nitropyridine (**24**)) showed a CNR initial rate between 3.5% and 40% when compared to that of CAM. Additionally, 3 compounds (2-nitrobenzesulfonamide (**6**), 4-nitrophenylbutyrate (**10**), and 2-nitro-4,5-dichloroaniline (**21**)) all had initial rates between 77% and 88% compared to that of CAM. Finally, and more interestingly, 4 compounds (3-(4-nitrophenoxy)propionic acid (**9**), 4-nitro benzyl alcohol (**11**), 6-nitrovetraldehyde (**15**), and 2-nitronaphthaldehyde (**16**)) reacted with initial rates greater than that of CAM; 157%, 122%, 132%, and 125%, when compared to 100% for CAM, respectively. From these data we can deduce a few criteria for the CNR: (i) the enzyme does not react well with aliphatic nitro compounds, and (ii) inserting a nitrogen atom into the aromatic system significantly harms the ability of the enzyme to reduce the compound effectively. Looking at the substituents located *para* to the nitro group in three (**9**-**11**) of the seven best compounds (**6**, **9**, **10**, **11**, **15**, **16**, and **21**) in which only a *para*-substituent exist, we observed that an ether, an ester, or an hydroxymethyl group directly attached to the aryl group allow for the activity of the CNR to be retained. When looking at compounds **6** and **7**, we find that a sulfonamide in *ortho* of the nitro group is well tolerated, whereas the same sulfonamide is not as well tolerated at the *para*-position. With compounds **15**, **16**, and **21**, we find that having additional substituents at the *ortho*-, *meta*-, and *para*-positions of the aryl ring can be well tolerated by CNR. All combined, these data further confirm the promiscuity of the CNR enzyme. From these data we can also conclude that substituting the phenyl ring of CAM with a pyridyl group could help reduce the action of CNR on the antibiotic. Additionally, increasing the number of substitutents of the phenyl ring of CAM may have a similar effect

Knowing how the purchased nitro-containing compounds behaved with CNR, we next examined the ability of the reductase to reduce nitro groups on synthetically generated compounds similar to CAM in structure. To investigate the broad application of CNR for nitro reduction and potential deprotection, we synthesized three main classes of 4-nitrobenzyl derivatives, notably 4-nitrobenzyl ethers, esters, and thioethers (Scheme 2 and Fig. S59). With these synthesized compounds, we wanted to establish the effect of a benzylic oxygen atom, either in an ether (compounds from series **26**) or ester (compounds from series **27**) linkage, as well as that of a benzylic sulfur atom in a thioether linkage (compounds from series **28**) on the reduction activity of CNR. Various esters of CAM have

previously been designed as potential prodrugs with useful oral administration,[42] making this functional group an interesting choice in the design of CAM derivatives. Furthermore, CAM heterodimers, in which CAM was linked to another antibiotic (neomycin B, tobramycin, or clindamycin) through an ether or thioether linkage, exhibited improved antimicrobial properties,[23, 43] encouraging us to include ether and thioether functional groups in our study.

Briefly, the syntheses of all compounds were performed as follows. The 4-nitrobenzyl ethers **26a,b,d-k,o,q** were obtained in 3-71% yields by heating 4-nitrobenzylbromide at reflux with the corresponding alcohols in the presence of silver oxide (Scheme 2A). The alcohols utilized in this study included aliphatic (ethyl and propyl), cyclic (cyclobutylmethyl, cyclopentylmethyl, cyclohexylmethyl, and cyclopentyl), and aromatic (benzyl, 4-bromobenzyl, 4-chlorobenzyl, 4-nitrobenzyl, 4-chloro-2-fluorobenzyl, and phenyl) alcohols. The 4-nitrobenzyl esters **27c-i,k-n**, on the other hand, were prepared by heating 4-nitrobenzylbromide with the corresponding carboxylic acids in the presence of potassium carbonate and isolated in 35-88% yields (Scheme 2B). The substituents included propyl, cyclohexylmethyl, cyclobutyl, cyclopentyl, cyclohexyl, 1-adamantyl, phenyl, 4-bromophenyl, 4-chlorophenyl, 4-chloro-2-fluorophenyl, and lithocholyl groups. Finally, refluxing an ethanolic solution of 4-nitrobenzylbromide with benzyl mercaptan, 4-bromobenzyl mercaptan, 4-chlorobenzyl mercaptan, 4-chloro-2-fluorobenzyl mercaptan, or cyclohexanethiol afforded the 4-nitrobenzyl thioethers **28g-i,k,p** in 26-73% yields (Scheme 2C). The choice of substituents ranged from a small-sized ethyl group to a bulky litocholyl group allowing us to investigate the steric effect of these substituents on nitroreduction. For complete experimental protocols and characterization of all compounds synthesized, please see the Supporting Information.

As with the 25 purchased compounds (**1-25**), we evaluated the initial rate (first 5 min) of nitroreduction of compounds **26a,b,d-k,o,q**, **27c-i,k-n**, and **28g-i,k,p** by CNR by UV-visible assays (Fig. 3B and Table S1). The initial rate of CNR was set to 100% for the known substrate CAM for comparison purposes. Prior to performing an in-depth SAR analysis, we first looked for general trends. Overall, compounds **26b**, **27e**, **27g**, **27h**, **27k**, and **271** showed no modification (0% activity) by CNR at this time interval, while compounds **26d**, **26e**, **26f**, **26g**, **26j**, **26q**, **27c**, **27d**, **27f**, **27i**, and **27n** were modified at a rate that was less than 50% the rate of CAM (Fig. 3B). With the exception of compounds **26a** (56%), **26o** (67%), and **28p** (62%), all other compounds (**26h**, **26i**, **26k**, **27m**, **28g**, **28h**, **28i**, and **28k**) showed reactions that were similar or faster than that of CAM. Before taking a closer look at the structures of the nitro compounds, it is safe to say that most ester-based compounds did not show a significant conversion in the time monitored with the exception of compound **27m**, the adamantly ester. This compound seemed to work slightly better (120%) than CAM.

We next took a close look at the SAR, by comparing the various R groups within a same linkage type (*e.g.*, ether, ester, or thioether). Looking more closely at the R groups of the ethers, propyl (**26b**, 0%), methylcyclobutyl (**26d**, 7.5%), methylcyclopentyl (**26e**, 21%), methylcyclohexyl (**26f**, 7.0%), benzyl (**26g** 29%), and 4-nitrobenzyl (**26j**, 21%) were all very poor substrates. On the contrary ethyl (**26a**, 56%), 4-bromobenzyl (**26h**, 116%), 4-chlorobenzyl (**26i**, 90%), 4-chloro-2-fluorobenzyl (**26k**, 88%), cyclopentyl (**26o**, 67%), and

phenyl (**26q**, 45%) were all good to moderate substrates. Based on these data it would seem that any longer or distal cyclic alkyl groups, bulky groups, or electron-withdrawing groups had a significant impact on the reduction activity of the enzyme when reducing the 4-nitrobenzyl ether compounds tested here. In the case of the ester linkage, we interestingly observed that only the adamantyl moiety of **27m** resulted in a compound in which the nitro group was easily reduced by CNR. These data indicate that an ester linkage is detrimental to CNR activity. The thioether compounds (**28**) had less variability with only the cyclohexyl compound (**28p**, 62%) showing a reduced initial rate compared to CAM.

We finally performed a more in-depth SAR analysis by comparing compounds with the same R groups but different linkage types. By doing so, we wanted to answer the question: Is one type of linkage favored over another when it comes to nitro reduction by CNR? We compared three pairs of ethers and esters (**26d** and **27d** with R = cyclobutyl; **26e** and **27e** with R = cyclopentyl; **26f** and **27f** with R = cyclohexyl), and found them to be all poor CNR substrates. In all cases, the reduction of the nitro group in ether-containing molecules (**26**) was faster than (**d** and **e**) or equal to (**f**) the reduction of the nitro group in ester-containing compounds (**27**). This is further validation that the ester is not the best linkage for CNR activity. We also compared four groups with identical R chains (**g**, **h**, **i**, and **k**) in all three families (**26**, **27**, and **28**). For the compounds with R = benzyl (**g**), we observed that the thioether linkage in **28g** was much more conducive to CNR activity than the ether linkage in **26g**, which itself was better than the ester in **27g**. When examining the other three R groups (**h**, **i**, and **k**), we found that both the ether (**26**) and thioether (**28**) linkages were conducive to CNR activity, while the ester linkage (**27**) was not. Overall, we conclude that the best linkage for the reduction of these molecules is the thioether, followed closely by the ether, and lastly by the ester.

Now knowing that CNR could reduce a variety of nitro-containing compounds, we wanted to determine if CNR could reduce the nitro group in the synthesized molecules to an amine, and if the resulting 4-aminoether/thioether/ester would then spontaneously collapse to the corresponding alcohol, thiol, or benzoic acid. Bioreduction of this type has been seen before in an *E. coli* NADH-dependent nitroreductase.[44] To assess the product of the reactions we followed the protocol published for a similar reductase.[45] Large-scale reactions were performed using CNR to reduce three compounds (**26h**, **27h**, and **28h**), representative of each family synthesized with the same 4-bromophenyl group. The reactions were incubated overnight at 37 °C. After a full 24 h of incubation, the reaction mixtures were extracted into EtOAc to remove NADP(H) and other biomolecules from the reactions. The residues from the extractions were analyzed by LCMS. The LCMS trace was scanned for the possible reduction analogues, the amine, and the free alcohol/thiol/carboxylic acid (reduction and deprotection masses are shown in Table 1). In general, we observed a reduction of the 4-nitrobenzyl ether (**26h**) and the 4-nitrobenzyl thioether (**27h**) to the corresponding 4-aminoether and 4-aminothioether (Table 1 and Fig. S60). In the original publication documenting the reduction of CAM,[41] the authors predicted that the nitro group went through the nitrosyl (N=O) moiety, and the hydroxylamine (NHOH), along with other intermediates, before reaching the final amine. While analyzing the MS data for the large-scale reaction of compound **26h**, in addition to the reduced compound, we were also able to

see a peak congruent with the hydroxylamine derivative. This observation confirms the existence of the hydroxylamine intermediate originally proposed for CAM and opens the possibility of this enzyme to reduce hydroxylamines. The 4-nitrobenzyl ester (**28h**), which did not display detectable initial rate with CNR by UV-visible assays, surprisingly, was not only reduced, but the compound spontaneously collapsed to yield the corresponding benzoic acid. While this data does not agree with the UV-visible assay, the length of incubation is significantly different. To confirm that the observation of the carboxylic acid is real and not an artifact of the mass spectrometry experiments, samples of the reaction were injected onto RP-HPLC at various times during the reaction to monitor the progression. A significant new peak was not seen until 3 h, which agrees with a decrease in the signal from the starting material (Fig. S61). In an effort to confirm that this is a general trend for the esters and not specific to compound **27h**, we also investigated by LCMS the 4-chloro-2-fluoro- (**27k**) and 4-chloro-substituted (**27i**) derivatives and observed the corresponding benzoic acids for these compounds. The 4-nitrobenzyl group is a common protecting group for carboxylic acids. Traditionally, the 4-nitrobenzyl ester, when used as a protecting group, is removed by electrolysis, $SnCl_2$, sodium dithionite, or catalytic hydrogenation with Pd-C.[46] CNR uses no metals or harsh chemicals, just NADPH to reduce this nitro group to the amine. The instability of 4-aminobenzyl esters leads them to spontaneous deprotection. While further studies are needed to fully understand the scope of this CNR enzyme, this is a novel method of completely removing the 4-nitrobenzyl protections on carboxylic acids.

In conclusion, we have described the synthesis of twenty-eight 4-nitrobenzyl derivatives and evaluated the ability of CNR to modify these compounds and an additional twenty-five commercially available nitro-containing compounds. While the substrate preference of CNR towards the synthesized 4-nitrobenzyl derivatives varied across the range, we found that CNR could reduce many of the tested nitro-containing molecules with different functionalities. We also discovered that CNR could be used to completely remove the 4-nitrobenzyl protecting group from carboxylic acids. This opens the door to the potential use of CNR as a chemoenzymatic tool for the preparation of arylamines or deptrotection of 4-nitrobenzyl protected acids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AG(s)	aminoglycoside(s)
AAC	aminoglycoside acetyltransferase
AME	aminoglycoside-modifying enzyme

ANT	aminoglycoside nucleotidyltransferase
APH	aminoglycoside phosphotransferase
CAM	chloramphenicol
CAT	chloramphenicol acetyltransferase
CNR	chloramphenicol nitroreductase
CoA	coenzyme A
CPT	chloramphenicol phosphotransferase
EtOAc	ethyl acetate
Et_2o	diethyl ether
LCMS	liquid chromatography-mass spectrometry
MeOH	methanol
NADP(H)	β-nicotinamide-adenine dinucleotide phosphate (reduced)
NMR	nuclear magnetic resonance
RP-HPLC	reversed-phase high pressure liquid chromatography
TLC	thin-layer chromatography

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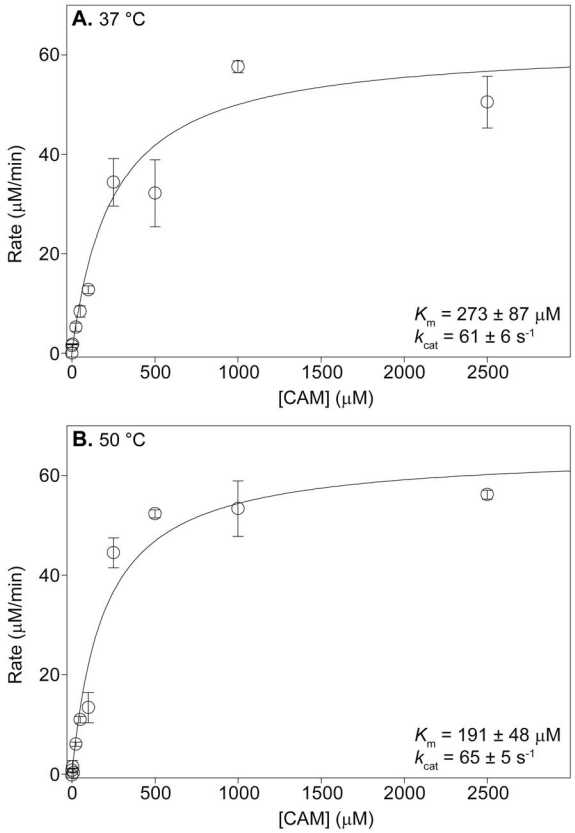


Fig. 1.

Michaelis-Menten curves for the kinetic parameters of CNR with CAM as a substrate at **A.** 37 °C and **B.** 50 °C.

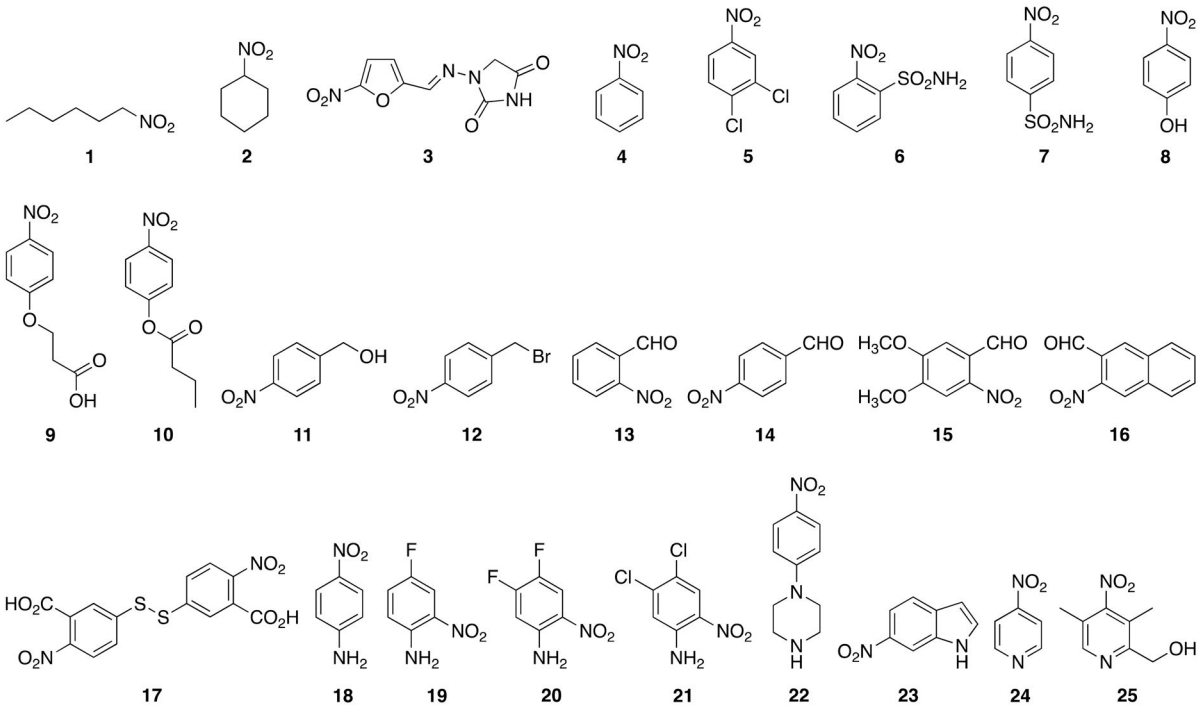


Fig. 2:
Structures of commercially available nitro-containing molecules tested with CNR.

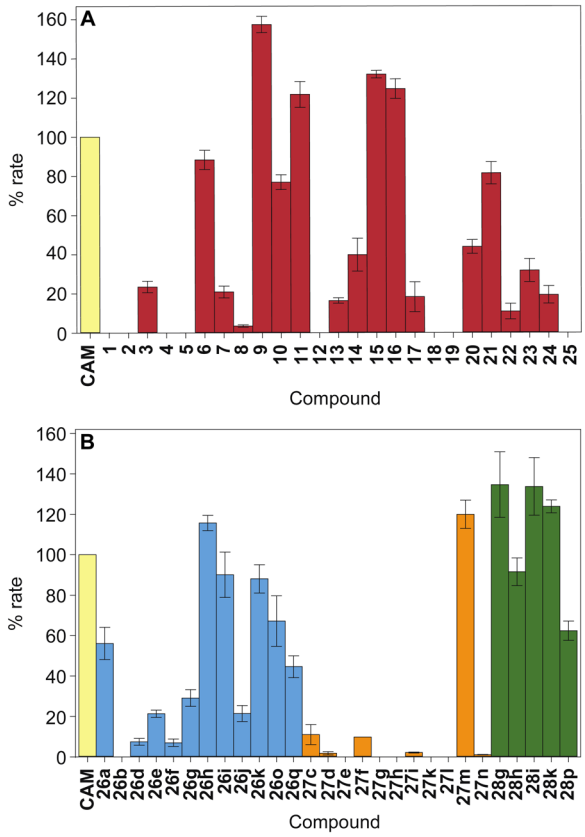
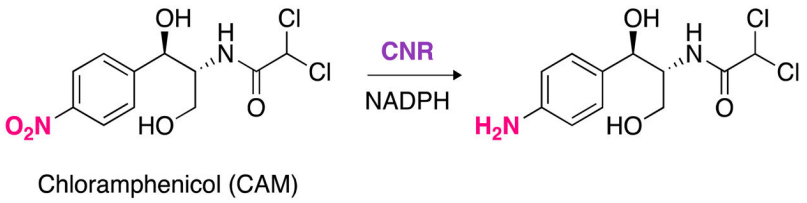


Fig. 3.

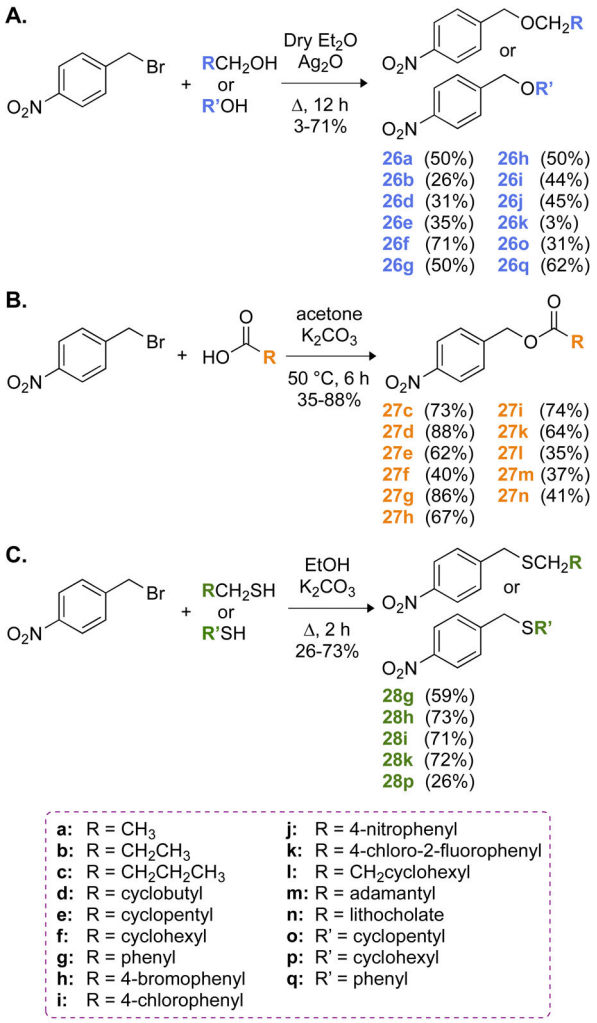
CNR activity against **A.** the commercially available nitro-containing molecules tested in this study and **B.** the *p*-nitrobenzyl derivatives generated in this study. *Note*: The exact values used to generate this figure are presented in Table S1.



Chloramphenicol (CAM)

Scheme 1.

Structure of chloramphenicol (CAM) and schematic representation of its reduction by the chloramphenicol nitroreductase (CNR) resistance enzyme.



Scheme 2.

Synthetic schemes for the preparation of **A.** 4-nitrobenzyl ether derivatives **26a,b,d-k,o,q**, **B.** 4-nitrobenzyl ester derivatives **27c-i,k-n**, and **C.** 4-nitrobenzyl thioether derivatives **28g-i,k,p**.

Table 1:

Masses (*m/z*) observed in the large-scale reactions with CNR for various compounds.

Compound	*m/z* observed	*m/z* calculated for reduction	*m/z* calculated for deprotection
26h	291.0[a]	291.0	185.0
27h	199.0[b]	305.0	198.9
27i	155.1[b]	261.1	155.0
27k	173.0[b]	279.1	173.0
28h	308.0[c]	307.0	200.9

[a] positive mode, M^+ ;

[b] negative mode, $[M-H]^-$;

[c] positive mode, $[M+H]^+$.