Accessing active fragments for drug discovery utilising nitroreductase biocatalysis

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Abstract

Biocatalysis has played a limited role in the early stages of drug discovery. This is often attributed to the limited substrate scope of enzymes not affording access to vast areas of novel chemical space. Here, we have shown a promiscuous nitroreductase enzyme (NR-55) can be used to produce a panel of functionalised anilines from a diverse panel of aryl nitro starting materials. After screening on analytical scale, we show that sixteen substrates could be scaled to 1 mmol scale, with several poly-functional anilines afforded with ease under the standard conditions. The aniline products were also screened for activity against several cell line of interest, with modest activity observed for one compound. This study demonstrates the potential for nitroreductase biocatalysis to provide access to functional fragments under benign conditions.

Introduction

Biocatalysis, the use of enzymes for chemical synthesis, has become an increasingly important tool for compound synthesis in the past 20 years.[1] As directed evolution has become a mature discipline,[2] coupled with the increasing computational power that can be used alongside to inform evolution,[3–6] the number of chemical transformations that are possible by employing enzymes has significantly increased. This is further consolidated by more cost-effective and rapid metagenomic analyses of environmental genetic data,[7] giving access to practically unlimited numbers of enzyme sequences and therefore potential synthetic diversity.

The power of enzyme-supported synthesis has been realised within chemical manufacturing, with numerous large-scale processes now involving directed evolution campaigns to deliver process-suitable biocatalysts.[8] This includes contributions for transaminase,[9] keto reductase,[10] lipase,[11] imine reductase,[12,13] amine oxidase,[14] and recently even whole biocatalytic cascades.[15] A recent analysis showed the average cost to bring a new medicine to market was $1.1bn,[16] between 2009 and 2018, highlighting the need for new methodologies which can offer both new chemical modalities and more efficient synthetic routes. However, there has been less attention paid to the use of biocatalysis for early-stage drug discovery, although several recent perspective articles have highlighted the benefit that enzymes could play in the search for new drugs.[17–20] Our group recently made the argument specifically that fragment-based drug discovery (FBDD) offers an ideal vehicle to employ biocatalysis in drug discovery,[17] as well as providing a convenient and cheap way to quickly develop a panel of chemical compounds. The approach taken in FBDD involves identifying small molecules that weakly bind to targets, which can then be synthetically elaborated into more complex, drug-like molecules.[21–24] Some of the key properties highlighted as important for fragments by Murray and Rees include diverse polar groups and multiple synthetically accessible vectors for fragment growth.[21] Due to the highly selective nature of biocatalysts (chemo, stereo, regio), poly-functional fragments are more easily accessed than protecting-group dependent synthetic approaches, thus affording fragments which potentially allow access to new chemical space (Figure 1). Our specific approach described herein aims to develop routes to common pharmacophoric molecules, namely anilines, by using biocatalysis to improve their synthesis, and give access to this potentially novel chemical space.

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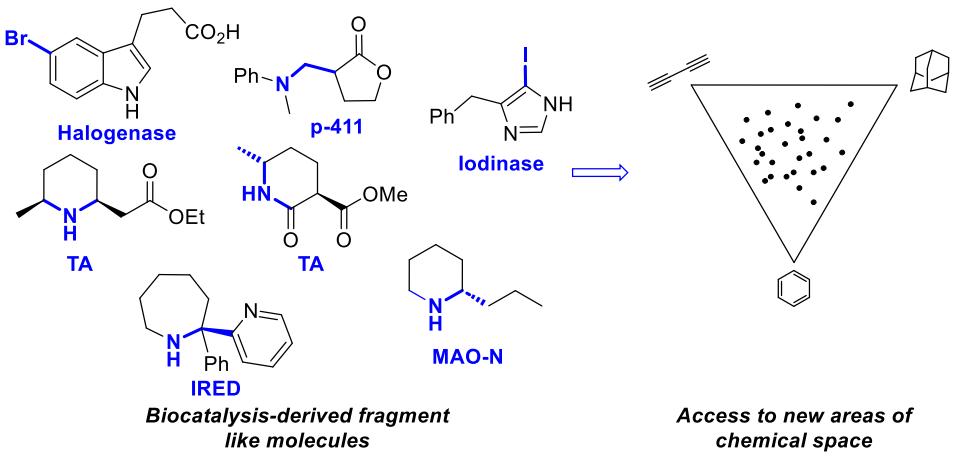
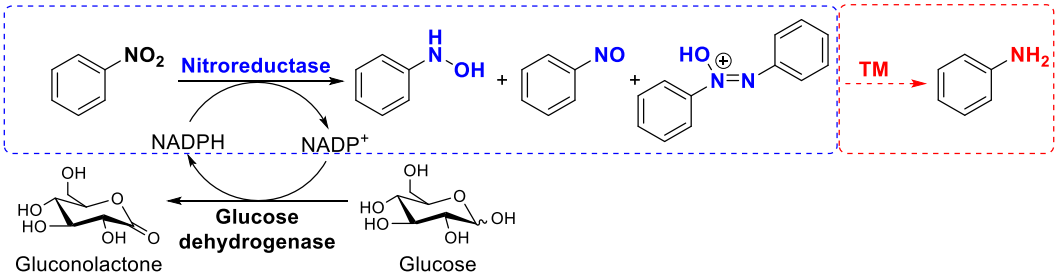


Figure 1. Fragments that can be synthesised using biocatalysis have potential to enable access to new chemical space. TA = transaminase, IRED = imine reductase, MAO-N = amine oxidase from *Aspergillus niger*.

Aniline moieties are present in ~25% of all pharmaceuticals, usually as components of aryl amides, so it is important that new routes to enable their simple syntheses are developed to allow the generation of more molecularly diverse bioactive molecules. The number of reactions performed in the chemical industry to synthesise aryl amides is significant, with previous analysis showing ~6% of all discovery phase reactions involved *N*-arylation.[25] A common approach to aniline synthesis is the reduction of nitroaromatics, usually via precious-metal mediated hydrogenation with H_2 gas. The case for the biocatalytic replacement of this reaction is strong due to safety concerns with using highly flammable gas. In contrast, nitroreductase (NR) enzymes offer a room-temperature, aqueous alternative, which uses glucose as the ultimate reductant via an NADPH/GDH recycling system. The high chemoselectivity of NRs allows for the synthesis of polyfunctional aniline compounds directly from the nitroaromatics in conjunction with a chemical co-catalyst which effects reduction of the hydroxylamine N-OH bond (Scheme 1).[26–28] The favourable reactivity of these enzymes over their chemo-catalytic counterparts has seen a recent increase in interest in using them as sustainable biocatalysts for the synthesis of a range of nitrogen-functionalised aromatics.[29,30]

The approach described herein involves the generation and subsequent biological testing of a series of aniline fragments from the corresponding nitroaromatics, which have all been obtained by facile reduction using NRs.[28] This procedurally simple process is demonstrated on a panel of 34 substrates, and paves the way for both a blueprint for generating modest fragment panels using biocatalysis, as well as more sustainable methodology to replace precious-metal dependent hydrogenations. We have already demonstrated the transfer of NRs into continuous reactors,[31] further highlighting the potential towards bioprocess optimisation in future.



Scheme 1. Nitroreductase mediated generation of nitroaromatics with coupled NADPH recycling and transition metal (TM) co-catalysed reduction of biotransformation products.

Results and discussion

An in-house compound collection containing over 30 distinct aryl nitro compounds was used for biocatalysis using NRs, which included a range of functionalities, different substitution patterns and heteroaromatic groups. Our initial goal was to determine the activity of a specific NR enzyme, a commercially available NR-55 (supplied by Johnson Matthey), towards our substrate panel and determine the synthetic generality.

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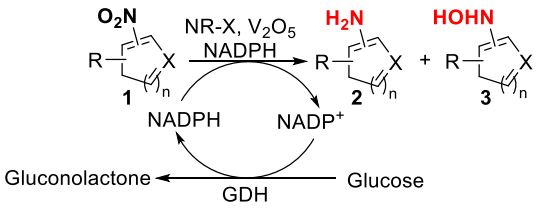
Initial analytical scale reactions (Scheme 2, analytical conversions shown in blue) were based on conditions reported previously,[28,31] using NR-55 and glucose dehydrogenase-101 (GDH-101, also supplied by Johnson Matthey) in lyophilised CFE format at concentrations of 5 mg mL^{-1} and 1 mg mL^{-1}, respectively. The substrate concentration of the entire panel was set to 50 mM, irrelevant of solubility of the substrate at this concentration, as per our previous observations of the enzyme being able to tolerate this, with 10% DMSO to aid solubility. Reactions were run for two hours and analysed by comparison of the ^{1}H NMR spectra of the starting material and crude product to determine conversion. As shown below, the NR-55 displayed a broad substrate scope towards aryl nitros (Scheme 2).

Chiefly, nitrogen-containing heterocycles **2a** and **2b** afforded quantitative conversions, and good conversions were observed for halogenated analogues **2c-2g** and **2j**. Interestingly and importantly, polyfunctional substrates were well tolerated with perfect functional group chemoselectivity observed in all instances. Of particular note are the phenolic ester **2h**, benzyl alcohol derivative **2i**, and the range of carboxyl esters and acids with several other groups.

In addition to the substrates which were successfully reduced, we have shown the remainder of the panel for comparison. Substrates **1t-y** were interesting in that some reactivity was observed using NR-55, however, the products of the reactions were not the desired ones and remained uncharacterised due to issues with scaled-up reactions. For example, 4-nitrophenylboronic acid **1t** afforded a product which did not appear to retain *para*-substitution upon analysis of the ^{1}H NMR spectrum. Perhaps unsurprisingly, the reactive isocyanate **1u** produced a complicated ^{1}H NMR spectrum (absent of starting material) and scale up of that particular substrate afforded a very poor mass recovery (>5%). Reactions of **1v-y** appeared to show some product on analytical scale, but once again scaling of the reactions afforded poor mass recovery so were deemed unsuccessful. The remaining substrates **1z-ah** were screened but showed no activity. This indicates NR-55 was limited to the substrates which worked, however screening libraries of NR variants or directed evolution could deliver other NRs which are fully active towards the other substrates.

Nonetheless, our results demonstrate the broad activity present within NR-55, contravening a common perception regarding biocatalysts that they act on individual substrates only. Whilst all compounds analysed were nitroaromatics, they varied in ring size, substitution pattern, aromatic electronic properties and orthogonal functional groups demonstrating the diversity of reactivity available to individual NRs. Being active towards 25 substrates out of 34 is akin to the substrate scope observed in many synthetic methodology studies,[32] showing the versatility and potential of biocatalysis for diversity generation.



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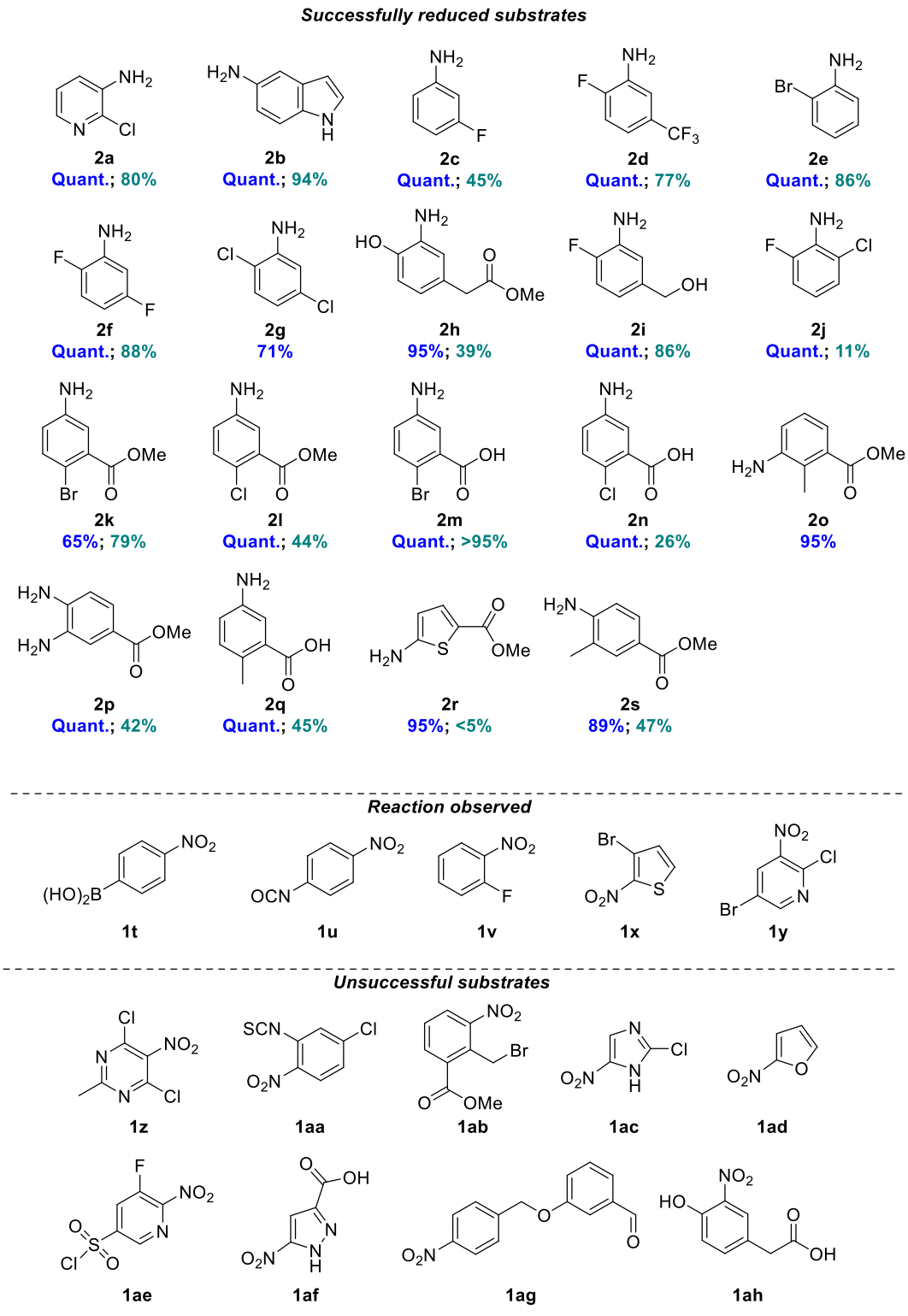

Scheme 2. The nitroreductase/V_2O_5-mediated reduction of a range of aromatic nitro compounds. Analytical scale (conversion in **blue**): General conditions: NR-55 (5 mg mL^{-1}, CFE), GDH-101 (1 mg mL^{-1}, CFE), V_2O_5 (2 mM), Nitroaromatic (50 mM), Glucose (200 mM), NADPH (1 mM), KPi buffer (250 mM, pH 7.5), DMSO (10% v/v), total volume = 1 mL; Preparative scale (isolated yield in **green**): general conditions as analytical, but total volume = 20 mL. Analysis of reaction by ^1H NMR.

With these initial analytical scale results in hand, the majority of the panel was scaled up to 20 mL preparative reactions (not **2g** or **2o** due to insufficient material). The reaction time was extended to 16 hours to allow full conversion to be observed for those substrates which did not complete within 2 hours. The substrate concentration was once again set at 50 mM, and NR-55 used at 5 mg mL^{-1}. Interestingly, the same conversion success was not translated to the larger scale with reduced yield in some reactions. This was due to several factors, including substantially more protein being in the

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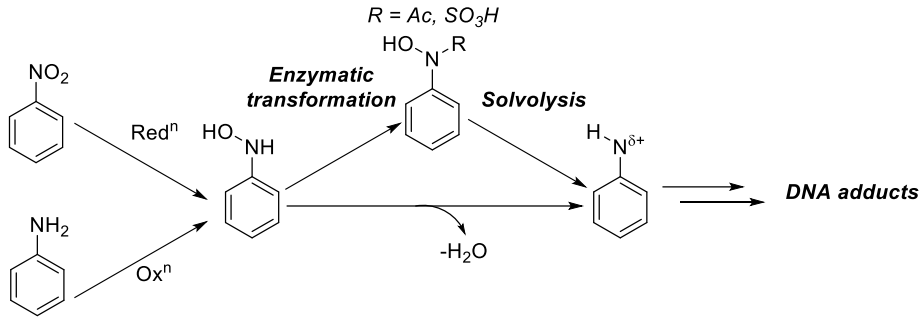
reactions and the fact that no mass-based yields were determined for the analytical scale reactions, therefore not identifying substrates which may not have fully converted. Some such examples included **2j** (11% yield), **2n** (26% yield) and most starkly **2r**, where only a few milligrams of an impure sample could be isolated. That being said, the preparative scale reactions were still able to successfully reduce 16 substrates on 20 mL scale at 50 mM concentration (1 mmol of substrate), with yields of at least 42% (yields range 11-97%). This provided, in almost all instances, at least 50 mg of the aniline fragments for biological screening.

Biological testing

In a cellular context, aryl amines can cause carcinogenesis due to their electrophilic nature by forming covalent bonds with DNA.[33,34] The initial step in the bioactivation is the oxidation of exocyclic amine nitrogen to form the corresponding hydroxylamine, which then undergoes heterolytic bond cleavage in mildly acidic aqueous environment, forming a reactive arylnitrenium ion.[34] This ion can also be formed by *N*-acetyltransferases or sulfotransferases as shown in **Error! Reference source not found.** (see ref. 45 and references therein).[35,36]



Scheme 3. Demonstration of metabolic activation of aromatic nitrogen compounds towards nitrenium ion formation. The δ^+ on the exocyclic nitrogen was calculated and statistically analysed for our panel of aniline substrates.

In order to assess the biological activity and toxicity of the compounds, we conducted biological assays. Disk diffusion assays were carried out using *E. coli* and *S. epidermis*, with no activity evident at the highest tested concentration of 20 mM in *E. coli*. However, anti-microbial activity was observed for **2h** and **2k** in *S. epidermis*, with the latter giving a clear dose response signal as seen in Figure 2 (see further detail in the SI).

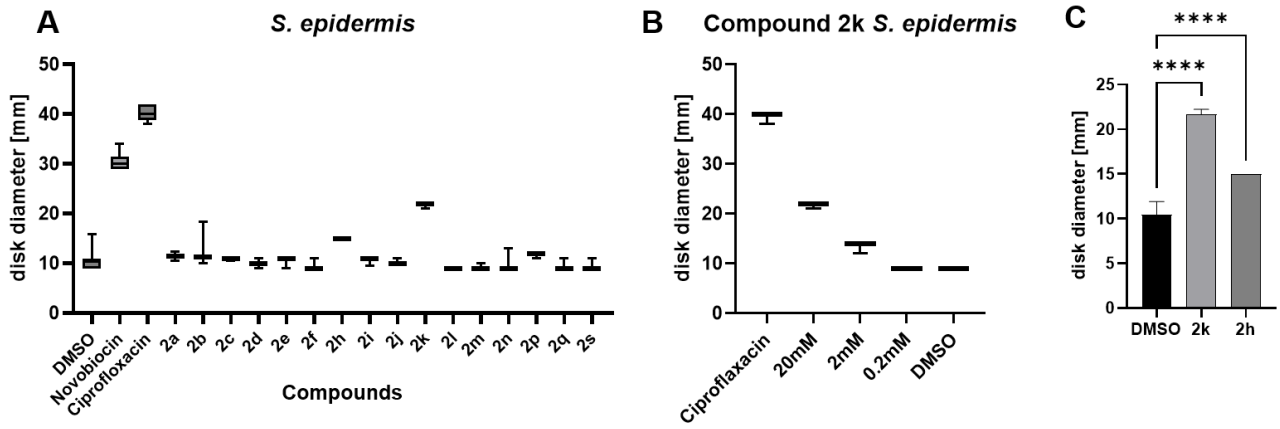


Figure 2. Kirby-Bauer disk diffusion susceptibility test against *S. epidermis.* (A) Kirby-Bauer disk diffusion susceptibility test of test compounds at 20mM with Novobiocin and Ciprofloxacin as controls. Shown are mean values of three biological repeats with standard deviation. (B) Antibiotic susceptibility testing of compound **2k** against *S. epidermis* at different concentrations with Ciprofloxacin as control. (C) Statistical analysis of antibiotic testing of compounds **2k** and **2h** using ANNOVA. Shown are mean values and standard deviations from three biological repeats. P-values are indicated by stars, with * ($P \leq 0.01$), ** ($P \leq 0.001$) and *** ($P \leq 0.0001$).

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To confirm the absence of toxicity the compounds were further tested against two human cancer cell lines (PC3, prostate and MDA-MB-468, breast) and a mosquito cell line (U4.4) using the MTT and Srb assays. No, or modest, effects were seen at the highest concentration of 200 µM, indicating that the generated compounds are unlikely to have formed cancerogenic compounds. Finally, a select array of compounds was tested in a cell cycle assay with no, or little, effect observed (see figure S1 in the SI and the accompanying text), again supporting the notion that these compounds are largely non-toxic to the human and mosquito cell lines tested.

Discussion

Using our approach for biocatalysis-inspired fragment generation more generally (i.e., applying to different enzyme classes) offers the potential to move into completely new areas of chemical space with unbiased chemistry. This will mean new molecular architectures could be discovered, offering potentially unique platforms to create novel pharmaceuticals. With this in mind, access to inhibitors of intractable targets may become possible for FBDD, with one such challenging area being protein-protein interactions.[37,38]

The polyfunctional anilines generated in this study present numerous synthetic handles, offering vectors for additional growth and therefore represent the potential impact biocatalysts can make in the synthetic elaboration of active fragments. In addition, the high chemoselectivity allows more efficient routes to be designed towards fragments with additional synthetic handles (e.g., amino, carboxyl and halogen groups **2k** – **2n**). Furthermore, many of the fragments are synthetically tractable using not only biocatalysis (e.g., biocatalytic reductive amination), but also using synthetic catalysts (e.g., cross-coupling via halogen).

Whilst the use of biocatalysis in early-stage drug discovery settings has been increasingly considered in recent years,[17–20] there is yet to be a large-scale implementation of biocatalysis in synthetic chemistry. There are numerous reasons this could be, including a lack of knowledge leading to perceptions of narrow scope, or the idea that recombinant proteins are expensive and hard to access without specialist microbiological knowledge. The high attrition rate in drug discovery means that investment in new routes may be difficult to justify, so use of the original, robust 'med chem' routes offers a significant risk mitigation.[39] However, adoption of biocatalysis within early-stage discovery and incorporation into the routes towards final targets after lead compound validation would likely lead to an increase in utilisation of biocatalysts overall as they would become better understood and easier to access. Moreover, biocatalysis may offer a chance to optimise and economise certain traditional 'med chem' approaches which would greatly enhance the drug discovery process.

More specifically, our study aimed to show that biocatalysis has a role to play in fragment-based drug discovery.[17] The selection of NRs allowed several aspects of biocatalysis to be highlighted in our study:

1. **Chemoselectivity**: aryl nitro reduction is typically carried out using platinum group metals under hydrogenation conditions. This approach, whilst successful, is often non-selective towards sensitive groups (i.e., halogens),[40] which can play important roles in subsequent functionalisation of active fragments. The NRs proceed with perfect selectivity, offering a route to polyfunctional fragments from readily available feedstocks.
2. **Broad substrate scope**: whilst not all enzymes will have a broad substrate spectrum, the off-the-shelf choice of the NR-55 used here showed that some biocatalysts do have promiscuity in-built, allowing a generalist catalyst to be used. Furthermore, the development of metagenomic screening kits and new HTS methodology circumvents many of the problems associated with narrow substrate scope. The wide availability of metagenomically identified enzyme families offers new catalysts in vast abundance, once again mitigating against low activity for specific substrates.[41]
3. **Efficiency**: reactions were often complete within two hours under biological conditions, at reasonably high substrate concentrations (50 mM). This level of efficiency (low energy, fast reaction times) matches that of many synthetic catalysts, thus removing the barrier for rapid progress required within the realms of early-stage discovery.
4. **Scalability**: our study demonstrates that biocatalysis reactions have the scope for upscaling, which could be improved through bioprocess optimisation. This opens up the possibility of

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implementation in large-scale drug discovery thus increasing its applicability and economic benefit, for example through continuous processing.[31]

Conclusion

We have shown that a panel of aniline fragments derived from biocatalytic nitro reduction have the potential to offer unique starting points for bioactive molecules. The panel includes a range of functionality, including synthetically tractable handles such as a carboxyl (ester) groups and halogens. This is important for the future derivatisation of the active fragments towards the synthesis of pharmaceutically active molecules.

In conclusion, our study highlights the untapped potential biocatalysts present in FBDD and compound development, both in easy access to novel chemical space as well as optimisation of already present synthetic processes with economic benefits. The wide variety of enzymes available for biocatalysis of synthetic substrates allows for wide applicability as well as innovation in synthetic processes, which may unlock previously intractable targets for drug discovery.

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Methods

Biotransformation method

To an Eppendorf vial containing KPi buffer (540 µL, 250 mM, pH 7.5) was added KPi buffer (250 mM, pH 7.5) solutions of NR55 (100 µL of a 50 mg mL^{-1} stock solution, final conc. 5 mg mL^{-1}), GDH101 (100 µL of a 10 mg mL^{-1} stock solution, final conc. 1 mg mL^{-1}), V_2O_5 (100 µL of a 20 mM stock solution, final conc. 2 mM), glucose (100 µL of a 2 M stock solution, final conc. 200 mM), $NADP^+$ (10 µL of a 100 mM stock solution, final conc. 1 mM). The reaction was then initiated by addition of a DMSO or MeCN solution of the nitro compound (50 µL of a 1 M stock solution, final conc. 50 mM). The vials were sealed and placed in an incubator at 35 °C with 200 rpm stirring for the required time to allow the reaction to go to completion. The reaction mixture was then quenched with an aqueous solution of NaOH (20 µL, 5 M), and EtOAc (1 mL) was added and the solution vortexed for 10 seconds. The reaction was centrifuged for 2 minutes at 14,000 rpm, the organic phase decanted and then analysed by ^{1}H NMR, GC-FID, GC-MS, or a combination thereof.

Cell culture

MDA-MB-468 and PC-3 cells were originally purchased from American Type Culture Collection (ATCC) and were a kind gift from Alan Richardson and Gwyn Williams (Keele University). MDA-MB-468 cell lines were grown in Roswell Park Memorial Institute medium (RPMI 1640; Corning # 10-040-CV) with 10% fetal bovine serum (FBS; Clone, GE Healthcare Lifesciences, #SH30109), 2 mM L-Glutamine (200mM, Gibco, # A29168-01) and 50 µg mL^{-1} (50 IU mL^{-1}) penicillin/streptomycin (Sigma, # P4458). PC-3 cells were grown in nutrient mix F-12 (Kaighn's modification, Corning, # 10-025-CV) supplemented with 10% fetal bovine serum and 50 µg mL^{-1} (50 IU mL^{-1}) penicillin/streptomycin (Sigma, # P4458). The cells were grown at 37 ºC in 5% CO2 and trypsinised (Trypsin, Gibco, # 25200-056) to subculture. U4.4 cells were a gift from Rennos Fragkoudis at Pirbright institute and maintained at 28 °C with 5% oxygen. U4.4 cells were grown with EMEM (Sigma, #M-4655) or DMEM F-12 (Gibco, # 11320-033) supplemented with 10% FCS (HyClone, GE Healthcare Lifesciences, #SH30109), 1% non-essential amino acids (Gibco, #11140-025) and 1% penicillin/streptavidin (Sigma, #P4333).

MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was conducted according to established protocols.[42] Briefly, cells were seeded in the inner wells of a 96-well plate at a density of 50,000 cells mL^{-1} with 100 µL cell solution per well. 200 µL of PBS was placed into the outer wells.

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Plates were incubated at 37 ºC for 2 days before treatment with compound. Then, 100 µL of compound dilutions, 1% DMSO control or 5-fluorouracil positive control (1 mM to 2 µM) was placed in each well and plates incubated for another 2 days. Compounds were diluted in culture media to a final concentration of highest (200 µM or 100 µM; 1:100) and lowest (2 µM and 1 µM; 1:10,000). DMSO control was done in 6 technical replicates and compounds in 4 or 5 technical replicates. Three biological repeats were run. On day 4, 20 µL of MTT reagent (5 mg mL^{-1} in PBS, Invitrogen Molecular Probes # M6494) was added to each of the wells and plates incubated for 3-4 hours. Then, all media was carefully removed without disturbing the cells, 100 µL of solubilisation solution (40% v/v DMF, 4% v/v acetic acid, 16% w/v sodium-dodecyl sulfate) added and plates placed on rocker for 10 minutes to dissolve formazan crystals. Plates were measured on a plate reader at dual wavelength, 570 nm and 630 nm.

Srb assay

Srb (Sulforhodamine B) assay was conducted according to established protocols.[43] A cell density of 70,000 cells mL^{-1} was used and 50 µL of cell suspension seeded in the inner wells of a 96-well plate. 200 µL of PBS was placed into the outer wells. Then, 50 µL of compound dilutions, 1% DMSO control or 5-fluorouracil positive control (1 mM to 2 µM) was placed in each well and incubated at 37 °C for 4 days before measurement. Compounds were diluted in culture media to a final concentration of highest (200 µM or 100 µM; 1:100) and lowest (2 µM and 1 µM; 1:10,000). DMSO control was done in 6 technical replicates and compounds in 4 or 5 technical replicates. Three biological repeats were run. Then, 26 uL 50% (w/v) TCA was added and the plate incubated at 4 °C for 1 hour. The plates were washed four times by submerging the plate in a tub with slow-running tap water and excess water removed by gently tapping the plate into a paper towel. After the last wash the plate was allowed to air-dry at room temperature. 50 µL of 0.04% (w/v) SRB solution (Invitrogen # S1307) was added to each well and the plate incubated at room temperature for 1 hour. Plates were washed four times with 200 µL of 1% (v/v) acetic acid to remove unbound dye. Plates were allowed to air-dry at room temperature before adding 50 µL to 100 µL of 10 mM Tris base solution (pH 10.5) to each well and shaking the plate on an orbital shaker for 10 minutes to solubilize the protein-bound dye. Absorbance was measured at 510 nm in a microplate reader.

Antibiotic susceptibility testing

Antimicrobic testing was performed using the Kirby-Bauer disk diffusion method using 9mm disks (CAT no) on Miller-Hinton agar (CAT no). 200 µL of *S. epidermis* or *E. coli* solution were spread onto MH agar and left to dry for 2 minutes. Disks were soaked with 30-50 µL of DMSO, compound at concentrations of 20 mM or 10 mM (compound 1), or positive control and placed onto the agar. Positive controls used were Novobiocin at 200 mg L^{-1} and Ciprofloxacin at 0.5 mg L^{-1} for *S. epidermis*, and ampicillin at a concentration of 400 mg L^{-1} and Ciprofloxacin at 0.5 mg L^{-1} for *E. coli*. Plates were placed upside-down in a 37 °C incubator and incubated for 24 hours. The diameter of each disk including exclusion zone caused by antimicrobic activity was determined using a ruler. Data from three independent biological assays were averaged and standard deviations determined. Statistical analysis was performed using ANNOVA.

Cell cycle assay

Cells were seeded at 500,000 cells per well in 6-well plates and grown until a confluency of around 80% was reached after 2 days. Cell medium was replaced with media containing cell arresting compounds at the appropriate concentration (50 µM for 5-fluorouracil and 200 µM for test compounds). Control wells contained 1% DMSO. The cells were incubated for 24-30 hours before being harvested by removing the media and briefly washed with PBS before adding 1 mL PBS and scraping the cells off the plastic surface. Cells were collected by centrifugation for 5 minutes at 300g, PBS taken off, fixed in 700 µL ice-cold 70% ethanol, then stored at -20 ºC until analysis. Cells were further treated according to instructions for the Muse Cell cycle kit (Luminex, #MCH100106) and analysed using the Muse cell analyser (Luminex). The percentage of cells in G0/G1, S and G2/M phases was determined. Experiments were run in biological triplicates, and mean values and standard deviation determined. Graphs were produced and data fitted using a sigmoidal fit in Graphpad.

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