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Efficient Discovery of Bioactive Scaffolds by Activity-Directed Synthesis

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Abstract

A novel discovery approach – activity-directed synthesis – is described in which reactions with alternative possible outcomes are steered towards functional products. Arrays of catalysed reactions of α-diazo amides, whose outcome was critically dependent on the specific conditions used, were performed. The products were assayed at increasingly low concentration, with the results informing the design of a subsequent reaction array. Finally, promising reactions were scaled-up and, after purification, sub-micromolar ligands based on two scaffolds with no previous annotated activity against the androgen receptor were discovered. By analogy with biosynthetic pathway evolution, the approach enables the emergence in tandem of both bioactive small molecules and associated synthetic routes. The highly general approach might be applied in the discovery of novel and diverse chemotypes with a very broad range of biological functions.

Introduction

The astonishing functional and structural diversity of natural products continues to provide tremendous inspiration in drug discovery and chemical biology.[1-3] Natural products arise through the evolution of biosynthetic pathways, driven by functional benefit to the host organism.[4,5] In stark contrast, synthetic routes to bioactive small molecules are almost always developed in isolation from the assessment of their function.

In many discovery programmes, bioactive small molecules are initially identified by high-throughput screening, and, to facilitate this approach, cheminformatic

approaches have been developed to assess the scaffold diversity of screening libraries.[6] The annotated bioactivity of known scaffolds can also facilitate the exploration of chemical space, and can provide inspiration in the design of bioactive molecules based on related scaffolds.[7,8] The optimisation of small molecule function is generally undertaken through iterative rounds of design, synthesis and assaying.[9] To expedite ligand optimisation, a narrow toolkit of reliable synthetic methods with predictable outcomes has emerged to support medicinal chemistry.[10,11] Established medicinal chemistry approaches[12] may have inadvertently steered molecular design towards a subset of molecular scaffolds, and tended to exacerbate chemists' exceptionally uneven historic exploration of chemical space.[13] The emergence of diversity-oriented synthesis[14-16] has expanded the diversity of small molecule screening collections, but is still reliant on an expanded toolkit of synthetic methods with inherently predictable outcomes.

Although function has steered the directed evolution of biosynthetic pathways, for example to produce carotenoids with specific colours,[17] the emergence of chemical syntheses has rarely been directed by the function of the resulting products. Chemical strategies which link structure and function include the *in situ* synthesis of enzyme inhibitors from building blocks within active sites.[18-20] In addition, dynamic combinatorial chemistry exploits reversible reactions to template the formation of cognate ligands for specific molecular targets.[21] To apply such approaches in bioactive small molecule discovery, the challenge of developing reactions that operate efficiently under buffered aqueous conditions must be met. To date, only a few reactions have been developed that operate efficiently under conditions compatible with folded proteins, fundamentally limiting the chemical diversity of the small molecules that may be discovered.

Herein, we describe a novel discovery approach in which bioactive small molecule function directs the emergence of an associated synthetic route. We specifically

chose to exploit catalysed reactions with alternative possible outcomes that might be steered by the specific catalyst and reaction conditions used. It was anticipated that such reactions would enable exploration of the relevance of a wide range of molecular scaffolds to a required biological function.

In each round, an array of reactions was designed and, after scavenging, the products were assayed at increasingly low concentration for agonism of the androgen receptor. The results informed the design of a subsequent reaction array until, finally, promising reactions were scaled-up to reveal, after purification, bioactive small molecule ligands (Fig. 1).

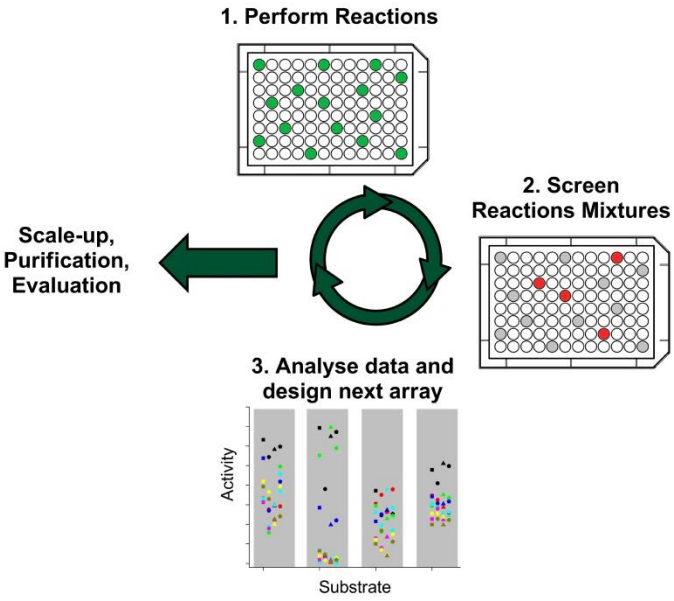
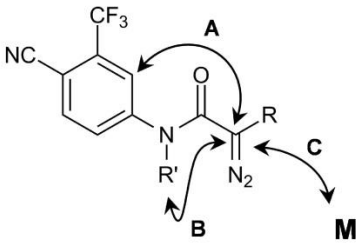


Figure 1: Overview of activity-directed synthesis. In each round, arrays of reactions are performed. The resulting product mixtures are assayed at increasingly low concentration, and can inform the design of subsequent reaction arrays. Finally, promising reactions are scaled-up, and the products purified, identified and evaluated.

Crucially, the approach enabled the discovery of ligands with sub-micromolar activity, based on two scaffolds with no previously annotated activity against the androgen receptor. To our knowledge, the study constitutes the first example in which the emergence of synthetic routes conducted under non-aqueous conditions was directed by the biological activity of the resulting small molecule products.

Results

We chose to exploit catalysed reactions of diazo compounds because alternative outcomes are possible with many substrates, and yet may often be controlled through judicious choice of catalyst and reaction conditions. For example, α-diazo carbonyl compounds are well known to participate in many intermolecular[22] reactions (e.g. C–H, N–H and O–H insertions; cyclopropanations; ylide formation/cycloaddition) as well as intramolecular variants that form alternative ring sizes.[23-25] Such inherently unpredictable reactions are generally unsuitable in current ligand discovery approaches which typically involve the synthesis and purification of arrays of small molecules. However, such reactions are ideal in activity-directed synthesis since their outcome may often be controlled by appropriate catalysts and ligands,[26,27] and synthetically-accessible bioactive molecules based on alternative scaffolds might be discovered.



	R	
R'	Ac	H
Pr	**1**	**2**
CH_2Ph	**3**	**4**
$(CH_2)_2Ph$	**5**	**6**
$(CH_2)_2CH=CH_2$	**7**	**8**
$(CH_2)_2iPr$	**9**	**10**
$CH_2CONMe(CH_2)_2CH=CH_2$	**11**	**12**
$(CH_2)_2OMe$	**13**	-
4-pyridylmethyl	**14**	-
$(CH_2)_2CN$	**15**	-
propargyl	**16**	-

Figure 2: Structures and possible fates of α-diazo amides. Possible fates include: intramolecular reaction with the appended *N*-aryl ring (A); intramolecular reaction with the R' side chain (B); and intermolecular reaction with a different molecule, **M** (C).

We investigated whether activity-directed synthesis could enable the discovery of novel chemotypes from a fragment found in a wide range of androgen receptor modulators.[28-32] Initially, we prepared[33] twelve α-diazo amides **1**-**12** (Figure 2) which each bear the 4-cyano-3-trifluoromethylphenyl group and a variable R' group. Some possible intra- and intermolecular reactions that these substrates might undergo are outlined in Figure 2. The range of R' substituents was chosen to increase the scaffold diversity of possible cyclisation products (e.g. formed by intramolecular C–H insertion, cyclopropanation, or ylide formation/cycloaddition). We performed an array of reactions in a 96-well plate in which the diazo substrate (100 mM in dichloromethane) and catalyst (1 mol%; for structures, see Supplementary Data Fig. 1) were varied. After 48 hr, the crude reactions were scavenged to remove metal contaminants (for optimisation, see Supplementary Data Fig. 2), evaporated, and assayed for agonism of the androgen receptor (total concentration of products: 10 μM in 1% DMSO in pH 7.5 aqueous buffer) (Figure 3, Panel A and Supplementary Data Table 1). Activity was determined using a commercially available assay with a time-resolved FRET readout.[34] Crucially, we had already established that the diazo substrates were all inactive under the conditions of the assay (Supplementary Data Fig. 3). Four substrates (**1**, **3**, **6** or **7**) yielded products that were highly active: these substrates were fed into the design of the subsequent reaction array, together with, as a control, two substrates (**11** and **12**) that had not yielded active product mixtures.

In round two, we performed an array of reactions in which the substrate (**1**, **3**, **6**, **7**, **11** and **12**), catalyst (1 mol%) and solvent (dichloromethane, THF, toluene or ethyl acetate) were varied. The reactions were evaporated, scavenged and assayed at ten-fold lower concentration (total concentration of products: 1 μM in 1% DMSO in pH 7.5 aqueous buffer). Crucially, the substrates **11** and **12**, that

were included as controls, did not yield active product mixtures in round two. Furthermore, the activity of the product mixtures derived from the other substrates was critically dependent on the specific catalyst and reaction conditions used. For example, the most active product mixtures were derived from reactions of the substrates **1** and **3**, but only when a specific class of catalyst (rhodium carboxylates) was used in specific solvents (dichloromethane, toluene or ethyl acetate) (Figure 3, Panel B and Supplementary Data Table 2). These data suggest that the fate of these substrates may depend critically on the catalyst used, with only a specific class of catalyst steering the reaction towards active products.

In round three, we exploited the substrates **1** and **3**, as well as four additional structurally-related substrates **13**-**16**. We performed a reaction array in which these six substrates were varied, together with the solvent (dichloromethane, toluene or ethyl acetate) and catalyst (six rhodium carboxylates including the two most promising catalysts from the previous round). The reaction products were assayed at ten-fold lower concentration (total concentration of products: 100 nM in 1% DMSO in pH 7.5 aqueous buffer) (Figure 3, Panel C and Supplementary Data Table 3). Again, for three substrates (**1**, **3** and **15**), reactions were only steered towards active products by a narrow range of catalysts and reaction conditions.

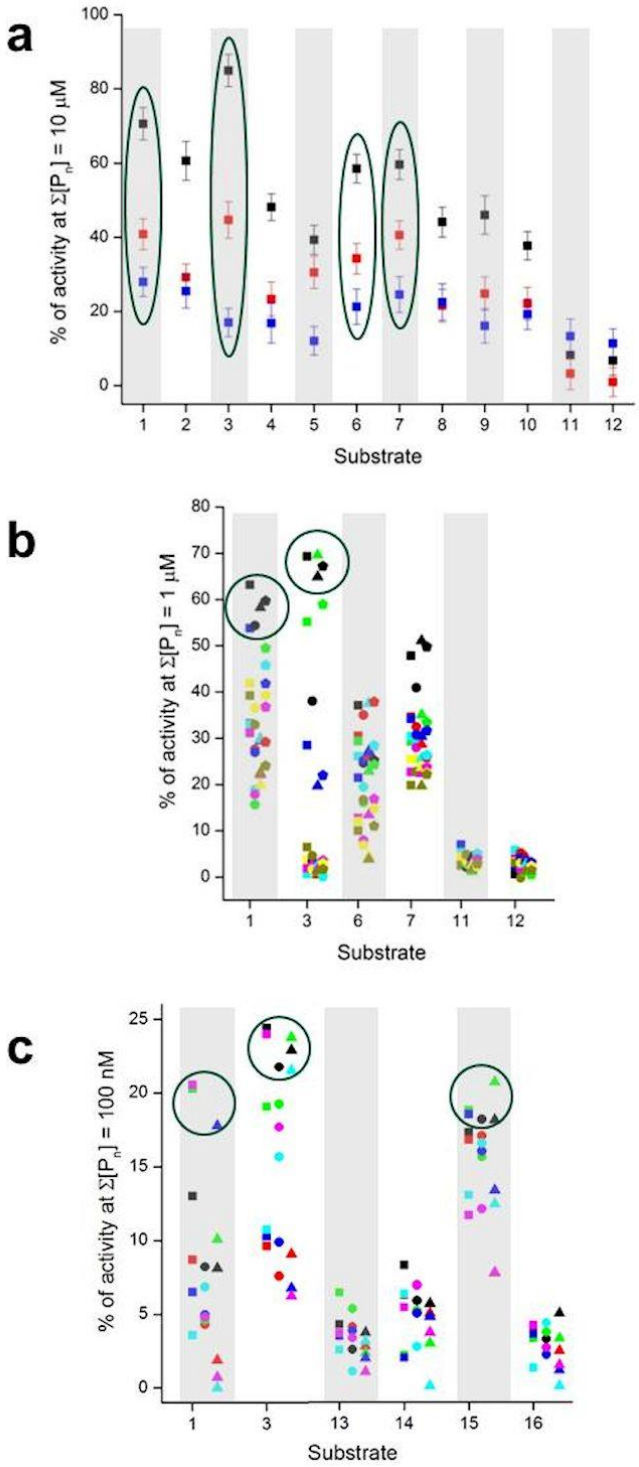
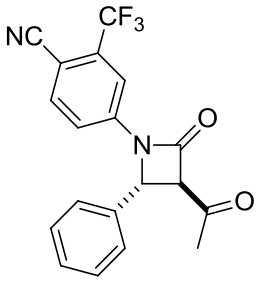
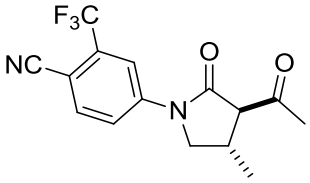
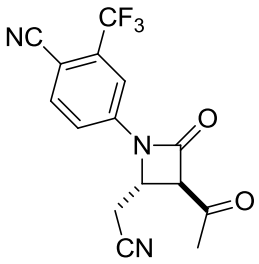


Figure 3: Activity of the products derived from reaction arrays. A: In round 1, the substrates **1**, **3**, **6** and **7** yielded active product mixtures, and were exploited in round 2. B: In round 2, substrates **1** and **3** yielded active product mixtures when treated with rhodium carboxylate catalysts in dichloromethane, toluene or ethyl acetate. C: In round 3, eight promising reactions were selected for scale-up on the basis of the activity of their products. Biological activity is expressed relative to 5 μM testosterone. See Supplementary Data Tables 1-3 for the catalysts (represented by different colours) and solvents (represented by different shapes) used. Experiments

were performed in duplicate. Error bars are omitted from Panels B and C for clarity (typical error 1.5-4.5%, see Supplementary Data Tables 1-3).

Finally, on the basis of active product mixtures identified in round 3, we scaled up eight reactions whose products had had the most promising biological activity (Table 1 and Supplementary Data Table 4). In each case, a major product was purified, and its structure was elucidated. The three distinct major products were assayed at a range of concentrations for agonism of the androgen receptor (Table 1 and Figure 4) (for the structure and activity of the reference ligands testosterone and flutamide, see Supplementary Data Fig. 4). To confirm its activity, the most potent ligand, **17**, was also prepared using an independent synthetic route (Supplementary Data Fig. 5, Panel A); this sample was found to have identical activity to that prepared by $Rh_2(esp)_2$-catalysed cyclisation of the substrate **3** (Supplementary Data Fig. 5, Panel B). The β-lactams **17** and **19** were full agonists of the androgen receptor, whilst the γ-lactam **18** functioned as a partial agonist.

Table 1: Yield and activity of the products of selected reactions that were scaled-up (see also Supplementary Data Table 4).

Entry	Reaction conditions[a]	Product	Yield / %	EC_{50} / nM
1	**3**, 1 mol% $Rh_2(esp)_2$, EtOAc	 **17**	75	340 ± 30 (agonist)
2	**1**, 1 mol% $Rh_2(esp)_2$, CH_2Cl_2	 **18**	90	470 ± 40 (partial agonist[b])
3	**15**, 1 mol% $Rh_2(esp)_2$, EtOAc	 **19**	78	440 ± 60 (agonist)

[a]esp **=** α,α,α′,α′-tetramethyl-1,3-benzenedipropionate, [b]Maximum activity was 50% of that of testosterone.

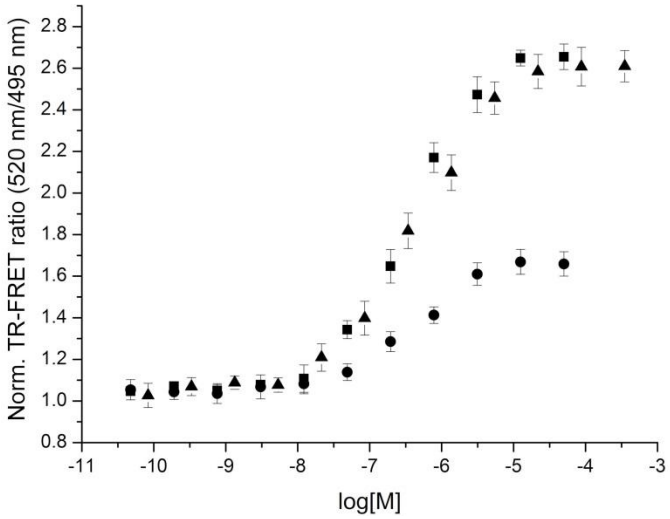


Figure 4: Dose-dependent agonism of the androgen receptor by the major products of scaled-up reactions (see Table 1). Squares: **17**; Circles: **18**; Trangles: **19**.

Discussion

Activity-directed synthesis provides a novel and highly efficient approach to the discovery of bioactive small molecules, together with associated synthetic routes. The discovery of reactions that yield active products is undertaken in parallel through iterative rounds of reactions and screening. Only reactions that yield highly active products are scaled up, and the products purified, identified and characterised. Resource is thus highly focused on bioactive reaction products. Conventional ligand optimisation approaches tend to invest similar resource in all compounds prepared: although parallel synthesis techniques are often exploited, the purification of the resulting arrays of compounds is usually undertaken in series (often using mass-directed liquid chromatography) before assays are undertaken in parallel and structure-activity relationships are formulated.

To enable the discovery of alternative bioactive chemotypes, activity-directed synthesis requires methods whose outcome may be varied through choice of the specific catalyst and reaction conditions used. We deliberately chose to exploit metal-catalysed reactions of diazo compounds on the basis of the range of intra- and intermolecular reaction types that are possible. To demonstrate the fitness of diazo chemistry for activity-directed synthesis, we showed that the fate of the

α-diazo amide **3** varies widely: in total, three distinct compounds were observed, whose distribution depended critically on the specific catalyst and reaction conditions used (Supplementary Data Fig. 6 and Supplementary Data Table 5).

Promising reactions were identified solely on the basis of the activity of the resulting product mixtures. To demonstrate that the activity of product mixtures provided insights into the activity of individual components, we purified and assayed the products of a limited number of reactions (Figure 5 and Supplementary Data Fig. 7). The $Rh_2(OAc)_4$-catalysed reaction of **8** (from round 1) gave low yields of the oxindole **20** (yield: 13%; EC_{50} = 10.1 ± 0.1 μM), the oxindole **21** (yield: 12%; inactive up to 1 mM) and the δ-lactam **22** (yield: 26%; EC_{50} = 0.71 ± 0.14 μM; partial agonist); the activity of this product mixture was detected (Supplementary Data Table 1), but did not quite reach the threshold that we used to select substrates for round 2. The $Rh_2(tfa)_4$-catalysed reaction of **3** in CH_2Cl_2 (from round 2; screened at a total product concentration of 1 μM), and the $Rh_2(OAc)_4$-catalysed reaction of **13** in CH_2Cl_2 (from round 3; screened at a total product concentration of 100 nM), gave the oxindoles **25** (yield: >92%; EC_{50} = 2.4 ± 0.5 μM) and **26** (yield: 85%; EC_{50} = 12.0 ± 0.3 μM) respectively: in each case, the activity of the product mixture was low (Supplementary Data Tables 2 and 3), reflecting the expected activity of these compounds at the concentrations screened. Thus, in addition to the active β- and γ-lactams (**17-19**) prepared from **1**, **3** and **15**, significant chemical space accessible to the substrates was thus explored, yet ultimately discarded, in the search for synthetically-accessible, bioactive compounds.

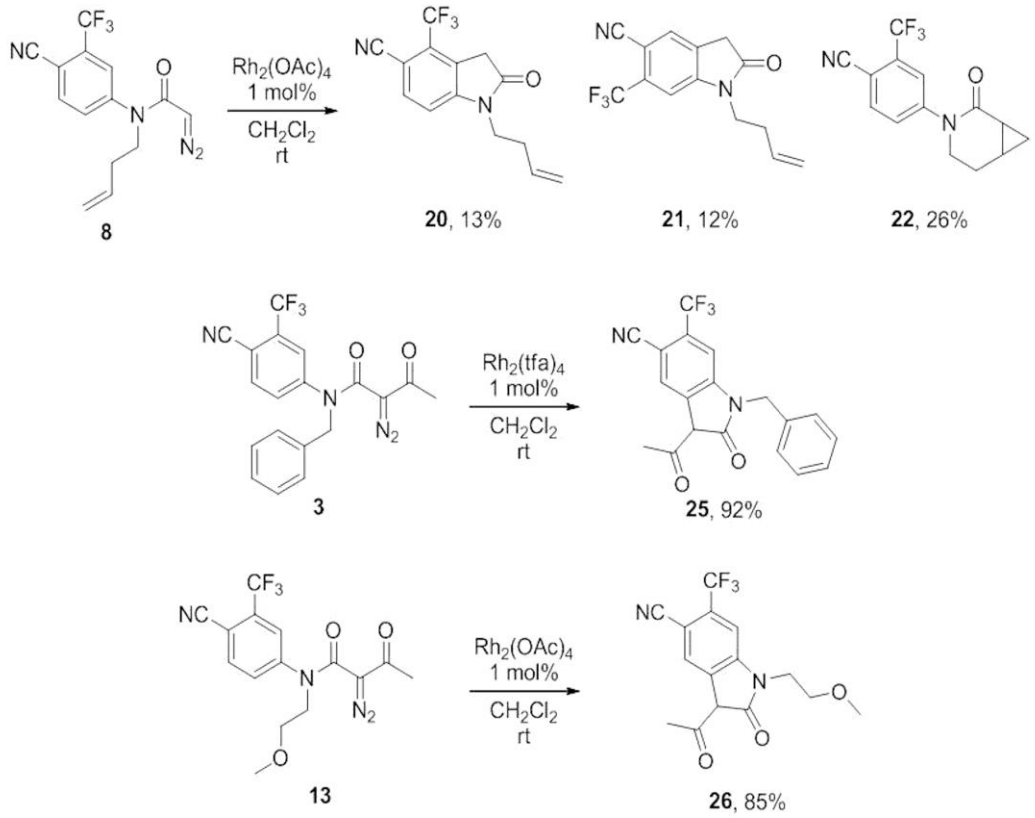


Figure 5: Alternative products accessible from the α-diazo amides **8**, **3** and **13** explored respectively in rounds one, two and three. The chemical space occupied by products – the oxindoles **20**, **21**, **25** and **26** and the δ-lactam **22** – was explored yet ultimately discarded in the search for bioactive, synthetically accessible compounds.

We analysed the impact of screening at ten-fold lower concentration in each round of activity-directed synthesis. Thus, we retrospectively assayed the product mixtures from rounds 2 and 3 at the total product concentrations used in the previous round (i.e. 10 μM and 1 μM respectively) (compare Panels B and C, Figure 3 with Panels b_1 and c_1, Supplementary Data Fig. 8). At the lower concentration, the dynamic range of the assay was greater, improving confidence that the most promising reactions were taken forward, either to feed into round 3 (from round 2) or for scale-up (from round 3).

The screening of mixtures has been previously adopted in discovery programmes,[35] and approaches to help identify the active components have been developed.[36] However, in activity-directed synthesis, the activity observed would

likely be influenced not only by the activities of the individual components, but also by their yield. It was therefore expected that the approach would allow the identification of reactions in which a substrate was steered towards the most active product. To test this, and to understand the basis of the discovery of the most active ligand **17**, we correlated retrospectively the activity of the product mixtures derived from **3** with its yield (Supplementary Data Fig. 9 and Supplementary Data Tables 6 and 7). In round 2, a low level of activity was observed for most product mixtures at total product concentration 1 μM, usually because the reaction had been steered towards the poorly active oxindole **25** (EC_{50} = 2.4 ± 0.5 μM) (Supplementary Data Fig. 8). However, under specific reaction conditions, a high level of activity was observed which correlated with a high yield of the β-lactam **17** (EC_{50} = 340 ± 30 nM). The optimisation of the yield of active components (such as **17**) greatly facilitated their purification and structural elucidation.

In addition to optimizing the yield of active products, activity-directed synthesis also enabled the discovery of structurally-related bioactive small molecules. The reactions performed in round 3 were inspired by the most promising substrates, catalysts and solvents used in round 2. Two of the reactions selected for scale-up involved the substrate **15** that had been first introduced in round 3, and both of these reactions gave the active β-lactam **19** in good yield (78% and 70%, Table 1 and Supplementary Data Table 4). The discovery of this bioactive molecule thus stemmed from a reaction in round 2 had yielded the structurally-related β-lactam (**19**) (Supplementary Data Table 4).

Activity-directed synthesis was exploited in the discovery of bioactive small molecules by development of a fragment. Overall, a directed approach enabled the discovery of three bioactive small molecules with sub-micromolar activity: the β-lactam **17** (EC_{50} = 340 nM), the γ-lactam **18** (EC_{50} = 470 nM) and the β-lactam **19** (EC_{50} = 440 nM). In each case, it was simple to identify the most active

component from the product mixture, a process that was greatly facilitated by the emergence of a high-yielding synthesis (highest yield of **17**: 75% from **3**; highest yield of **18**: 90% from **1**; highest yield of **19**: 78% from **15**). Notably, the optimal catalyst – $Rh_2(esp)_2$ – for the synthesis of the most active ligands (**17**-**19**) was designed rationally to catalyse C-H aminations,[37] and would unlikely have been exploited in a deliberate synthesis of β- or γ-lactams.

Activity-directed synthesis is broadly analogous to the evolution of a biosynthetic pathway because bioactive small molecules emerge in tandem with routes for their synthesis. Because the approach was underpinned by reactions with alternative outcomes, the discovery of bioactive compounds based on two distinct scaffolds was possible. Remarkably, neither of these chemotypes had previously annotated activity against the androgen receptor. In addition to metal-catalysed reactions of diazo compounds, it is likely that many other reactions that have alternative possible outcomes – such as metal- and organocatalysed inter- and intramolecular reactions – may also have value in activity-directed synthesis. By liberating chemists to exploit the most powerful transformations at their disposal, we anticipate that this highly general approach could be applied in the discovery of novel and diverse chemotypes with a very broad range of biological functions.

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Supplementary Information statement

Supplementary information is linked to the online version of the paper at www.nature.com/nature.

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Author contribution statement

A. N. and S. W. conceived, designed and supervised the project. G. K. undertook the experimental work. A. N., S. W. and G. K. analysed the results and wrote the paper.

Author information

The authors have no competing financial interests.

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