Miniaturization of Popular Reactions from the Medicinal Chemists' Toolbox for Ultrahigh-Throughput Experimentation

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Abstract. Miniaturization is a tactic employed in many technologies to accelerate discovery and enable new applications such as systems-level evaluation. The miniaturization of chemical synthesis to the limits of chemoanalytical and bioanalytical limits of detection could accelerate drug discovery by increasing the amount of experimental data collected per milligram of material consumed. Here we demonstrate the miniaturization of popular reactions used in drug discovery such as reductive amination, *N*-alkylation, *N*-Boc deprotection and Suzuki coupling for utilization in 1.2 µL reaction droplets. Reaction methods were evolved to perform in high-boiling solvents at room temperature, enabling the diversification of precious starting materials, such as the complex natural product staurosporine.

Chemical space exploration in drug discovery generally requires access to many molecules with diverse physicochemical properties.[1–3] A suite of trustworthy reactions – namely amide coupling, Suzuki coupling, heteroatom arylation such as Buchwald-Hartwig coupling, heteroatom alkylation by reductive amination, and *N*-Boc deprotection – have emerged as reactions of choice for pharmaceutical chemical space exploration (Figure 1).[1,4–6] These reactions are the workhorses of medicinal chemistry and allow many chemical analogs to be simultaneously prepared, either manually or automated in arrays of glass vials,[7,8] in wellplates,[6,9,10] or in flow.[11–13] Inspired by Moore's Law of transistor miniaturization,[14] label-free reaction miniaturization has emerged as a technology with considerable promise to enable systems-level studies of the seemingly infinite chemical and reaction condition space.[15] With reaction miniaturization, less starting material is needed to produce more reaction data, and the acquisition of each experimental data point requires less laboratory real estate, reaction vessel volume, and produces less hazardous waste. This optimization increases the overall efficiency and "greenness" of the exploration process.[16] Based on miniaturization principles developed to enable combinatorial chemistry and high-throughput experimentation (HTE) on a 24, 96 or 384 reaction array,[16,17] ultrahigh-throughput experimentation (ultraHTE)[18] was developed to perform reactions in 1,536 wellplates,[9,10,19,20] giving increased freedom to survey reacting substrates and reaction conditions in concert. However, while reaction miniaturization may bolster the synthesis of bioactive compound libraries[10] or the systems-level analysis of reaction performance,[18] few reactions are designed with "above-the-arrow" reaction conditions that favor miniaturization. It is therefore critical to develop easily miniaturized and automatable method variations of the most popular synthetic reactions.

The robotic liquid handlers typically used in low-volume reagent doing rely on involatile, uniform reagent stock solutions with low viscosity. Thus, high-throughput reaction methodology typically favors high boiling solvents that fully dissolve all reaction components. In ultraHTE,[18] a typical reaction volume is ~1 µL, and solvents and reagents generally have a low enough vapor pressure that they can be handled without significant evaporation during experiment setup. It is possible to handle volatile reagents or use volatile solvents, by using liquid handlers that can rapidly deliver 384 reagent doses at a time,[20] or by employing a large dead volume of reagent stock solution or running reactions at high dilution, typically in flow systems.[21] However, increasing dead

volume unnecessarily consumes precious starting materials, and reaction dilution is often detrimental to reaction kinetics. Therefore, the use of high boiling solvents facilitates reaction miniaturization; it is likewise a remit of green chemistry.[22,23] Solubility is also a key consideration in automated dosing. Techniques utilized in lower throughput reaction arrays to handle heterogenous reagent mixtures, such as slurry loading,[15] present significant engineering challenges during miniaturization. Therefore, selecting solvents that create homogenous reagent mixtures ensures precise liquid-handling for reliable and consistent results. Miniaturization of homogenous reactions that run to completion at room temperature is also desirable to avoid issues related to heat transfer, mass transfer and surface effects on insoluble particles, making analysis and eventual scale-up of such reactions straightforward.[24] Furthermore, reaction mixtures must be compatible with plastics that are routinely used as labware consumables on liquid handling robots – typically high-density polyethylene (HDPE), polypropylene (PP) and cyclic octane copolymer (COC). For these reasons, the initial development of nanoscale synthesis was predicated on fully homogenous reactions that could be run at room temperature in high boiling solvents like *N*-methylpyrrolidine (NMP), *N,N*-dimethylacetamide (DMAc) or dimethylsulfoxide (DMSO).[9,10] With this background we embarked on the miniaturization of popular reactions by identifying reaction conditions that would operate at room temperature, in high boiling solvents, in plastic labware.

We targeted the most frequently used reactions in medicinal chemistry (Figure 1)[1,4–6] for translation to the nanomole scale format. Building on our previous studies, which establish viable conditions for amide coupling[10] and C–N coupling[9,10,19] in a miniaturized library format, we turned our attention to automation friendly variations of Suzuki coupling, reductive amination, *N*-alkylation and Boc-deprotection. This menu of reactions would cover two-thirds of those currently used by medicinal chemists and vastly accelerate the exploration of chemical space, particularly through the merger of chemical synthesis to bioassays.[10]

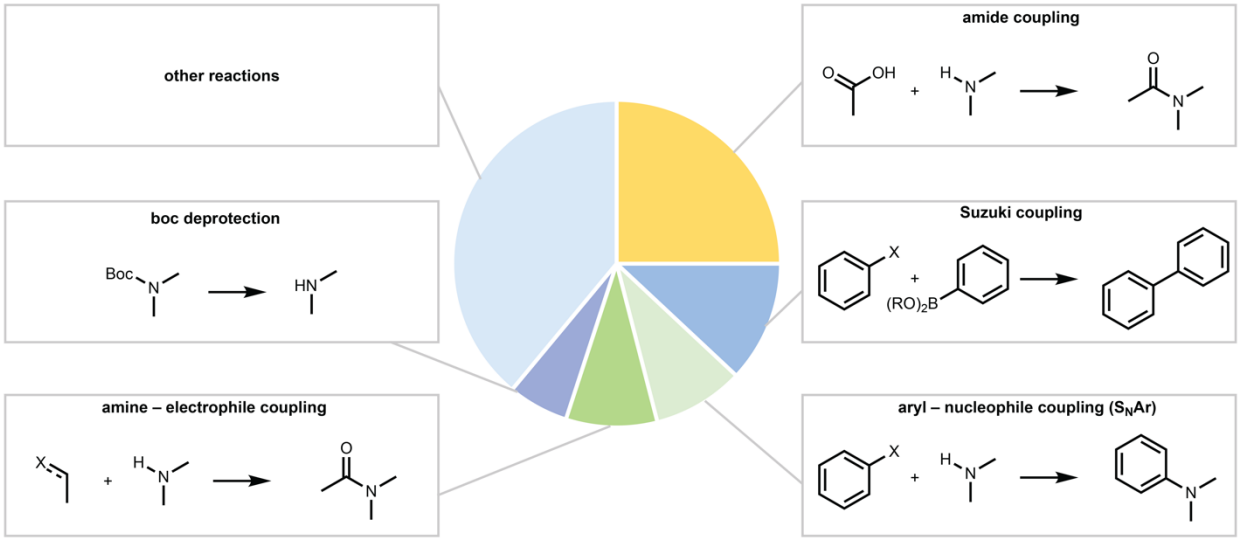


Figure 1. Popularity of common reactions in the synthesis of pharmaceuticals. Data collected from Ref. 5.

Given our earlier success in the miniaturization of Pd-catalyzed C–N coupling using soluble superbases,[9,10] we initiated our studies by targeting the Suzuki coupling – the second-most frequently used reaction in drug discovery. Our development of a Suzuki coupling protocol for ultraHTE was inspired by prior reports detailing the nanoscale use of Buchwald precatalysts with soluble organic bases.[9,25] We were particularly interested in studying Suzuki coupling to heterocyclic bromides **19** and **20** for our reported kinase inhibitor campaign,[10] as the biaryl coupling of these substrates was unsuccessful under previously reported nanoscale Suzuki conditions.[9] The XPhos G2 or G3 Buchwald precatalyst is known to readily couple aryl halides at room temperature; however, the preferred solvent for this reaction is THF,[26] which is too volatile to use in the nanoscale format and incompatible with the COC wellplates we first employed.[27] Additionally, aqueous K_3PO_4 is used as the base in these reactions. While mixtures of THF and aqueous K_3PO_4 form a biphasic mixture, we considered that aqueous K_3PO_4 may form a monophasic mixture or well-dispersed suspension in a polar aprotic solvent such

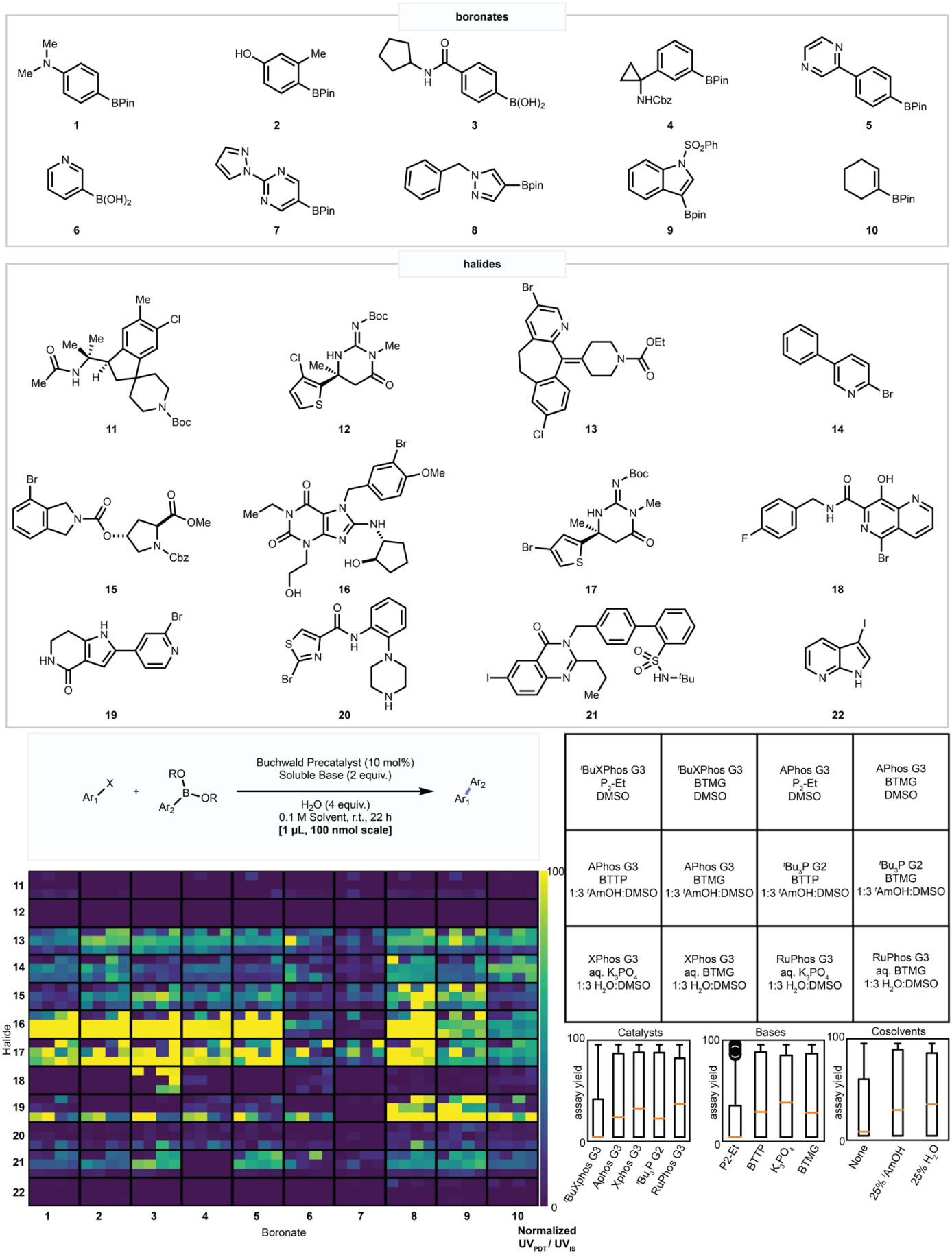


Figure 2. Nanoscale ultraHTE Suzuki coupling reaction performance; (top) boronates (**1**–**10**) and halides (**11**–**22**) used in the study; (bottom left) heatmap of conversion to product as determined by UPLC-MS relative to an internal standard; (bottom right) box-plots comparing the effects of base, cosolvent and catalyst on reaction performance.

as DMSO. We interrogated three solvent systems that contained water or an alcohol as a cosolvent, given the importance of hydroxyl groups as activators in Suzuki coupling.[28] Reactions using XPhos G3 or RuPhos G3 were dosed with an aqueous solution of K_3PO_4 or BTMG and were ran in 3:1 DMSO-water, whereas reactions with ^{t}BuXPhos G3, APhos G3 or ^{t}Bu$_3$P G2 were dosed with P_2-Et or BTMG, with four equivalents of water, and ran in 100% DMSO or 25% ^{t}AmOH in DMSO. These twelve reaction conditions were tested on eight previously reported Informer Halides (**11–13, 15–18** and **21**),[29] two simpler heterocyclic halides (**14** and **22**), and bromides **19** and **20** (Figure 2).[10] A corresponding array of boronates (**1**–**10**) included four diverse Informer Boronates[29,30] and six boronates of interest to our affinity ranking studies.[10] All 1,440 reactions (12 halides × 10 boronates × 12 reaction conditions) were performed in ultraHTE format in 1,536 wellplates and their performance was evaluated by UPLC-MS conversion to product compared to an internal standard.

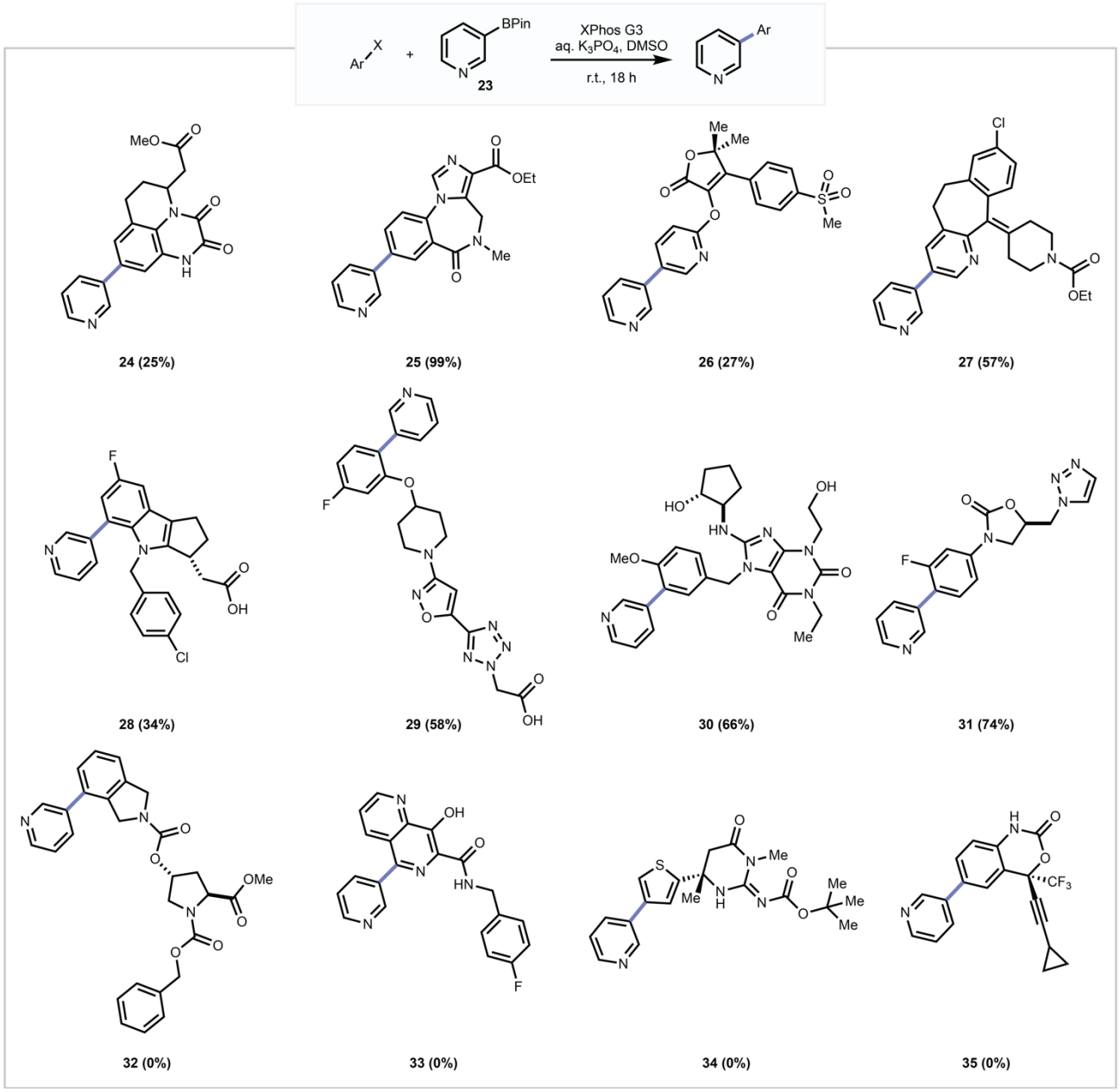
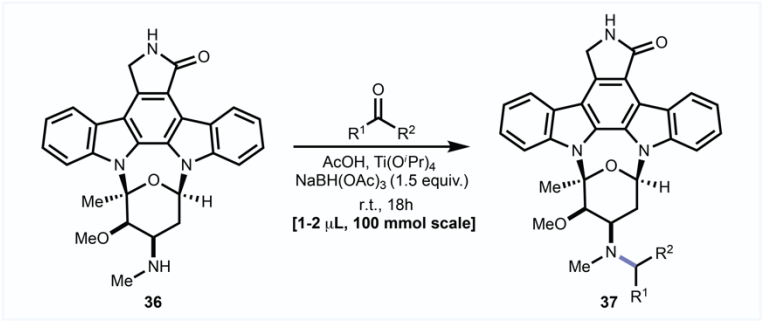


Figure 3. Selected examples of coupling Informer Library halides to 3-boronopyridine (**23**) on 50 µmol scale using automation friendly room temperature conditions (XPhos G3, aq. K_3PO_4, DMSO) optimized above. Yields are of purified products.

By surveying reaction space in this systems-level format, several trends emerge. For instance, analysis of average reaction performance against various reaction parameters reveals that K_3PO_4 is a preferred base, with BTTP and BTMG having comparable but slightly lower performance, and the previously reported P_2Et[9,25] being

the least successful in these Suzuki couplings. Additionally, anhydrous DMSO was considerably less productive than DMSO with ^{t}AmOH or water as cosolvent, which was anticipated.[28] In terms of the phosphine ligand, ^{t}BuXPhos did not perform well in our study, whereas APhos, $^{t}Bu_3P$, RuPhos G3 and XPhos all gave acceptable catalysis. Given these data, we chose XPhos with aqueous K_3PO_4 in DMSO as the ideal conditions for subsequent coupling studies. To test reaction performance on a more traditional reaction scale, a variety of high complexity halides from an Informer Library[29] were coupled to 3-boronopyridine (**23**) on a 50 µmol scale at room temperature (Figure 3). Of the 12 high complexity halides employed, eight produced the coupled product (**24**–**31**) in greater than 25% using XPhos with aqueous K_3PO_4 in 1:3 water:DMSO. We believe substrate decomposition contributed to the failure of **32**–**35**. Nonetheless, given the complexity of the substrates explored, we believe that the use of XPhos G3 with aqueous K3PO4 in DMSO are viable room temperature reaction conditions for automated library synthesis in pharmaceutical chemical space.

Reductive amination is another of the most popular transformations used in medicinal chemistry.[5,6] A common set of room temperature reaction conditions employs $NaBH(OAc)_3$[31] and acetic acid in 1,2-dichloroethane (DCE) or MeCN,[32] while DMAc, another popular solvent,[33] could be used for miniaturized synthesis. While reductive amination has been a popular choice for library synthesis in drug discovery on the milligram scale,[6,34] we set our ambitions on the microgram-scale diversification of the precious natural product staurosporine **36**. A preliminary study on the reductive amination of **36** with aldehydes and ketones revealed that $Ti(O^{i}Pr)_4$, a common additive,[35–37] was key to achieving high conversion. We therefore investigated the reductive amination of **36** with eight aldehydes and eight ketones (see Supplementary Information for structures) on the nanoscale across 48 reaction conditions (768 total reactions) to give analogues of type **37**, examining solvent, concentration, and reagent loading simultaneously (Figure 4). We found on average that between 3–5 equivalents AcOH and 2–3 equivalents of $Ti(O^{i}Pr)_4$ gave the best conversion across the panel of substrates tested, particularly using NMP as the solvent, in which staurosporine was observed to be more soluble than in DMAc. Given that homogeneous reactions are much more amenable to miniaturization, we proceeded with NMP as solvent for our next study. Ketones showed low relative conversion across the range of reaction conditions tested with amine **36**.



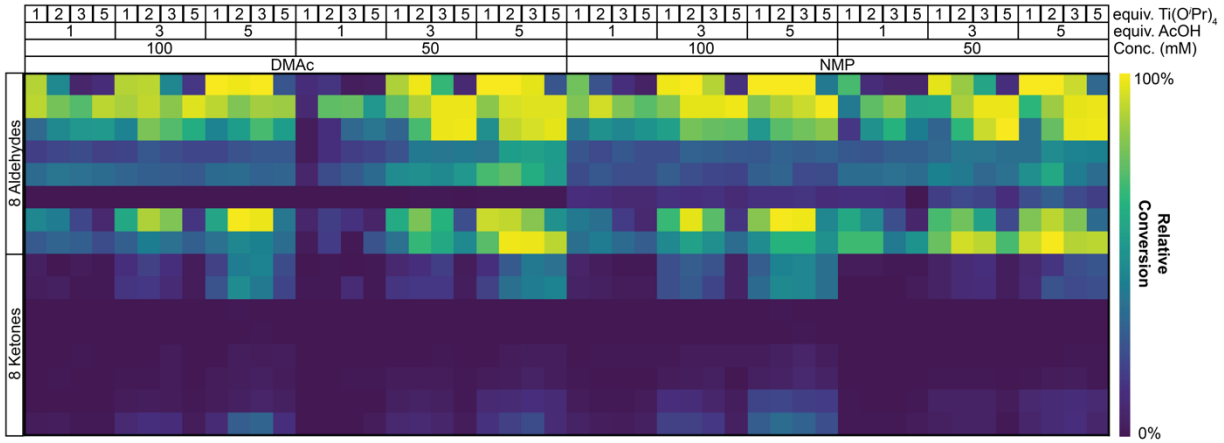


Figure 4. Nanoscale reaction optimization for the reductive amination of staurosporine **36** with 8 aldehydes, 8 ketones and 32 reaction conditions. Relative percentage conversion was determined by UPLC-MS.

Having explored many reaction conditions on few substrates, we next explored few reaction conditions on many substrates to build a library of staurosporine analogues through reductive amination with 48 diverse aldehydes (Figure 5). We selected eight conditions from our previous screen for the study [2 concentrations × 2 AcOH loadings × 2 $Ti(O^iPr)_4$ loadings × aldehydes] giving a total of 384 reactions. Of the 48 analogues targeted, 46 were successfully synthesized and detected by LCMS. Selected reactions were repeated on a 50 µmol scale and isolated yields of products (**39**–**44** and **S1**) varied from 38–89%, with some of the lower yields attributed to observed instabilities of the purified products upon solvent removal.

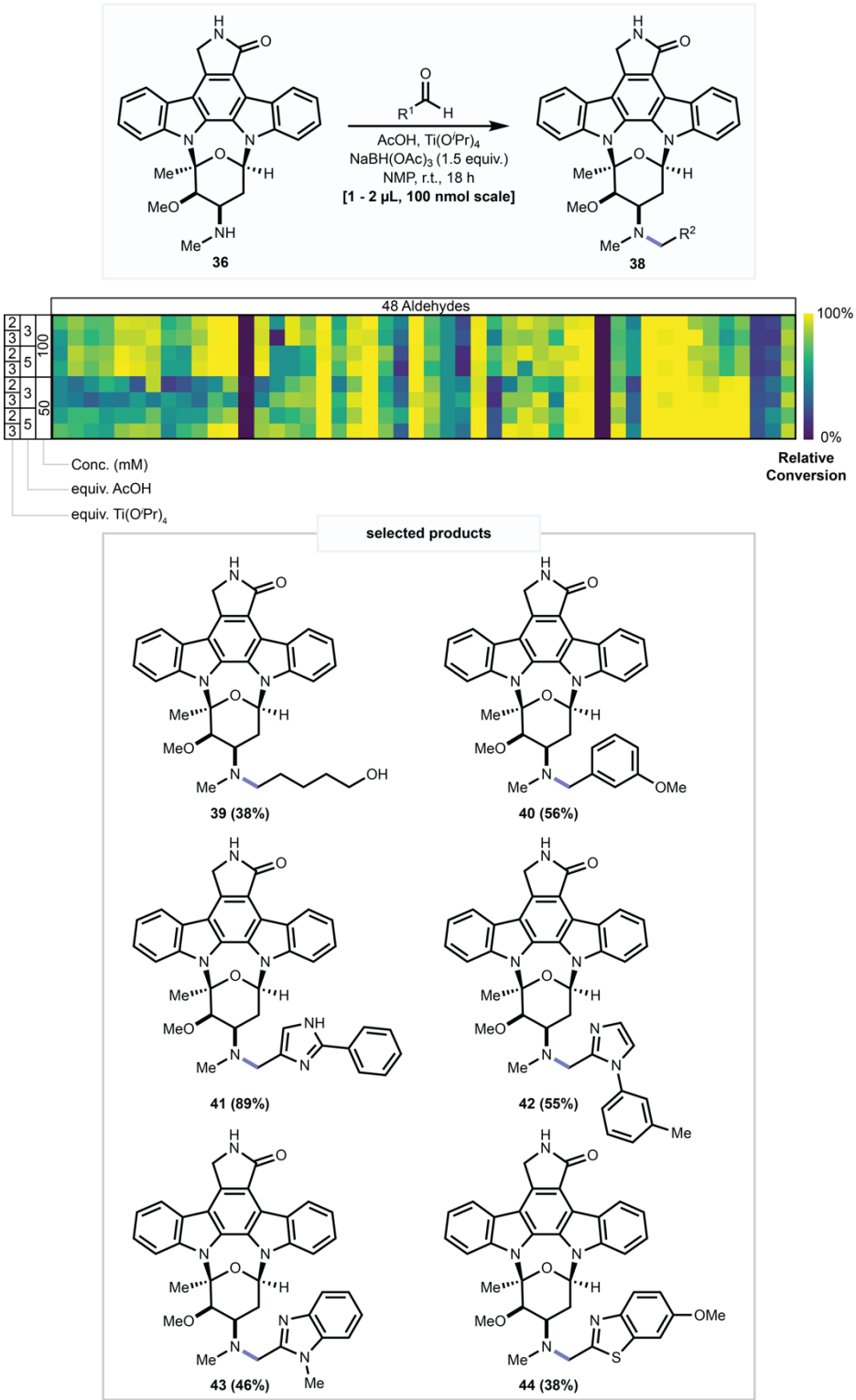


Figure 5. **Nanoscale staurosporine analog library synthesis via reductive amination.** (top) Heatmap of conversion to product for the reductive amination of staurosporine **36** with 48 aldehydes using 8 reaction conditions. Relative conversion was determined by UPLC-MS; (bottom) selected reactions were repeated on 50 µmol scale giving **37**–**42**. Yields are of purified products.

A pinnacle of nanoscale chemistry operations will be the advent of multistep synthesis. We demonstrate here a simple entry to multistep nanoscale synthesis in the form of a selective *N*-alkylation and *in situ* Boc-deprotection. Base promoted *N*-alkylations serve as a complementary approach to reductive amination. The *N*-alkylation of amide **45** with 12 primary, secondary, allyl and benzyl halides in the presence of 4 organic and organometallic bases was achieved on a 200 nmol scale (Figure 6). The products of these reactions are potent MK2 inhibitors.[38–40] The regioselective alkylation of the less nucleophilic amide *N*-atom was made possible through Boc-protection of the more nucleophilic piperazinyl nitrogen.

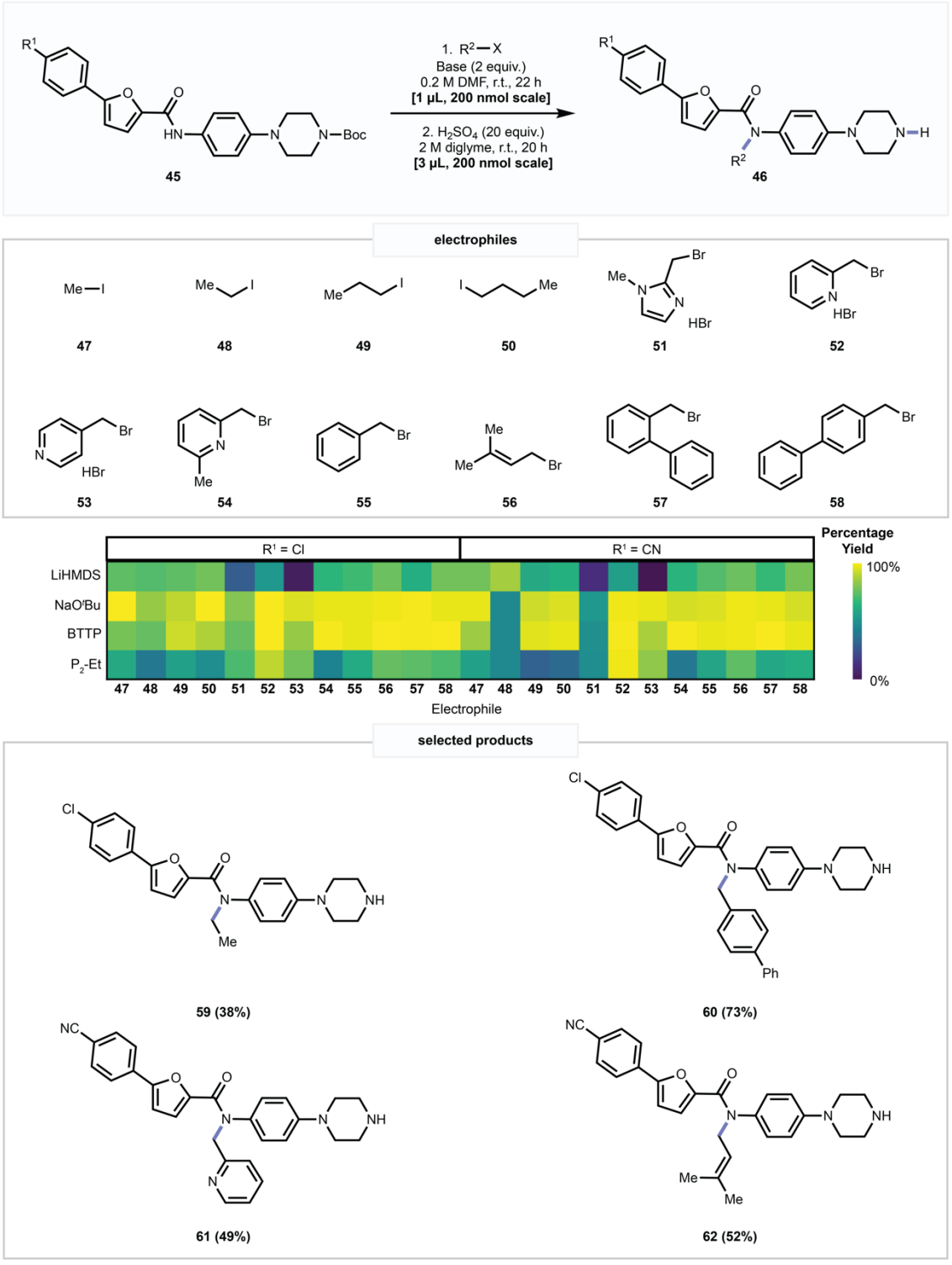


Figure 6. Two-step nanoscale synthesis of MK2-inhibitor analogs of type **46** via *N*-alkylation followed by Boc-deprotection; (top) Electrophiles **47**–**58** were coupled to **45**; (middle) heatmap of reaction performance determined by UPLC-MS relative to standard curves created from isolated products; (bottom) selected examples repeated on 40 µmol scale, Yields are of purified products.

The deprotection of Boc-groups is the fifth most popular reaction used in drug discovery (Figure 1),[5] and thus represents another critical addition to the miniaturized synthetic chemistry toolbox. Following a survey of acids, we found that quenching reactions with sulfuric acid smoothly effected deprotection giving MK2-inhibitor analogs of type **46**. While trifluoroacetic acid (TFA) was also a viable acid, our reactions were performed in an inert atmosphere glovebox on a robotic system each containing sophisticated metal components, so we wanted to avoid the use of volatile acids like TFA and selected H_2SO_4 as a non-volatile alternative.[41] The partnering of base-promoted *N*-alkylation with *in situ* deprotection marked our first successful operation including more than one reaction operation in the ultraHTE format. From a screen of two-step reaction conditions we identified NaO^{t}Bu as being the most productive base for the first step across the electrophile library (**47**–**58**). Therefore, we applied optimal conditions of NaO^{t}Bu in DMF followed by H_2SO_4 in diglyme to the multi-step synthesis of the 24 target compounds on the microscale, successfully isolating 21 products in yields of 12–73% (see Supplementary Information). Four such alkylated-deprotected compounds (**59**–**62**) are shown in Figure 6.

Chemical space exploration in drug discovery was a motivator in the development of the nanoscale synthesis platform.[9] It is therefore important that the favorite reactions from the medicinal chemist's toolbox are available in this miniaturized and automated format. This report showcases key principles required to pursue reactions on the nanoscale effectively by performing homogeneous reactions, in high boiling solvents, at room temperature upon a selection of biologically relevant substrates. As this miniaturized chemistry technology develops, there will be significant chemical and engineering challenges to overcome so that chemists can incorporate the full breadth of modern chemical reactions and achieve further integration with bioassays.[10] We envision that large virtual compound libraries could be enumerated and scored using chemoinformatics to satisfy diverse objectives, then selected compounds would be synthesized by matching each substrate pair to productive reaction conditions using the HTE technology described herein. Such systems-level investigations of bioactive chemical space with chemical synthesis can only be realized through effective reaction miniaturization.

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