Multistep Library Synthesis in Flow: Production of Matrix Libraries in an Assembly Line

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ABSTRACT

In drug discovery, automated library synthesis has traditionally focussed on established chemistry such as amide formation or Suzuki coupling. However, to explore a broader chemical space, recent automated protocols have emphasized on increasing the fraction of sp^3 hybridized carbons to move beyond the limitations of planar molecules. Herein we present a novel approach for multistep library synthesis in continuous flow, enabling the linkage of three different elements in a combinatorial way. To achieve this synthesis, we employed nine different reactions in various combinations. Initially, we constructed a full combinatorial library, followed by the development of multi-transformation libraries where disparate reactions were integrated into a single experimental setup, prioritizing chemistries capable of introducing $C(sp^3)$ fragments while maintaining efficiency. By integrating diverse reactions and optimization capabilities, our method offers a powerful means to expand chemical diversity in drug discovery libraries.

Introduction

Organic synthesis is pivotal in the multidisciplinary process of drug discovery.[1-3] In recent years, there has been a surge in interest among pharmaceutical companies in rapidly generating analogue libraries, leading to the emergence of automated synthesis protocols as a promising approach to accelerate medicinal chemistry programs.[4-6] Initially, these automation platforms primarily targeted the "big five" reactions, including amide formation, Suzuki coupling, nucleophilic aromatic substitution, sulfonylation, reductive amination, and alkylation.[7-9] Recently, novel automated protocols have been developed to expand the available chemical space, with a strong focus on preparing compounds with higher C(sp^3) fractions.[10] This has been achieved through various strategies, such as updating underused traditional cross-coupling[11] or exploring novel radical photoredox couplings.[12-17]

While most automated library synthesis methods are based on focused library approaches, allowing exploration of a selected vector[8-10,18], there has been limited description of multistep automated approaches capable of preparing a diverse range of compounds by handling different transformations.[19] Multistep synthesis enables the opportunity to combine various elements in a single exploration or apply diversity-oriented synthesis (DOS) strategies, facilitating the preparation of structurally diverse compounds in a single experiment.[20,21] The majority of these multistep approaches are flow-based and have been applied to the preparation of selected active pharmaceutical ingredient (API) and a small set of close analogues.[22-25] Three primary approaches have been employed for multistep synthesis: linear approaches, where elements are added in line to construct the target molecules, cyclic approaches, where elements are added sequentially, and radial approaches around a central switching station that combines non-simultaneous individual reactions for molecule construction (Fig. 1).[26,27]

The integration of flow chemistry for modular multistep synthesis with library synthesis presents an intriguing prospect for the rapid generation of analogues in a combinatorial manner, thereby unlocking new possibilities in Drug Discovery. Drawing an analogy to car

manufacturing, where various components are connected in an automated assembly line to construct the final car, we envision leveraging flow chemistry to combine different elements in a modular line. This approach would enable the synthesis of combinatorial libraries in an assembly line fashion (Fig. 1). The productivity of this automated approach could be assessed by measuring the number of products obtained per unit of time.

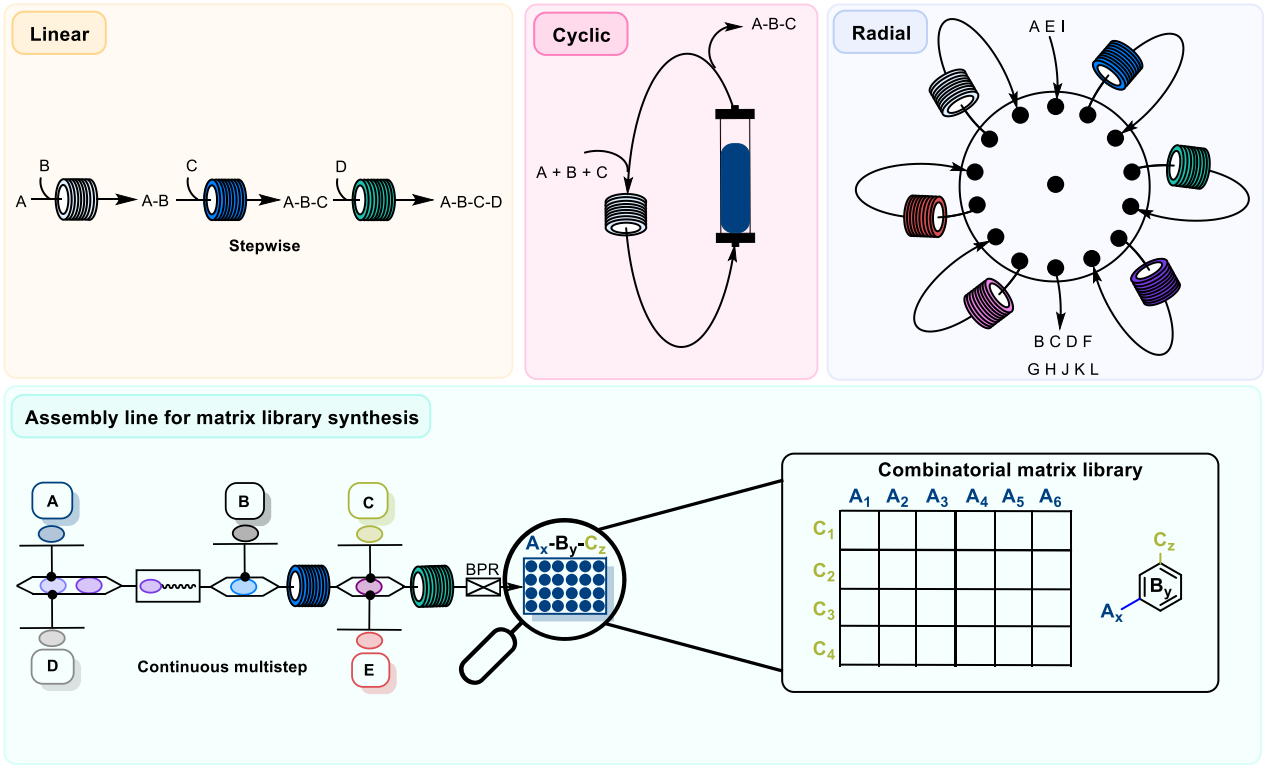

Figure 1, Previous work described for automated synthesis. Linear: Stepwise synthesis with intermediate purification. Cyclic, which is the synthesis of biopolymers such as peptide synthesis. Radial: combination of cyclic and linear synthesis approaches where flow modules surrounding a central core require minimal equipment while retaining maximum synthetic versatility. Assembly line: the current approach where a system resembling an assembly line produces molecules and large combinatorial matrix libraries.

Results and discussion

To investigate this approach, we opted for the Asia flow system from Syrris, featuring 6 injection ports that enable the addition of up to six different reactants or reagents in line under nitrogen atmosphere.[28] These ports are paired with chip or tube reactors capable of facilitating thermal reactions. The instrument enables the execution of multiple reactions in line through volumetric calculations, facilitating the addition of reactants or reagents at the appropriate times to enable the modular synthesis, resembling an assembly line.

The concept of adding new reagents into reaction slugs was initially pioneered by Dr. Djuric and co-workers at Abbott Laboratories (now AbbVie) using Conjure™ from Accendo Corporation.[29-31] These seminal studies showcased the potential of plug flow approaches for library synthesis, albeit with limitations in the diversity of chemistry that could be combined in line. Subsequent research led by Prof. Jamison demonstrated the capability of modular flow to facilitate assembly line-like approaches for the preparation of pyrazole cores, with application in API synthesis.[32]

Our objective was to integrate elements from both approaches within a single reactor to facilitate the preparation of combinatorial libraries, thereby accessing a broad area of chemical space in a single run. This approach enables the exploration of different substitution patterns, functionalities, distances and angles. Its potential applications in medicinal chemistry are significant, allowing for the rapid establishment of the structure-activity relationships (SAR), including additivity effects of different components in bioactive compounds, facilitating connectivity in fragment-based drug design[33], and preparing libraries for proteolysis targeting chimeras (PROTAC's).[34]

To validate our synthesis approach, we first conducted experiments to determine the suitable carrier solvent for performing sequential reactions. Drawing from the pioneering work of Dr. Djuric, we explored various perfluorinated solvents and injected slugs containing suitable colorant in a mixture of THF and DMF to assess cross-contamination. We observed a wetting effect on the surface of chip and tube reactors, resulting in carryover into the next slug.[35] Similar behaviour was observed when higher alkanes were employed as carrier solvent. However, when DMF was used as carrier solvent, no carryover effect was observed, and dispersion could be effectively controlled by the system.

Building on this discovery, we selected two well-established reactions commonly employed in our group: amide formation from esters with Lithium hexamethyldisilazane (LiHMDS)[36] and Negishi coupling[11] as the initial combination to be attempted as combinatorial

library approach. These methodologies were chosen for their ability to combine the use of challenging non-nucleophilic amines for amide formation with a method to introduce alkyl fragments and increase the fraction of $C(sp^3)$ or Fsp^3.[37] To assess cross-contamination using a sequence of these two reactions, we selected 4-amino-2-chloropyridine as the non-nucleophilic amine, while methyl 2-methoxybenzoate and methyl tetrahydro-2*H*-pyran-4-carboxylate were utilized as esters alternatively. The amide formed in line was then coupled with isopropylzinc bromide using $Pd(AcO)_2$ and RuPhos as catalyst. A total of 5 different inlets were utilized to introduce each element. The reactions were conducted without further optimization to evaluate the robustness and reproducibility of the system (SI Fig. S3). From this experiment, no cross-contamination was observed, and product conversions ranged from 42-58%, proving evidence of the reliability of the system (SI Fig. S4).

With the validated system in place, our focus shifted towards the creation of a combinatorial matrix library. Our strategy centred on utilizing Methyl bromobenzoate (Fig. 2) as the central core, strategically incorporating *ortho*, *meta* and *para* substitution to enhance molecular diversity (Core 1, 3 and 4, Fig. 2b). To amplify variation, methyl 6-bromonicotinate (Core 2, Fig. 2b) was included, featuring a nitrogen within the phenyl ring. To modify the left flank of the core, we utilized LiHMDS mediated amide coupling, with four anilines containing electron-withdrawing groups (Aniline 1-4, Fig. 2b). On opposite end, we employed the Negishi coupling, leveraging three distinct organozinc reagents to modify the (hetero) aryl bromide (a-c, Fig. 2b).

Our efforts resulted in the successful synthesis of an array of 48 compounds, of which 46 were successfully isolated. This achievement represents a staggering success rate of 96% translating to a productivity of 4 products per hour with a total run time of 12 h. The isolated amounts obtained would support further biological evaluation of the compounds. This experiment validated the combinatorial approach, demonstrating that molecules can be

synthesized by assembling 3 fragments in a single experiment, despite challenges associated with the use of organometallic reagents in flow.[21,38]

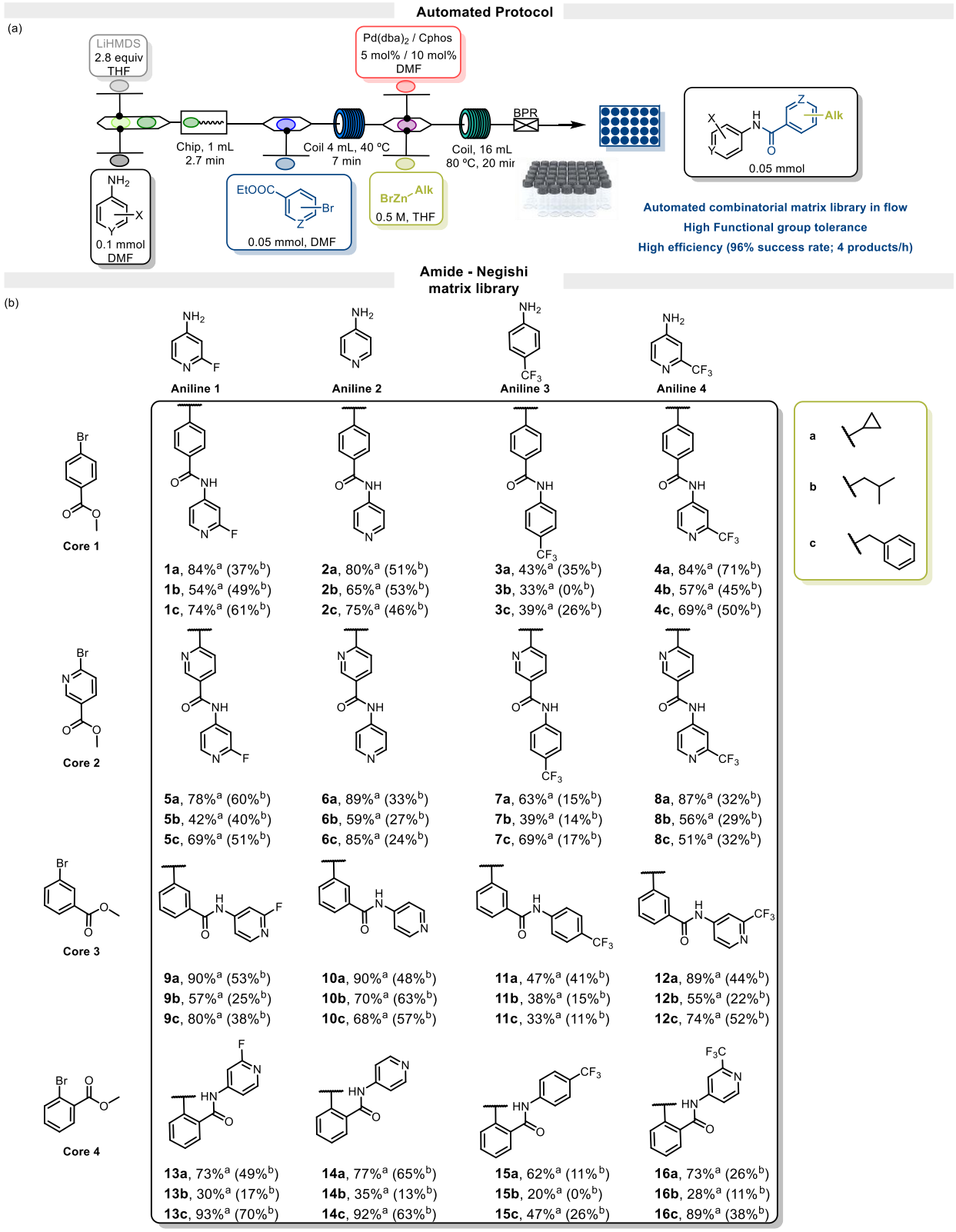

Figure 2, (a) schematic overview and picture of the 6-inlet ASIA system with 6 pumps, a chip reactor, 2 heated coil reactors, back-pressure regulator (BPR) and automated collector. (b) The combinatorial matrix library with 4 anilines, 4 bromo (hetero) aryl esters, and 3 organozinc reagents. [a] LCMS conversion [b] Isolated yield by HTP

Following the successful production of our initial library, we aimed to incorporate a photochemical transformation into the reaction sequence, recognizing the distinct advantages offered by flow chemistry in such reactions.[39,40] In our recent paper, we addressed reproducibility challenges in high-throughput photochemistry by leveraging amino radical transfer (ART) chemistry.[13] Notably, within the scope of our investigation, we discovered the compatibility of this chemistry with free amino alkyl pinacolboranes (amino-Bpins). This discovery offers the prospect of directly functionalizing the amine, thereby unlocking a broad spectrum of possibilities. The envisioned approach involves the ART chemistry in the initial step and subsequently harnessing the nucleophilicity of the free amine in the following step. We deliberated on telescoping the ART reaction with various processes, including amide coupling, reductive amination, alkylation, sulfonylation, nucleophilic aromatic substitution (SnAr), and urea formation with isocyanate.

We began assessing the validation and reproducibility of the newly incorporated ASIA photoreactor under non-optimized conditions, employing the model ART reaction with two distinct alkyl-Bpins: isopropyl and oxetane. Both reactions exhibited exceptional reproducibility, with standard deviations ranging from 0.35-0.46% over 12 and 24 repetitions (SI Fig. S7, S8, S9 and table S3). Additionally, we conducted a comprehensive cross-contamination test, reaffirming our earlier findings, revealing no instances of cross-contamination (SI Fig. S10). To ascertain the system's limits, we examined various injection volumes using two injection ports (SI Fig. S11). Through these experiments, we determined that the minimum injection volume per line is 125 µL, resulting in a total slug of 250 µL of an 0.1 M solution. These findings provide crucial insights into the operational capabilities of the system.

With these parameters in hand, we proceeded to examine the residence time of the ART reaction with the pyrrolidine-Bpin, mindful of the maximum temperature constraint of 25 ºC due to the cryo controller integrated into the photoreactor. Our initial investigation revealed that

a residence time of 60 minutes (SI Table S4) in the first step resulted in complete consumption of the starting material. Notable, the pyrrolidine-Bpin is supplied as a HCl salt, prompting us to explore whether increasing the morpholine equivalents was required to accommodate the salt and would enhance the reaction. However, increasing the morpholine in the mixture showed a slight decrease in conversion (SI Table S5).

With the optimized ART reaction, we proceeded with a direct in-line screening of coupling reagents for the amide coupling. Our analysis identified both Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium (HATU) and 1,1'-carbonyldiimidazole (CDI) as superior coupling reagents for this purpose (SI Fig. S12). To ensure complete conversion towards the amide, we employed 3 equivalents of acid and 6 equivalents of HATU and diisopropylethylamine (DIPEA), accounting for the excess morpholine (2 equiv.) introduced in the previous step.

Having established a fully telescoped in-line sequence, we selected eight different amino-Bpin (1-8, Fig. 3) building blocks to be combined with three acid counterparts, facilitating the execution of a matrix library utilizing the photochemical ART followed by amide coupling (Fig. 3). The library exhibited a commendable success rate of 88%, with 21 out of 24 compounds successfully isolated. **Bpin 4**, displayed no conversion towards the desired product (**27**-**29**), likely due to the unstable nature of the radical via clock reaction.[41] Nonetheless, all the other amino-Bpins demonstrated moderate to good conversions and were isolated successfully.

The total run-time of the library was 24 hours, translating to an average of one product per hour. Main productivity limitation was the fact that the second reaction only commenced after the first one entered the second coil reactor. Productivity could be further enhanced if modulation of the volume of carrier fluid between reaction slugs had been enabled, allowing the execution of more reactions in a single line.

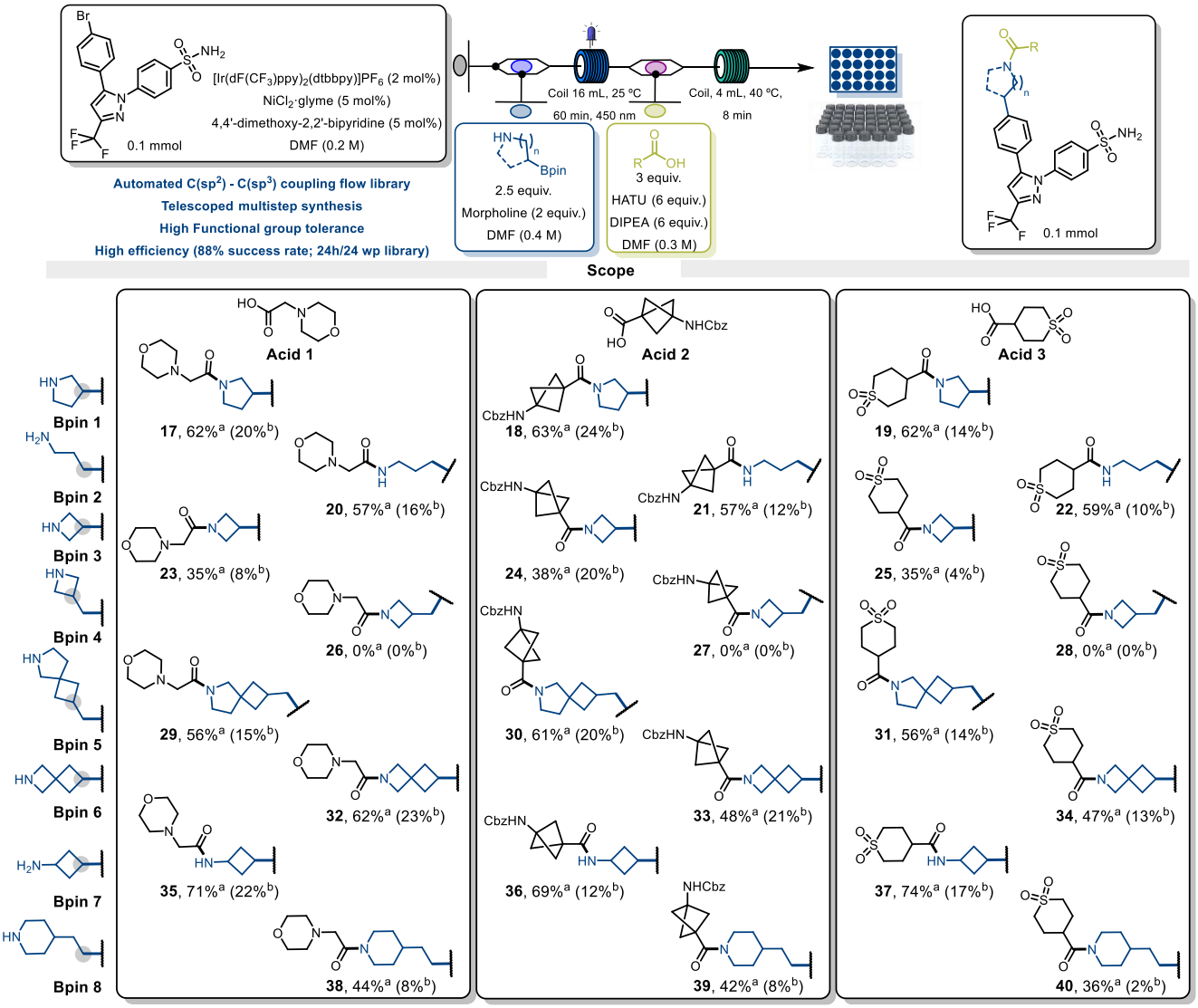

Figure 3, Combinatorial matrix library of ART chemistry telescoped with amide formation with HATU. Where 8 free amine bpins combined with 3 acids resulted in 24 unique products with a success rate of 88%. [a] LCMS conversion [b] Isolated yield by HTP

To expedite the exploration of the chemical space, additional chemistry was conducted following the ART step. Utilizing the exact same setup and reaction conditions, with the addition of one more injection line to introduce either the reducing agent or a base, we focussed initially on reductive amination. Common reducing agents for both aldehydes and ketones were screened directly in flow (SI Fig. S14). Among these agents, only sodium cyanoborohydride demonstrated full conversion to the desired products, providing to be fully soluble and compatible with DMF. To accommodate the diverse chemistry, the coil reactor was maintained at 60 ºC.

The reductive amination achieved full conversion for both aldehydes (Fig. 4, **41, 42**) and ketones (**43-48**). Subsequently, utilizing the same setup and reaction conditions, sulfonylation (**49-54**), alkylation (**55-58**), and nucleophilic aromatic substitution (**59-61**) were tested with DIPEA in the fourth line, demonstrating conversion to the desired product across all the reactions. While the urea formation with the isocyanate (**62-65**) necessitated shifting one line, the setup remained the same 3-line configuration required for amide coupling (**66-68**). We expanded the scope of amides to underscore matched pairs within the library. With this experiment, we have demonstrated the capability to prepare structurally diverse compounds in a single experiment to explore a substantial portion of the chemical space relevant to the SAR within a single experiment. All compounds from the 3 libraries above (Fig. 2-4) were purified by High-Throughput Purification techniques (HTP) using a MS-triggered preparative high-performance LC (HPLC) to deliver 95 compounds in suitable amount to support subsequent biological assays.[42]

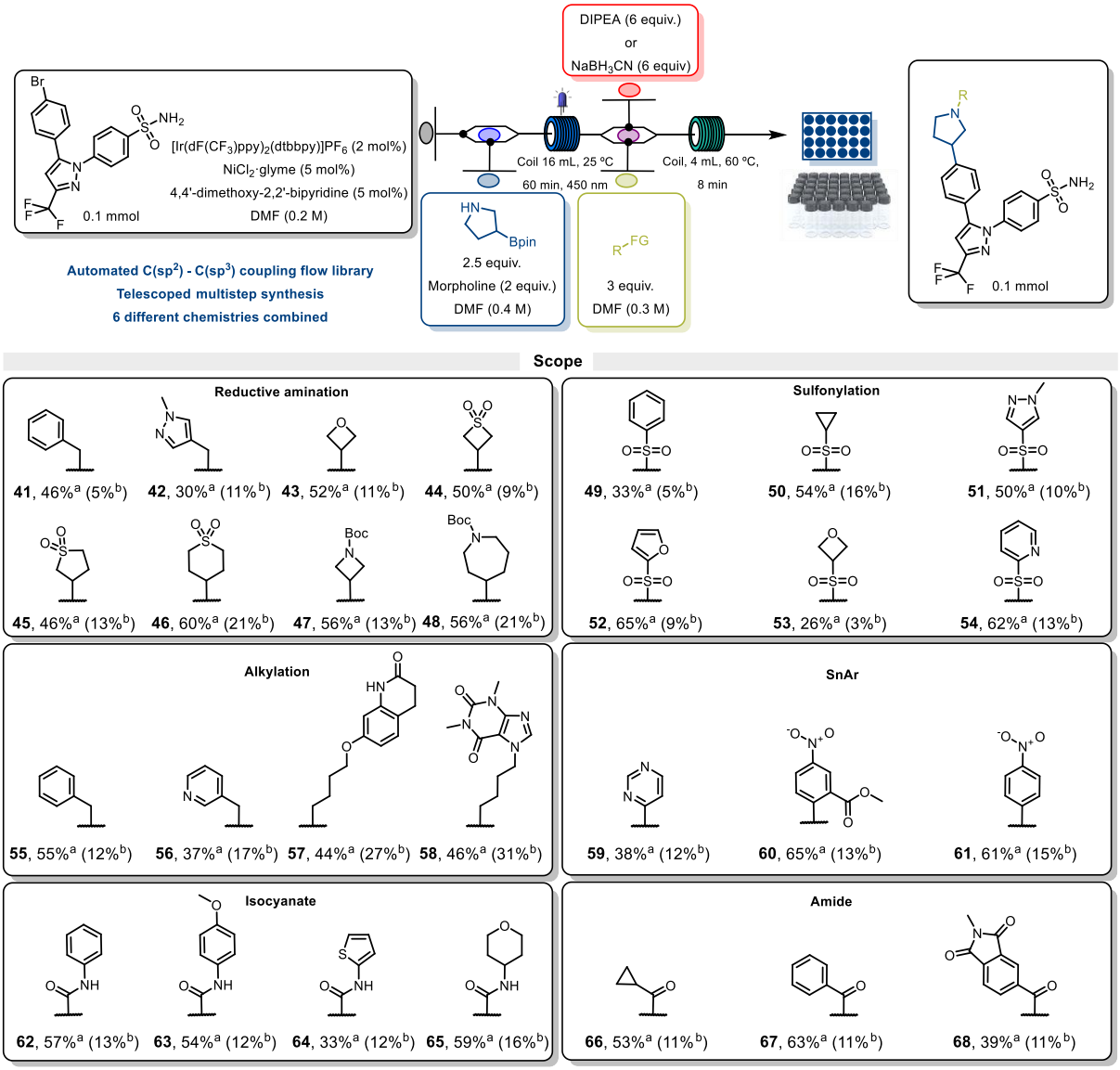

Figure 4, Combinatorial matrix library of ART chemistry telescoped with 6 different chemistries. Where the pyrrolidine bpin was combined with 6 different chemistries to highlight the diverse functional groups that can be introduced in the exact setup. Reductive amination: aldehyde or ketone (3 equiv.) with $NaBH_3CN$ (6 equiv.). Sulphonylation: Sulphonyl chloride and sulphonyl fluoride (3 equiv.) with DIPEA (6 equiv.). Alkylation: alkyl bromide (3 equiv.) with DIPEA (6 equiv.). Nucleophilic aromatic substitution (SnAr): Chloro or Fluoro (hetero)aryl (3 equiv.) with DIPEA (6 equiv.). Urea formation: Isocyanate (3 equiv.). Amide formation: Acid (3 equiv.) with HATU and DIPEA (6 equiv.). [a] LCMS conversion [b] Isolated yield by HTP

Conclusions

In conclusion, this paper presents a robust and versatile method for efficiently producing compounds in an assembly line fashion. This approach offers the flexibility to perform up to 9 different chemistries in the same set up to build a diverse set of compounds in library format by combining different fragments and different reactions in a single experiment. This will allow to modulate various aspects of the molecule, making it highly advantageous for constructing

combinatorial matrix libraries aimed at exploring the entire chemical space relevant to structure-activity relationship (SAR) studies in early drug discovery programs. Through the different experiments performed, we demonstrate the broad applicability, optimizing and showcasing chemistries from amide formation to ART photoredox catalysis. This assembly line approach has clear applications in small molecule drug discovery, PROTAC synthesis and fragment-based drug discovery, areas of research that involve linking fragments, making the versatility of our approach particularly valuable. Future development of this strategy to drug discovery will be matter of future publications.

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Data availability

The authors declare that the data to support the findings of this study are available within the paper and the Supporting Information (SI). Source data are provided with this paper.

Author Contributions

J.A. managed and supervised the project; B.P. lead the project and executed most of the experiments. I.A. performed the initial optimization and cross-contamination studies; S.C., M.L.L, J.M., J.E.G., contributed with significant scientific discussion and suggestions for the direction of the project. E.P. enabled first reaction combination of LiHMDS amide coupling with Negishi. R.R. performed final HTP of compounds. B.C.A. characterized the NMRs of selected compounds. J.A. and B.P. co-wrote the manuscript with input of I.A.. All authors discussed the results and reviewed the manuscript.

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