Automated synthesis of arrays of compounds

1. Rationale/Aim

The discovery of high-quality chemical probes generally requires iterative cycles of compound design, synthesis and evaluation. In the case of fragment-based probe discovery, starting points tend to be low-affinity fragments which have been discovered, for example, by high-throughput protein crystallography. These fragments may be developed into higher affinity compounds by fragment "growth", "merging" or "linking". Historically, the synthesis of designed series of molecules has tended to use the limited toolkit of robust reaction that is widely exploited in medicinal chemical and chemical biology. We have developed a platform for the automated synthesis of series of molecules that enables a broader range of reaction classes to be harnessed, particularly for the direct functionalization ("growth") of fragment hits.

2. Experimental conditions

2.1 Key Requirement:

Substrates, reagents and catalysts:

Substrates, reagents and catalysts for synthetic chemistry, prepared as stock solutions in barcoded vessels; typically, substrates and reagents are prepared as 0.5-2.0 M solutions, whereas sub-stoichiometric components are prepared at lower concentration (e.g. 10-20 mM for a catalyst used at 1 mol% loading).

Instruments and software:

- Automated chemistry robotic synthesizer, housed in a 3m x 1m enclosure, supplied by Zinsser Analytic. The synthesizer includes a solid handling platform (0.7 m wide) and automated chemistry (2 m wide with variable layout to house racks for reagents/stock solutions, reaction blocks and stations for reaction work-up via filtration or liquid-liquid phase separation). A separate module for off-deck execution of arrays of photochemical reactions was also supplied, enabling irradiation at numerous wavelengths (365, 385, 405, 420, 445, 527, 630 nm) at five distinct power levels (in the range 100-

305 mW/well). The software package of Zinsser Analytic comprises three programs with different purposes and capabilities. The “ZALayouter” (v 8.1.0) defines the physical layout of the instrument along with all components and dimensions to ensure that the digitally designed methods can be executed correctly. The “ZADesigner” software (v 8.1.0) is used for writing and/or simulating of synthetic methods, whereas the “ZARunner” module (v 8.1.0) physically executes synthetic methods.

- Waters Acquity H-CLASS UPLC equipped with a photodiode array (PDA) detector, an evaporative light scattering (ELS) detector and a SQD2 mass spectrometer detector with electrospray ionization. The “MassLynx” (v4.2) software comprises numerous sophisticated modules, each controlling specific parts of the system. The “Acquity Console Client” enables the instrument modules to be controlled independently, and the “inlet file” is used to design the chromatographic method by defining specific flow rates and gradient schemes. The specific chromatographic run (comprising file name, injection position, injection volume, chromatographic method, MS method, collection method and triggers) is defined *via* a “sample list”. Alternatively, analytical plates can be submitted and run as full batches utilising the “OA Login” and “OA manager” modules. The “FractionLynx” module controls a microlitre-scale fraction collector.
- Waters Autopurification system comprising a photodiode array (PDA) detector, an evaporative light scattering (ELS) detector and a SQD2 mass spectrometer detector with electrospray ionization. The software is analogous to the MassLynx (v4.2) on the UPLC system. The “fraction file” defines specific thresholds or timed events for collection, while the “sample list” offers multiple separate or combined collection triggers for mass-directed collection or collection via UV/VIS (specific wavelength or PDA) or ELS trace. Independent of triggers, the collection can be precisely initiated following a time program.
- The HTZ Selecta 500_{SX} weighing robot comprises a balance with 0.1 mg accuracy, three slots for fraction tube racks (for 84 tubes per rack) and a barcode scanner. Barcoded tubes are weighed out empty before performing preparative purification. The information about the weight is stored in a tab-delimited text-file linked to the specific barcode (tare). After purification and evaporation of solvents, the fraction tubes are weighed out again, storing the tare, the absolute weight of the fraction tube and the resulting weight of product on an output tab-delimited text-file. Software: CCX-BG run time (v 1.0.22340).
- GeneVac SP Scientific HT-6, 3i series, comprising six rack hangers and adapters for various containers (fraction tubes, shallow/deep well plates etc.). Software v01.11.

- Hamilton STAR liquid handling robot (ML_STAR 4.5.0.5217), comprising three tip carriers for 1000 µL, 300 µL and 50 µL pipette tips, two carriers compliant with Zinsser reaction blocks, shallow or deep well plates, allowing for processing of up to 384 reactions in one method, a solvent vessel carrier and a fraction tube rack carrier compliant with the racks used for the chromatographic systems and the weighing robot. The liquid handler pipette arm contains a channel of eight flexible pipettes as well as a full 96-pipette head to process samples and full plates. Software: Venus software package (v4.5) with the "Hamilton Method Editor" (v4.5.0.7977), which is used to design methods, the "Hamilton Run Control" (v4.5.0.7977) for the advanced execution of methods, and the "Hamilton MethodManager" (v4.5.0.7977), which works as a simplified GUI for less experienced users.

Calibration of quantification by evaporative light scattering detection:

Seven calibration mixes comprised 7-hydroxyethyl theophylline, hydrocortisone, dibenzyl 2,3-dihydroxysuccinate, dibenzyl succinate and dibenzyl phthalate with each compound at 12.5, 10, 7.5, 5, 2.5, 1.0 and 0.5 mg/mL. The calibration mixes were all prepared by weighing on a calibrated 1 decimal place balance and diluted to volume in 10 ml volumetric flasks with dimethylsulfoxide:water (80:20). Where necessary, flasks were immersed in an ultrasonic bath to aid dissolution.

The calibration should be routinely performed twice a year (every six months). 1 µL of each of the seven calibration concentration mixes were injected in triplicate onto the UPLC-MS system and the ELSD peak areas and retention times were manually extracted from the generated results files. Calibration equations were produced in Origin with retention time (minutes) as the X-axis, log ELSD peak area as the Y-axis and log mass concentration as the Z-axis. The calibration surface produced, was described by the following equation (see A. W. Squibb, M. R. Taylor, B. L. Parnas, G. Williams, R. Girdler, P. Waghorn, A. G. Wright and F. S. Pullen, *J. Chrom. A* 2008, **1189**, 101-108):

$$\log(\text{concentration}) = A + B \times \text{retention time} + C \times \log 10(\text{response area}) + D \times \text{retention time}^2 + E \times \text{retention time} \times \log 10(\text{response area}) + F \times (\log 10(\text{response area}))^2$$

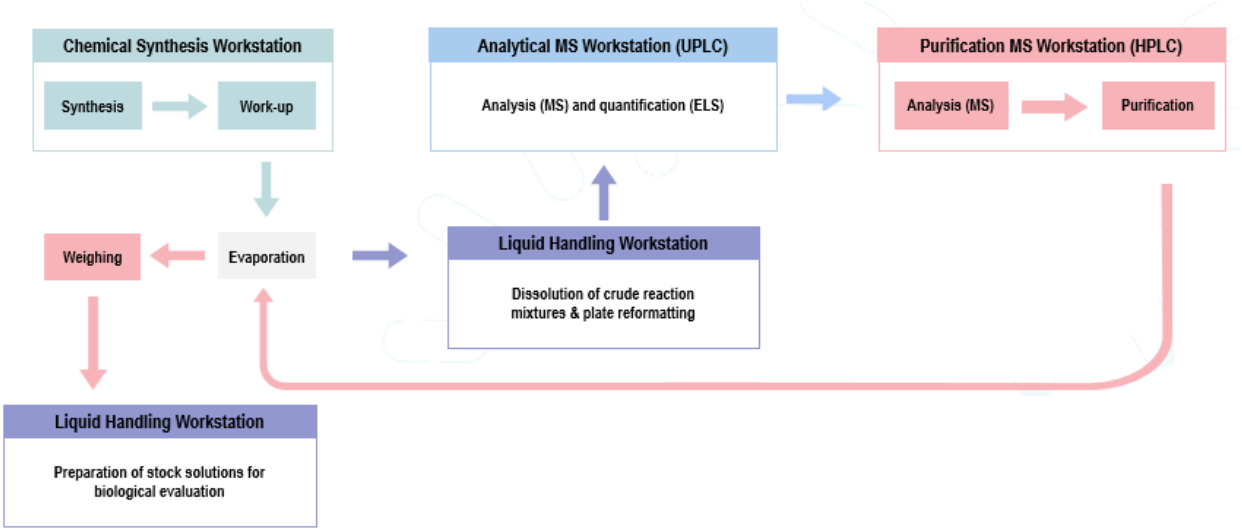
R^2 was calculated as a goodness-of-fit measure ($R^2 = 0.998$)

2.2 Key resources:

Commercially available starting materials were obtained from Sigma−Aldrich, Fluorochem, Acros, Apollo Scientific, Alfa Aesar and Strem. Anhydrous acetonitrile was purchased from Acros. All solvents used in purification were of chromatography or analytical grade.

3. Protocol

3.1 Workflow



3.2 Protocol:

1. Stock solutions of all components needed for automated reactions were prepared and stored in vessels; typically, substrates and reagents were prepared as 0.5-2.0 M solutions, whereas sub-stoichiometric components are prepared at lower concentration (e.g. 10-20 mM for a catalyst used at 1 mol% loading). For a reaction array used to exemplify the platform (that harnessed photoredox-catalysed dehydrogenative coupling reactions), the concentrations of the stock solutions were: hetarene substrates (0.5M in acetone); hydrogen donor co-substrates (2M in acetone); oxidants

(*tert*-butyl peroxyacetate or ammonium persulfate) (1M in acetone); acids (*p*-toluenesulfonic acid or trifluoroacetic acid) (1.2 M in acetone); photocatalyst (11.2 mM in acetone).

2. Three worklists were prepared to execute each individual array. The "Reagent Master" excel file is used to define barcodes for all substrate- and reagent stock solutions used (Zinsser method, index, SMILES, *M*, exact mass, stock concentration, solvent). The "Reagent Current" excel file delineates the physical position of each of the defined barcodes on the Zinsser deck. The "Reaction worklist" tab-delimited text-file defines the specific combinations of components to be explored in the array.
3. Reactions were typically performed on 100-500 µL scale in glass vials in 96-well plate format by sequential addition of components to vials. For a reaction array used to exemplify the platform, the stock solutions of the appropriate hetarene, co-substrate, acid and oxidant were added sequentially to vials. In general, reactions were executed on the robotic synthesis platform. However, photochemical reactions were subsequently executed off-deck in a bespoke module (for a photochemical array used to exemplify the platform, each well was irradiated at λ_{max} = 385 nm and 150 mW per well).
4. Depending on the used solvent system or the need for an aqueous work-up, the crude reaction mixtures were evaporated in the parallel evaporator. Alternatively, crude reaction mixtures were directly analysed and purified.
5. The evaporated products were redissolved in a fixed volume of DMSO using the liquid handling robot, and analysed by analytical UPLC. The analysis was performed with a positive and negative switching mode using a Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm × 1.7 µm) column and gradient elution with a binary solvent system (MeCN plus 0.1% formic acid and H_2O plus 0.1% formic acid; flow rate: 0.8 mL/min; total run length: 3.5 min). The column oven was set to 45 °C; the photodiode array detector scanned over a wavelength range of 210 to 500 nm; the ELSD nebuilser mode was set to cooling; the drift tube temperature was set to 35 °C; the gas pressure was set to 60 psi; and the ELSD gain was typically set to 200 with the data collection rate set at 40 Hz. The quantity of each of the products formed was estimated using evaporative light-scattering detection (ELSD) without the need for previously-prepared standards (see equation above).
6. Selected reactions, typically those that exceed a threshold yield of product, were purified by mass-directed HPLC. The system was run in positive mode using a Waters XBridge Prep C18 (100 mm × 19 mm × 5 µm) or Waters XBridge Prep C18 (50 mm × 19 mm × 5 µm) column and gradient elution with

a binary solvent system (MeCN plus 0.1% formic acid and H_2O plus 0.1% formic acid). The product fractions were evaporated in the parallel evaporator or lyophilised.

7. The amount of product formed was determined by automated gravimetric analysis of barcoded sample tubes before and after addition/evaporation of purified products.
8. Samples of the prepared products, typically 10 mM solutions in DMSO, were prepared using the liquid handling robot.