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Continuous Flow Synthesis of DABCYL Azo Fluorescent Quenchers

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ABSTRACT: A robust and inherently safer continuous flow process has been developed for the synthesis of DABCYL azo fluorescent quenchers. The conventional diazotization azo coupling sequence was successfully translated from hazardous batch conditions to a controlled multistep flow process, enabling the *in situ* generation and immediate consumption of reactive diazonium intermediates. Systematic optimization of temperature and residence time afforded yields up to 84% and throughputs of 17 g h^{-1} in single-step operation. The integrated two-step continuous system delivered DABCYL **3a** in up to 84% yield with a throughput exceeding 65 g h^{-1} and excellent reproducibility. All DABCYL derivatives exhibited broad visible absorption (418−532 nm) and high molar extinction coefficients (2.4−3.3 × 10^4 M^{-1} cm^{-1}), confirming their suitability as dark quenchers. The process demonstrates enhanced safety and throughput, providing a practical foundation for the potential development of industrial-scale manufacture of specialty DABCYL reagents and related azo compounds.

KEYWORDS: *continuous flow chemistry, process intensification, azo coupling, diazotization, DABCYL, fluorescent quenchers*

INTRODUCTION

Azo compounds represent one of the most versatile and widely utilized families of synthetic organic molecules, with applications extending from photoconductive pigments, paints, and cosmetics to inks, dyes, and color photography.[1−7] Their characteristic −N═N− azo linkage typically connects two aromatic groups, generating strong absorption in the visible region (400−700 nm). This absorption underpins their widespread use in the dye industry, where azo-based colorants account for more than 60% of all synthetic dyes produced globally.[8,9]

In recent years, azo chemistry has evolved beyond its traditional role in coloration to find important applications in chemical biology and molecular diagnostics. In particular, nonfluorescent azo derivatives such as DABCYLs have emerged as effective azo fluorescent quenchers (AFQs) in Förster resonance energy transfer (FRET) systems.[10] In these constructs, the azo chromophore functions as an efficient "on−off" fluorescence switch, suppressing emission from a proximal fluorophore until activation by an enzymatic or chemical trigger restores fluorescence. This mechanism underpins a variety of widely used diagnostic technologies, including real-time PCR and FRET-based nucleic acid detection assays.[11−13]

Among AFQs, the DABCYL family is distinguished by its strong absorption, high quenching efficiency, and structural simplicity, delivering performance comparable to more complex systems such as the Black Hole Quencher (BHQ) series.[14,15] Both DABCYLs and BHQs are azo-based, non-emissive quenchers, but they differ in their photophysical profiles and application space. DABCYLs typically exhibit a narrower absorption spectrum and are most effective in short-range (FRET or contact) quenching, making them particularly well suited to peptide substrates and intramolecular probe designs.[16,17] In contrast, BHQs display broader, higher-intensity absorption and enhanced quenching efficiency, enabling their use in highly sensitive and multiplexed assay formats.[2,3]

Despite these differences, DABCYL derivatives offer several practical advantages, including a readily derivatized scaffold, reliable performance in short-range systems, and extensive incorporation into legacy probes and validated assays, supporting continuity and reproducibility in established workflows.[16,17] Their widespread adoption and commercial significance underscore the need for scalable, efficient, and inherently safe synthetic routes. While our previous work has highlighted the challenges associated with accessing BHQ-type azo quenchers via continuous flow methodologies, often resulting in low yields, the development of a robust, high-yielding continuous flow approach to DABCYL synthesis, as reported herein, represents an important advancement with potential applicability across a broader range of azo compounds.

The synthesis of azo compounds typically follows a two-step sequence involving the diazotization of an aromatic primary amine at low temperature and its subsequent electrophilic coupling with an electron-rich aromatic nucleophile.[6,8,10,11,14,15,18−23] The azo group usually forms *para* to an activating substituent on the coupling partner. Azo coupling reactions can present significant manufacturing challenges due to their use of hazardous diazonium intermediates and exothermic reactions. While direct calorimetric measurements of the heat released specifically during azo coupling have not, to our knowledge, been reported, there are reports of

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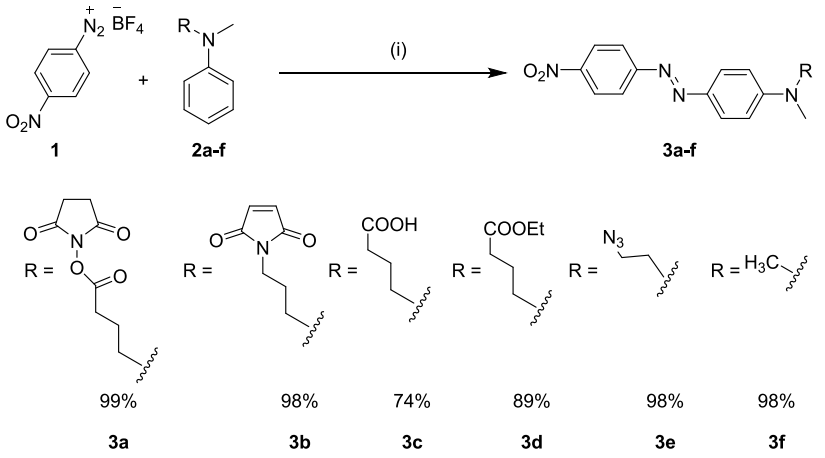


Figure 1. Synthesis of bioconjugatable DABCYL derivatives. (i) Diazonium **1** (0.45 mmol), aniline coupler **2a−e** (0.54 mmol), MeCN, 0 °C, 30 min, procedure adapted from Chevalier et al. and McCormack et al.[2,15].

calorimetric data concerned with the diazotization step and the thermal behavior of the diazonium intermediates. Diazotization reactions are generally exothermic, with reported reaction enthalpies of approximately −110 to −130 kJ mol^{-1}.[22,24] Diazonium salts are known to be intrinsically unstable and decompose exothermically ($\Delta H \approx 215$ J g^{-1}; onset ≈ 27 °C at 2 K min^{-1}).[25] Azo coupling reactions themselves can be considered exothermic as they convert high energy aryl diazonium ions into thermodynamically more stable azo compounds, consistent with thermochemical data for azobenzene derivatives.[25,26] Such exothermic reactions often necessitate strict control of mixing and addition rates to prevent local overheating, side reactions, or decomposition of the diazonium intermediate.[14,15,18−21] Industrial batch processes can suffer from poor heat and mass transfer, leading to gradients in temperature, pH, and reactant concentrations that compromise yield, product quality, and color consistency.[20,21] Moreover, diazonium salts themselves can be hazardous, thermally unstable, and potentially explosive.[6,22,23] Continuous flow processing has emerged as a powerful alternative to conventional batch synthesis, offering enhanced control, safety, and scalability. This is the case for the preparation of azo compounds, particularly in handling hazardous or unstable diazonium salt intermediates safely through on demand generation and immediate consumption. By confining reactive species within small-volume flow channels and enabling precise regulation of parameters such as temperature, pressure, and residence time, continuous flow minimizes the risk of accumulation and uncontrolled decomposition of such intermediates. The inherently high surface area to volume ratio promotes efficient heat and mass transfer, resulting in improved reproducibility and reaction efficiency. Moreover, the modular nature of flow systems facilitates rapid process optimization and straightforward scale up through extended operation or reactor numbering-up, making this technology especially well-suited for the safe and efficient synthesis of complex organic molecules, including azo based quenchers like the DABCYLs.[21−23,27−29]

Our recent study demonstrated the complexity of continuous flow BHQ-2 quencher synthesis and underscored the intrinsic difficulties of azo dye formation, including under flow conditions.[15] Persistent triazene byproduct formation and low overall yields significantly hindered scalability and prevented successful telescoping of the BHQ-2 synthetic sequence. These findings highlight the limitations of BHQ-2 synthesis and emphasize the need to explore alternative azo quenchers, including DABCYL derivatives.

We aim to address these challenges by developing a robust and inherently safer continuous flow process for the synthesis of DABCYL azo quenchers. Particular emphasis was placed on translating the conventional diazotization azo coupling sequence, typically performed under batch conditions and involving hazardous diazonium intermediates, into a controlled flow process that enables *in situ* generation and immediate consumption of the diazonium reactive species. This approach aimed to enhance both operator safety and process reproducibility, while minimizing the risks associated with diazonium accumulation. In parallel, the study sought to optimize reaction conditions such as solvent, temperature, and residence time, and establish a reliable workup method suitable for continuous operation. The broader objective was to develop a selective, and high yielding continuous flow platform for the efficient synthesis of DABCYL derivatives, establishing a foundation for sustainable, industrially viable manufacturing of azo quenchers.

RESULTS AND DISCUSSION

Reaction Development

Initial reactions were first performed in a stepwise batch manner to establish reaction conditions, baseline yields and selectivity, and gather an understanding of solubility and process parameters to be used in the design of a robust continuous flow synthesis. In our study, commercially available 4-nitrobenzenediazonium tetrafluoroborate **1** was coupled with six aniline derivatives **2a−f** to afford the corresponding DABCYLs **3a−f**, Figure 1. The aniline couplers **2a−e** were synthesized as previously described by this group, see Supporting Information.[15] This azo coupling proceeded via a SEAr type reaction between an aryl diazonium salt and the activated (electron rich) aromatic partner.[15] Various workup strategies were evaluated to isolate the DABCYL products efficiently, including solvent evaporation, extraction, and buffer-induced precipitation. Among these, precipitation using sodium acetate buffer (0.1 M, pH 4.0) proved the most effective, providing pure DABCYLs in high isolated yields (Table 1). The products were subsequently fully characterized,

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and the corresponding analytical data are provided in the Supporting Information.

Table 1. DABCYL Batch Synthesis[a]

DABCYL no.	Solvent	Yield
3a	MeCN	99%
3b	MeCN	98%
3b	DMF	96%
3b	Cyrene	85%
3c	MeCN	74%
3d	MeCN	89%
3e	MeCN	98%
3f	MeCN	98%

[a]Reaction time: 30 min; reaction temperature: 0 °C.

While acetonitrile yielded high product conversion, the limited solubility of DABCYL derivatives in this solvent presented a potential limitation for continuous flow adaptation and the development of a process with high throughput.

To overcome the limited solubility of the reaction components in acetonitrile, alternative solvents—DMF and Cyrene—were evaluated for their ability to dissolve both the starting materials and the DABCYL product. These solvents were assessed in the synthesis of DABCYL derivative **3b** and benchmarked against the standard acetonitrile conditions, Table 1.

Use of DMF resulted in excellent solubility for both reactants and product, affording compound **3b** in a high isolated yield of 96%. Encouraged by this performance, we next examined Cyrene, a known greener alternative to DMF. Cyrene also provided good solubility for both substrates and product; however, a reduction in yield (85%) was observed, possibly due to its slightly lower miscibility with the aqueous buffer during workup. Furthermore, Cyrene's higher viscosity raised concerns regarding potential issues under continuous flow operation, such as increased back pressure or inefficient mixing.

The batch reactions established reliable reaction conditions and an efficient workup via buffer induced precipitation. Solvent evaluation confirmed solubility as a critical process parameter and although acetonitrile gave high conversions, its limited ability to dissolve DABCYL products pose challenges for translation to a flow process. In contrast, DMF provided superior solubility and yields, while Cyrene's higher viscosity and lower miscibility introduced practical constraints for continuous flow. Based on these observations, DMF was selected as the preferred solvent for subsequent optimization of a continuous flow synthesis of the DABCYL derivatives.

Photophysical Characterization of DABCYL Derivatives

The photophysical properties of the synthesized DABCYL derivatives were subsequently evaluated, and the data are summarized in Table 2. For several compounds, these measurements are reported here for the first time. UV−vis absorption spectra recorded in acetonitrile (Figure 2) revealed that all DABCYL derivatives display broad absorption bands spanning the 418−532 nm region of the visible spectrum. The absorption maxima (λ_{max}) for the series were centered at 466−481 nm, with full widths at half-maximum ($\Delta\lambda_{1/2}$) of 109−112 nm, consistent with the extended conjugation characteristic of aromatic azo chromophores.

Table 2. Photophysical Properties of Compounds 3a−f[a]

DABCYL no.	λ_{max}	$\varepsilon(M^{-1}\cdot cm^{-1})$	$\Delta\lambda_{1/2max}$
3a	477 nm	32,725	419−529 nm (110 nm)
3b	478 nm	33,220	420−529 nm (109 nm)
3c	481 nm	31,120	422−531 nm (109 nm)
3d	480 nm	32,335	420−532 nm (112 nm)
3e	466 nm	30,085	410−521 nm (110 nm)
3f	478 nm	24,430	418−530 nm (112 nm)

[a]All spectra samples were run at 0.02 mM concentration in MeCN at 25 °C.

All derivatives exhibited high molar extinction coefficients (24,430−33,220 M^{-1} cm^{-1}), indicative of strong light absorption. These properties are particularly advantageous for fluorescence quenching applications, as they enable broad spectral overlap with common fluorophores and facilitate effective nonradiative energy transfer. Importantly, the consistency of absorption profiles across the series confirms that structural variation in the coupling partner did not compromise the chromophoric integrity of the azo. The combination of broad visible absorption, high molar absorptivity, and stability underscores the suitability of these DABCYL derivatives as dark quenchers in optical probe and biosensor applications.

Single Step Azo Coupling Reaction: Continuous Flow Optimization

A continuous-flow protocol for the synthesis of DABCYL azo quenchers was developed using a Vapourtec R-series flow reactor equipped with dual piston pumps, a 1 mL PFA coil reactor (I.D. = 1 mm), and a 6 bar back-pressure regulator (BPR). The coiled reactor was temperature controlled by an insulated glass manifold, and the delivery of reagents was maintained by two independent piston pumps operating at defined flow rates. All reactor components were constructed from PFA to ensure full chemical compatibility. A schematic of the experimental setup is shown in Figure 3.

To enable systematic optimization, the concentrations of 4-nitrobenzenediazonium tetrafluoroborate **1** and the aniline coupling partner **2c** were maintained at 0.1168 M throughout all experiments. This fixed stoichiometry allowed the effects of residence time and temperature on reaction performance to be isolated during a design of experiments (DOE) screening, while minimizing waste and undesired byproduct formation. The DOE design comprised seven experiments (four corner points and three center points) to explore the experimental space and assess reproducibility. The results, summarized in Table 3, present the isolated yields of the target azo compound **3c** under each condition.

An inverse correlation between reaction temperature and yield was observed. Elevated temperatures led to a decrease in isolated yield, most likely due to the thermal instability of diazonium salt **1**. Conversely, higher flow rates (shorter residence times) resulted in improved isolated yields. This trend is consistent with the fast kinetics of the azo coupling reaction and improved micromixing under flow conditions. Rapid coupling and immediate product precipitation resulted in enhanced overall yields. These relationships are illustrated in the response contour plot (Figure 4).

Previous work by the group on BHQ2 type azo systems showed that the methoxy substituent pattern on the aryl ring of the aniline couplers had a significant impact on the azo coupling reaction.[15] When the methoxy substituent was *meta*

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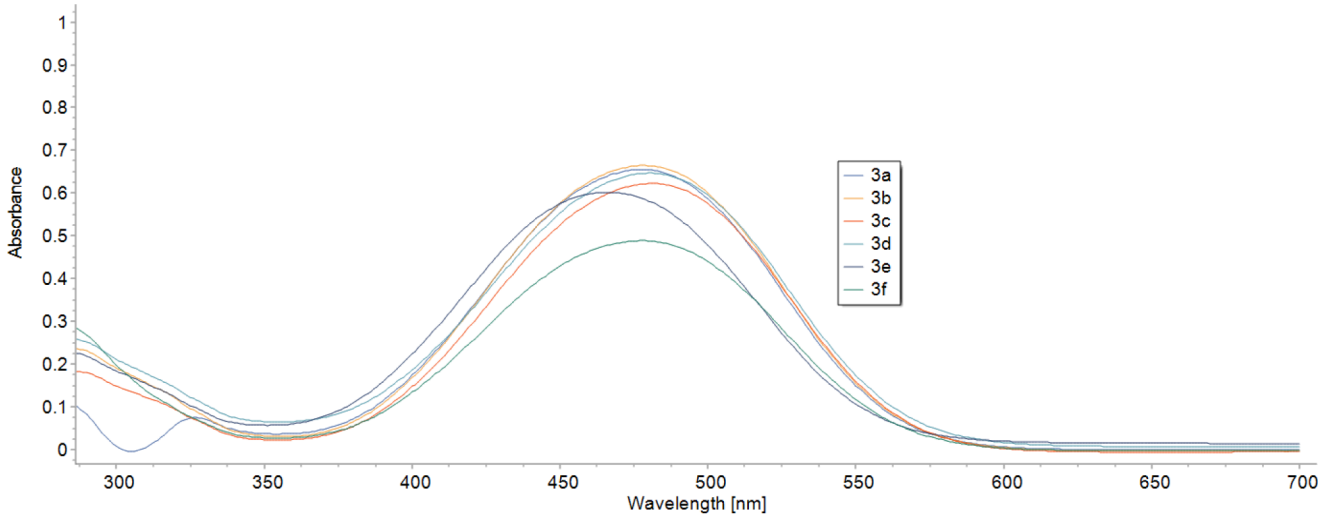


Figure 2. Normalized absorption spectra of bioconjugatable DABCYL derivatives recorded in MeCN at 25 °C.

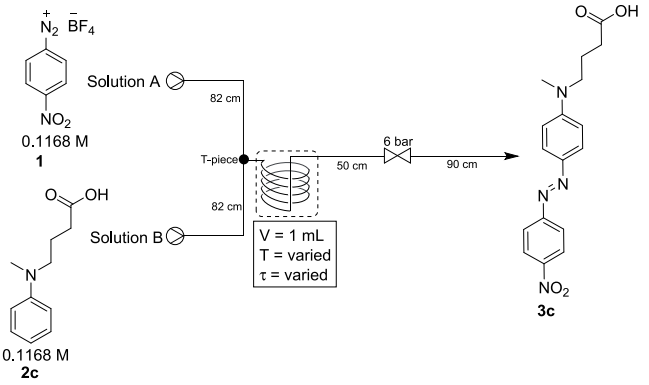


Figure 3. Schematic setup used for the two factor DOE screening of DABCYL **3c**.

to the aniline group it hindered azo formation and resulted in significant amounts of triazene formation from direct nucleophilic attack by the aniline nitrogen. When the methoxy substituent was *ortho* to the aniline group it hindered triazene formation and resulted in higher yields of the azo product.[15] With the DABCYL systems reported herein, there was no methoxy substituent and the presence of triazene product was not recorded.

The highest isolated yield obtained under continuous-flow conditions was 84%, compared with 74% under the corresponding batch process. This 10% improvement underscores the advantages of continuous processing, including enhanced control over reaction parameters, improved thermal management, and increased reproducibility. The superior performance under flow demonstrates the potential for a robust and inherently safer process for the generation and consumption of reactive diazonium intermediates.

Based on the DOE findings, maximum isolated yields were achieved at ambient temperature and minimal residence time. Conducting the reaction at 20 °C provided an energy-efficient and operationally safe approach. The maximum practical flow rate achievable with the current setup was a combined 12 mL min^{-1} (6 mL min^{-1} per reagent stream), corresponding to a residence time of approximately 5 s within the 1 mL reactor coil. These optimized conditions, residence time = 5 s; temperature = 20 °C, were adopted for the subsequent synthesis of the full series of DABCYL derivatives.

With the optimized parameters established, the continuous synthesis of DABCYL derivatives **3a–f** was carried out using the same single-step reactor configuration (Figure 5). Each reaction was conducted in triplicate to evaluate reproducibility and process robustness. Under the optimized conditions, high isolated yields with excellent purity were obtained for all derivatives (Table 4). ^{1}H NMR analysis confirmed the absence of detectable impurities or side products (see Supporting Information).

The process demonstrated impressive productivity, with throughput values ranging from 8.95 g h^{-1} to 17.04 g h^{-1}, depending on the specific derivative. The consistency observed across replicate runs further underscores the reproducibility and reliability of the continuous-flow platform.

Table 3. Two Factor DOE Screening Experimental Conditions and Their Corresponding Results of DABCYL 3c[a]

Experiment no.	Solution A flow rate (mL min^{-1})	Solution B flow rate (mL min^{-1})	Residence time (s)	Residence time (min)	Reactor temperature °C	Isolated yield (%)
1.1	6	6	5	0.083	75	54
1.2	0.325	0.325	92.3	1.538	48	54
1.3	6	6	5	0.083	20	84
1.4	0.166	0.166	180	3	20	64
1.5	0.325	0.325	92.3	1.538	48	50
1.6	0.325	0.325	92.3	1.538	48	51
1.7	0.166	0.166	180	3	75	32

[a]Reactor volume was 1 mL.

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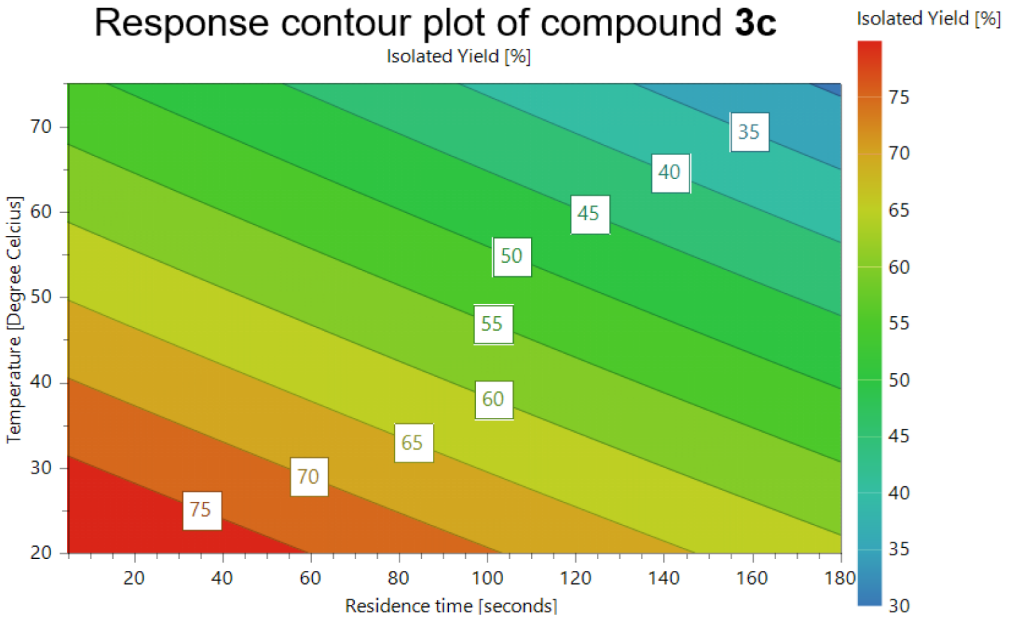


Figure 4. Response contour plot from the DOE screening of compound **3c**, generated from the DOE software MODDE13.

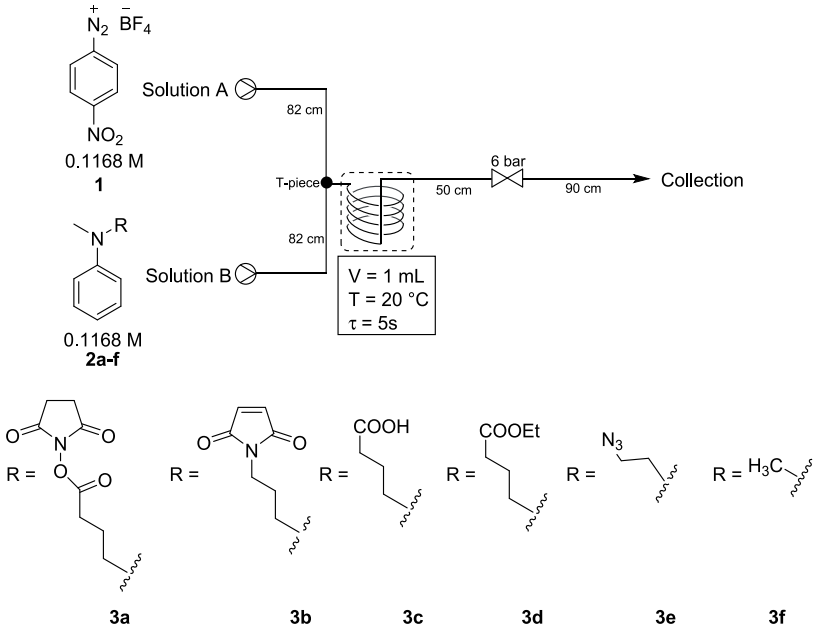


Figure 5. Schematic of experimental setup for the synthesis of DABCYLs **3a–f**. Collection was into vials containing a set volume of NaOAc buffer (0.1 M, pH 4.0), see Supporting Information for more detail.

Table 4. Experimental Conditions and Results for the Synthesis of DABCYLs 3a–f[a]

DABCYL no.	Isolated yield (%) $n = 3$	Average throughput (g h^{-1})
3a	92 ± 1%	17.04
3b	90 ± 2%	14.82
3c	91 ± 2%	13.104
3d	82 ± 1%	12.83
3e	91 ± 1%	12.48
3f	79 ± 2%	8.95

[a]*Solution A flow rate: 6 mL min^{-1}, Solution B flow rate: 6 mL min^{-1}, reactor volume: 1 mL, residence time: 5 s, reactor temperature, 20 °C.

Multistep DABCYL Synthesis: Continuous Flow Optimization

Following the success of the single-step continuous-flow synthesis, the next objective was to transition to a multistep continuous-flow process in which the 4-nitrobenzenediazonium salt **1** would be generated *in situ* and immediately consumed in the subsequent azo coupling reaction. This approach was designed to enable the safe production and instantaneous utilization of the reactive diazonium intermediate, while maintaining high yields, efficient throughput, and excellent product purity. Because diazonium generation is inherently hazardous and has been linked to runaway reactions in industrial batch settings, it was critical to develop a

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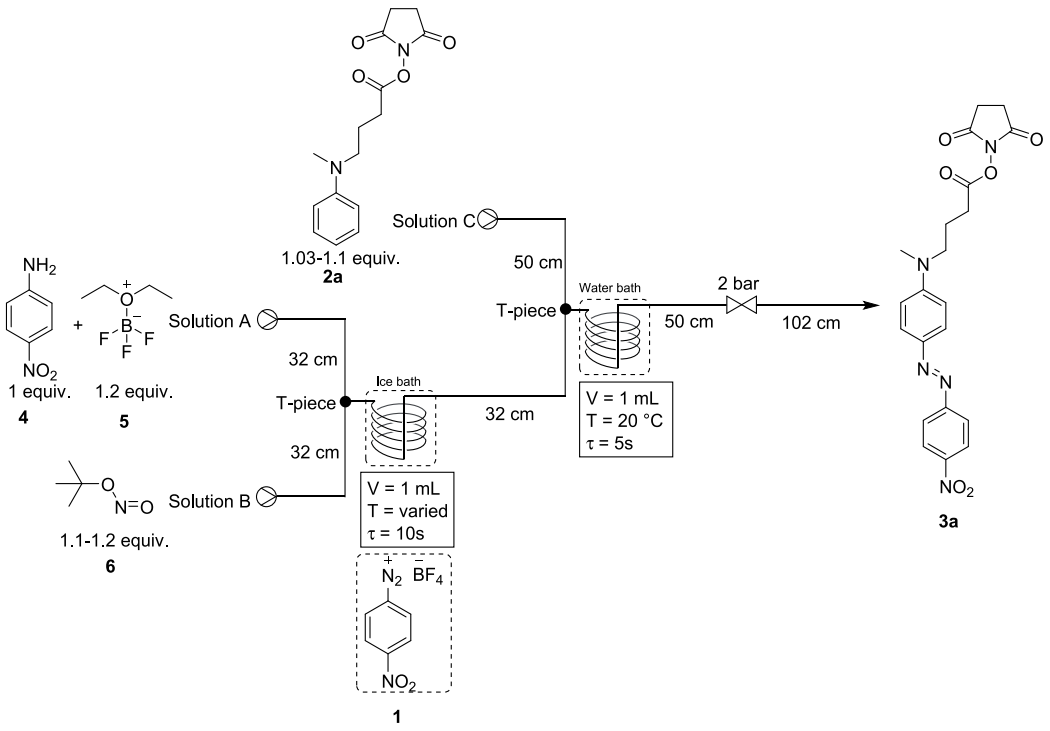


Figure 6. Schematic setup of the multistep continuous flow synthesis of DABCYL **3a**. Collection was into vials containing a set volume of NaOAc buffer (0.1 M, pH 4.0), see Supporting Information for more detail.

Table 5. Experimental Conditions and Results for the Multistep Continuous Flow Synthesis of DABCYL 3a[a]

Experiment no.	1	2	3	4
Diazotization Reactor temperature (°C)	0	20	0	0
Azo coupling Reactor temperature (°C)	20	20	20	20
Solution A concentration (M)	0.2257	0.2257	0.7008	1.168
Solution B concentration (M)	0.2467	0.2467	0.841	1.402
Solution C concentration (M)	0.1168	0.1285	0.3854	0.6424
Isolated yield (%) $n = 3$	83 ± 2%	84 ± 3%	79 ± 3%	74 ± 1%
Average throughput (g h^{-1})	15.0	15.2	43.8	68.3

[a]Solution A flow rate: 3 mL min^{-1}, Solution B flow rate: 3 mL min^{-1}, Solution C flow rate: 6 mL min^{-1}, diazotization reactor volume: 1 mL, azo-coupling reactor volume: 1 mL, diazotization residence time: 10 s, azo-coupling residence time: 5 s.

controlled and inherently safer process for diazonium handling under flow conditions.[30,31]

DABCYL **3a** was chosen as the model compound for the multistep continuous-flow study. This derivative exhibited excellent photophysical characteristics and high yield in the single-step flow synthesis, making it an ideal candidate for the multistep process optimization. The residence time for the azo coupling step (second step) was kept at 5 s, corresponding to a total flow rate of 12 mL min^{-1}, consistent with the optimized single-step process. To achieve a 12 mL min^{-1} azo coupling flow rate, the diazotization first step was set at a total flow rate of 6 mL min^{-1}, with the residence time controlled via reactor volume adjustment.

Literature precedent on diazonium generation under flow conditions indicated that at this scale, a residence time of 10 s is typically sufficient for complete conversion, while maintaining the reaction below 20 °C minimizes thermal decomposition and side reactions.[23,27,31,32] Accordingly, the diazotization step was carried out in a 1 mL PFA coil reactor (I.D. = 1 mm) maintained at 0−10 °C to provide a balance between reactivity and stability of the diazonium intermediate.

The multistep flow system comprised two Vapourtec SF-10 peristaltic pumps connected to the diazotization coil (1 mL, PFA, I.D. = 1 mm), which was temperature controlled in an ice bath, coupled directly to a Vapourtec R-series reactor for the azo coupling step (1 mL, PFA, I.D. = 1 mm) maintained at 20 °C in a water bath. A schematic of the multistep setup is shown in Figure 6.

The reaction conditions and results for four triplicate experiments evaluating the continuous multistep synthesis of **3a** are shown in Table 5, Experiments 1 and 2.

At 0 °C (Experiment 1), the telescoped two-step sequence afforded 83% isolated yield with a small standard deviation of 2%, confirming excellent reproducibility. The average throughput across triplicates was 15.0 g h^{-1}. In Experiment 2, the diazotization temperature was increased to 20 °C, and the equivalents of coupling partner **2a** were increased slightly (from 1.03 to 1.10) to ensure complete consumption of the diazonium intermediate. The yield remained high (84%) with a standard deviation of 3%, and the throughput essentially unchanged at 15.2 g h^{-1}. These results confirmed that both low and moderate temperatures provided efficient and reprodu-

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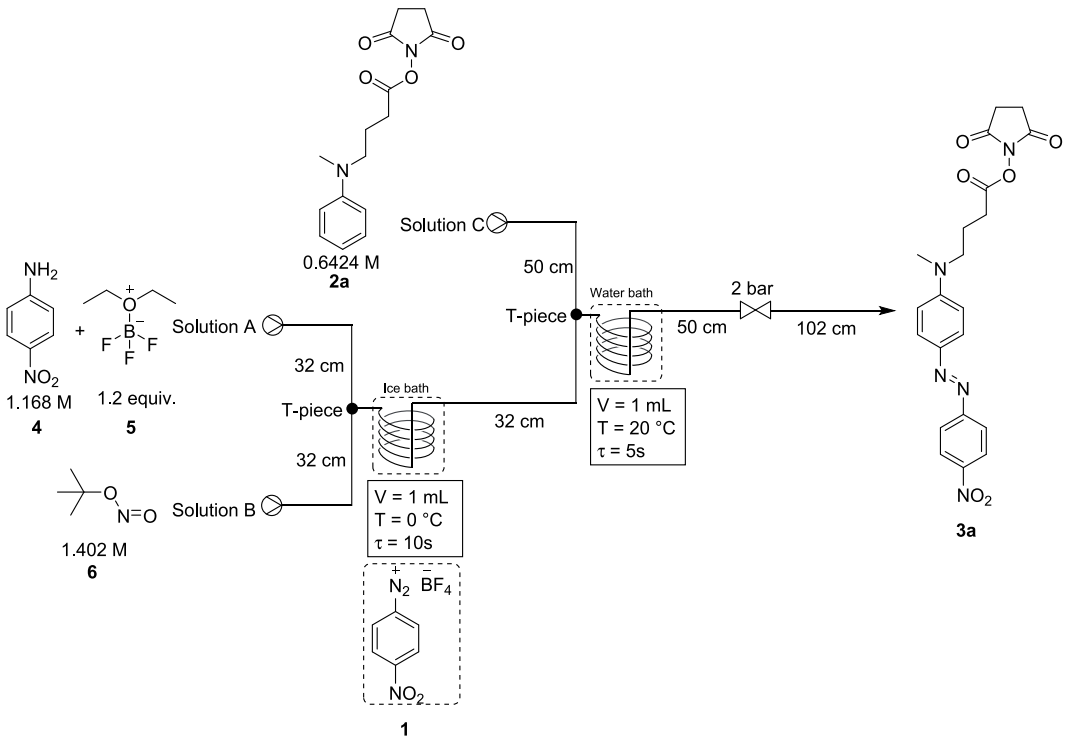


Figure 7. Final optimized intensified multistep continuous flow setup used for the synthesis of compound **3a**.

cible diazonium formation and coupling under continuous operation.

Multistep DABCYL Synthesis: Continuous Flow Intensification

Having established a robust and reproducible multistep process for DABCYL **3a**, attention turned to process intensification to maximize throughput. In Experiment 3 (Table 5), the reagent concentrations were increased to improve productivity. We did not systematically investigate shorter residence times at lower concentrations. Our experimental strategy was instead guided by a process intensification perspective, with a primary focus on maximizing productivity (space time yield) and minimizing solvent usage.

Although all components displayed good solubility in DMF, compound **2a** proved to be the solubility limiting reagent. Under these conditions, a modest reduction in yield to 79% (standard deviation 3%) was observed, likely resulting from mass transfer limitations, localized heating, or less efficient precipitation during workup. Despite this, throughput increased dramatically to 43.8 g h^{-1}, representing nearly a 3-fold improvement relative to Experiments 1 and 2.

Experiment 4 (Table 5) further intensified the process, approaching the solubility limit of **2a** and the maximum feasible operational flow rate for the system. The isolated yield decreased slightly to 74% (standard deviation 1%), but reproducibility remained excellent. Final reagent concentrations were as follows: Solution A = 1.168 M, Solution B = 1.402 M, and Solution C = 0.6424 M. These conditions delivered a high average throughput of 68.3 g h^{-1}, Figure 7.

To evaluate longer term performance, the optimized process was operated under steady-state conditions for 15 min. The reaction output was collected directly into sodium acetate buffer (0.1 M, pH 4.0) under continuous stirring. After steady state was achieved, 160 mL of reaction mixture was collected, corresponding to a theoretical product yield of 23.09 g. Following precipitation, filtration, and washing, 16.27 g of pure 2,5-dioxopyrrolidin-1-yl (E)-4-(methyl(4-((4-nitrophenyl)-diazenyl)phenyl)amino)butanoate (DABCYL **3a**) was isolated, corresponding to an isolated yield of 70%.

This result equates to an effective throughput of 65.1 g h^{-1}, further validating the operational stability of the intensified process. The minor decrease in yield (from 74% to 70%) likely reflects practical workup inefficiencies associated with isolating larger product quantities in an academic laboratory setting. Importantly, no reactor blockages or performance degradation were observed during the extended run, demonstrating the robustness and reliability of the multistep continuous-flow platform. Product identity and purity were confirmed by ^{1}H NMR and LC-MS analysis.

Azo fluorescent quenchers such as DABCYLs are widely used as specialty reagents in molecular biology and diagnostic probes, and are typically prepared at milligram to gram laboratory scale for probe development and biochemical assay research.[33,34] Commercial oligonucleotide synthesis reagents are supplied at gram to kilogram quantities, and patented industrial oligonucleotide manufacturing processes operate at tens of grams to tens of kilograms per batch,[35] consistent with fine chemical production of high-value materials in multi-purpose facilities.[36] This is in contrast to commodity azo dyes produced on multi tonne scale.[37,38] Within this context, the demonstrated continuous flow process, delivering 20 g of product at a productivity of ~65 g h^{-1}, represents a practical foundation to develop industrial manufacture processes for such specialty reagents. The 15 min steady state window was constrained by reagent availability rather than process limitations, with no evidence of fouling, blockage, or instability observed. Extrapolation to a 2 h continuous run would yield ~ 130 g from a single reactor, while established flow scale out strategies such as numbering up enable proportional increases in throughput; for example, operation of 10 parallel reactors

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would deliver ~ 1.3 kg in a 2 h campaign.[39] These projected outputs align with reported production volumes for specialty oligonucleotide reagents. Moreover, the compact reactor footprint, combined with the inherent safety of *in situ* diazonium generation and consumption, makes this approach attractive relative to traditional batch synthesis, particularly given the safety concerns associated with diazonium intermediates and large scale azo coupling.

Collectively, these results demonstrate that significant quantities of DABCYL **3a** can be produced rapidly, reproducibly, and with improved safety and under continuous flow conditions.

CONCLUSIONS

A robust and inherently safer continuous-flow platform for the synthesis of DABCYL azo quenchers has been successfully developed and demonstrated. Through systematic optimization, both single-step and multistep telescoped flow processes were established, enabling the *in situ* generation and immediate consumption of diazonium intermediates. This strategy effectively mitigates the risks traditionally associated with diazonium chemistry while providing precise control over reaction parameters. The resulting process delivered high isolated yields, excellent reproducibility, and throughputs up to 65.1 g h^{-1}, illustrating its potential to be developed into an industrial relevant process for specialty DABCYL reagent synthesis.

The developed continuous process represents a significant advancement over conventional batch methodologies, offering superior safety and process efficiency. Its modular configuration allows for straightforward adaptation to diverse aniline coupling partners, facilitating the rapid synthesis of structurally varied DABCYL derivatives. The high molar absorptivity and consistent photophysical characteristics of the products confirm their suitability as fluorescence quenchers in probe and diagnostic applications.

From a sustainability perspective, the study also highlights the need to replace or minimize the use of DMF, which, while providing excellent solubility and reactivity, poses environmental and regulatory concerns.[40] Greener alternatives such as Cyrene showed promise during preliminary studies but proved challenging under continuous conditions due to viscosity and workup limitations. Future work will therefore focus on identifying more sustainable solvent systems or adapting the process to slurry-compatible reactor technologies, as recently exemplified in the literature, to maintain efficiency while improving the environmental profile of the synthesis.[41]

Overall, this work demonstrates the value of continuous-flow chemistry as a powerful enabling technology for hazardous and exothermic transformations, exemplified here by azo coupling. The approach delivers a safe, efficient, and high-throughput route to DABCYL-based quenchers and establishes a foundation for the sustainable manufacture of functional azo materials for chemical biology, diagnostics, and related applications.

EXPERIMENTAL SECTION

General Information

Reagents and reactants which were commercially available were purchased from Sigma-Aldrich, Fluorochem or TCI, and used without further purification.

NMR spectra were recorded using Bruker Ascend 500 spectrometer at 293 K operating at 500 MHz for the ^{1}H nucleus, 125 MHz for the ^{13}C nucleus, and 471 MHz for the ^{19}F nucleus. All chemical shifts were referenced relative to the relevant deuterated solvent residual peaks or TMS. Deuterated solvents used were $CDCl_3$, MeOD, CD_3CN and DMSO. Assignments of the NMR spectra were deduced using ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR, along with 2D-NMR experiments; ^{1}H−^{1}H COSY, ^{1}H−^{13}C HMBC, ^{1}H−^{13}C HSQC, and ^{1}H−^{19}F HOESY. The following abbreviations were used to explain the observed multiplicities; s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), pt (*pseudo* triplet). Chemical shifts were given in ppm downfield from the internal standard and coupling constants were given in Hz. ^{13}C NMR spectra were recorded with complete proton decoupling. ^{19}F NMR spectra were recorded with proton and carbon decoupling.

High-resolution mass spectrometry (HRMS) was performed in Maynooth University (MU) and the University of Bath (UoB). HRMS in MU was conducted on an Agilent-LC 1200 Series coupled to a 6210 or 6530 Agilent Time-Of-Flight (TOF) mass spectrometer equipped with an electrospray source in both positive and negative (ESI ±) modes. Infrared spectra were recorded on a PerkinElmer Spectrum 100 FT-IR spectrophotometer using a smart endurance single bounce diamond, attenuated total reflection (ATR) cell. Spectra were recorded in the region of 4000−600 cm^{-1} and were obtained by the coaddition of 4 scans with a resolution of 4 cm^{-1}.

Continuous flow apparatus: The Vapourtec R2C+ model, model R4 and SF-10 peristaltic pumps were used for all the continuous flow synthesis. All tubing used was either PFA or PTFE and had a 1 mm I.D. with 1.6 mm O.D. T-pieces and other fittings were made of PTFE material.

Batch Synthesis of DABCYLs 3a−f, General Procedure

4-Nitrobenzenediazonium tetrafluoroborate (0.107 g, 0.45 mmol, 1 equiv) was dissolved in MeCN (5 mL). The resulting reaction mixture was stirred at 0 °C for 15 min. A solution of the corresponding aniline coupler (0.157 g, 0.54 mmol, 1.2 equiv) in MeCN (0.7 mL) was added dropwise, and the resulting mixture was stirred at 0 °C for 30 min. The product was precipitated by the addition of NaOAc buffer (0.1 M, pH 4.0, 70 mL). The red/orange solid was recovered by filtration, washed with a H_2O (50 mL), and finally dried under vacuum to give the title compound.

2,5-Dioxopyrrolidin-1-yl (*E*)-4-(Methyl(4-((4-nitrophenyl)diazenyl)phenyl)amino)-butanoate (3a)

Yield 0.197 g, 99%; R_f 0.44 (Petroleum ether-EtOAc 1:1 v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (d, J = 9.1 Hz, 2H, H14), 7.98−7.88 (m, 4H, H10 and H13), 6.79 (d, J = 9.3 Hz, 2H, H9), 3.59 (t, J = 7.0 Hz, 2H, H6), 3.13 (s, 3H, H7), 2.87 (s, 4H, H1), 2.71 (t, J = 7.0 Hz, 2H, H4), 2.11 (quint, J = 7.2 Hz, 2H, H5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.2 (C2), 168.3 (C3), 156.9 (C15), 152.4 (C8), 147.6 (C12), 144.1 (C11), 126.4 (C10), 124.8 (C14), 122.8 (C13), 111.7 (C9), 51.3 (C6), 39.0 (C7), 28.4 (C4), 25.8 (C1), 22.2 (C5); IR (ATR) 1732, 1604, 1508, 1339, 1193, 1144, 1070, 858, 829, 644 cm^{-1}; HRMS calcd for $C_{21}H_{21}N_5O_6$ m/z: $[M + H]^+$, 440.1494, found 440.1564 [Diff (ppm) = 0.56].

(*E*)-1-(3-(Methyl(4-((4-nitrophenyl)diazenyl)phenyl)-amino)propyl)-1*H*-pyrrole-2,5-dione (3b)

Yield 0.174 g, 98%; R_f 0.47 (Petroleum ether/EtOAc 1:1 v/v); ^{1}H NMR (500 MHz, DMSO) δ 8.36 (d, J = 9.1 Hz, 2H, H12), 7.94 (d, J = 9.2 Hz, 2H, H11), 7.84 (d, J = 9.2 Hz, 2H, H8), 7.03 (s, 2H, H1), 6.87 (d, J = 9.4 Hz, 2H, H7), 3.56−3.44 (m, 4H, H2 and H4), 3.07 (s, 3H, H5), 1.82 (quint, J = 7.4 Hz, 2H, H3); ^{13}C NMR (125 MHz, DMSO) δ 171.1 (CO), 156.2 (C13), 152.5 (C6), 146.9 (C10), 142.8 (C9), 134.6 (C1), 126.0 (C8), 125.0 (C12), 122.5 (C11), 111.7 (C7), 49.2 (C2/C4), 38.3 (C5), 35.0 (C2/C4), 25.7 (C3); IR (ATR) 3103, 2919, 1705, 1507, 1218, 1059, 856, 695, 535 cm^{-1}; HRMS calcd for $C_{20}H_{19}N_5O_4$ m/z: $[M + H]^+$, 394.1437, found 394.1518 [Diff (ppm) = 2.37].

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(*E*)-4-(Methyl(4-((4-nitrophenyl)diazenyl)phenyl)amino)-butanoic acid (3c)

Yield 0.114 g, 74%; R_f 0.61 (1% MeOH in DCM); ^{1}H NMR (500 MHz, DMSO) δ 12.25 (s, 1H, br, H1), 8.36 (d, J = 9.1 Hz, 2H, H12), 7.94 (d, J = 9.1 Hz, 2H, H11), 7.85 (d, J = 9.3 Hz, 2H, H8), 6.91 (d, J = 9.3 Hz, 2H, H7), 3.50 (t, J = 7.4 Hz 2H, H4), 3.08 (s, 3H, H5), 2.30 (t, J = 7.4 Hz, 2H, H2), 1.79 (quint, J = 7.4 Hz 2H, H3); ^{13}C NMR (125 MHz, DMSO) δ 174.3 (CO), 156.3 (C13), 152.7 (C6), 146.8 (C10), 142.7 (C9), 126.1 (C8), 125.1 (C12), 122.5 (C11), 111.6 (C7), 51.0 (C4), 38.3 (C5), 30.7 (C2), 21.9 (C3); IR (ATR) 2895, 1692, 1557, 1142, 950, 857, 824, 685, 535 cm^{-1}; HRMS calcd for $C_{17}H_{18}N_4O_4$ m/z: $[M + H]^+$, 343.1328, found 343.1408 [Diff (ppm) = 2.18].

Ethyl (*E*)-4-(Methyl(4-((4-nitrophenyl)diazenyl)phenyl)amino)-butanoate (3d)

Yield 0.149 g, 89%; R_f 0.82 (Petroleum ether/EtOAc 1:1 v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (d, J = 9.1 Hz, 2H, H13), 7.91 (m, 4H, H9 and H12), 6.78 (d, J = 9.1 Hz, 2H, H8), 4.15 (q, J = 7.1 Hz, 2H, H2), 3.52 (t, J = 7.2 Hz 2H, H5), 3.10 (s, 3H, H6), 2.39 (t, J = 7.2 Hz, 2H, H3), 1.98 (quint, J = 7.2 Hz 2H, H4), 1.27 (t, J = 7.1 Hz, 3H, H1); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.1 (CO), 157.0 (C14), 152.5 (C7), 147.5 (C11), 143.9 (C10), 126.3 (C9), 124.8 (C13), 122.8 (C12), 111.6 (C8), 60.8 (C2), 51.9 (C5), 38.8 (C6), 31.4 (C3), 22.4 (C4), 14.4 (C1); IR (ATR) 2886, 1732, 1600, 1507, 1327, 1103, 856, 823, 685, 535 cm^{-1}; HRMS calcd for $C_{19}H_{22}N_4O_4$ m/z: $[M + H]^+$, 371.1641, found 371.1772 [Diff (ppm) = 2.30].

(*E*)-*N*-(2-Azidoethyl)-*N*-methyl-4-((4-nitrophenyl)diazenyl)-aniline (3e)

Yield 0.144 g, 98%; R_f 0.77 (Petroleum ether/EtOAc 1:1 v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.34 (d, J = 9.2 Hz, 2H, H10), 7.94 (overlapping signals, d, J = 9.2 Hz, 2H, H6/H9; d, J = 9.2 Hz, 2H, H6/H9), 6.81 (d, J = 9.2 Hz, 2H, H5), 3.69 (t, J = 6.0 Hz, 2H, H2), 3.56 (t, J = 6.0 Hz, 2H, H1), 3.18 (s, 3H, H3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.8 (C11), 152.0 (C4), 147.7 (C8), 144.3 (C7), 126.3 (C6), 124.9 (C10), 122.9 (C9), 111.8 (C5), 51.8 (C2), 49.1 (C1), 39.4 (C3); IR (ATR) 2086, 1597, 1510, 1290, 1129, 944, 857, 829, 685, 542 cm^{-1}; HRMS calcd for $C_{15}H_{15}N_7O_2$ m/z: $[M + H]^+$, 326.1287, found 326.1367 [Diff (ppm) = 2.49].

(*E*)-*N*,*N*-Dimethyl-4-((4-nitrophenyl)diazenyl)aniline (3f)

For **3f**, the product was precipitated by the addition of NaOAc buffer (0.1 M, pH 4.0, 25 mL). The solid was recovered by filtration, washed with a mixture of MeCN/H_2O (1:1, v/v, 35 mL). The solid on the sintered glass filter was dissolved using DCM (25 mL) through the sintered glass funnel into a clean Büchner flask. The solvent was removed under reduced pressure to obtain the product as a dark red/brown solid.

Yield 0.120 g, 98%; R_f 0.30 (Petroleum ether/EtOAc 9:1 v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (d, J = 9.1 Hz, 2H, H8), 7.97−7.88 (m, 4H, H4 and H7), 6.77 (d, J = 9.1 Hz, 2H, H3), 3.14 (s, 6H, H1); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.9 (C9), 153.5 (C2), 147.5 (C6), 143.9 (C5), 126.2 (C4), 124.9 (C8), 122.8 (C7), 111.6 (C3), 40.5 (C1); IR (ATR) 2917, 1598, 1507, 1324, 1231, 1094, 855, 823, 687, 542, 515 cm^{-1}; HRMS calcd for $C_{14}H_{14}N_4O_2$ m/z: $[M + H]^+$, 271.1117, found 271.1193 [Diff (ppm) = 1.45]. Matches literature data.[42]

General Procedure for Single-Step Continuous Flow Synthesis of DABCYL 3a−f

Continuous Flow Reactor Setup. A Vapourtec R-series flow reactor equipped with piston pumps, a coiled reactor made of PFA tubing (1 mL volume, I.D. 1 mm), and a BPR (6 bar) was used. The coiled reactor was temperature controlled by an insulated glass manifold and delivery of the reactants to the coiled reactor was controlled by two piston pumps set at particular flow rates. All tubing used was made from PFA with an I.D. of 1 mm.

Preparation of Reagents. Solution A (Diazonium Salt): 4-Nitrobenzenediazonium tetrafluoroborate (0.692 g, 2.92 mmol, 1 equiv) was dissolved in DMF (25 mL). The resulting reaction mixture was stirred at 0 °C for 15 min, making a stock solution of 0.1168 M concentration. This was placed into a Vapourtec reagent bottle, labeled A. Solution B (Coupler): Aniline coupler (2.92 mmol, 1 equiv) in DMF (25 mL) making a stock solution of 0.1168 M concentration. Both reagent bottles and solvent reservoir bottle (DMF, 40 mL), were sonicated for 10 min prior to start of run to ensure full solubilization of reagents and removal of dissolved air. Solution A was placed on ice and bottle wrapped in aluminum foil to keep the reagent cooled and block out light.

Continuous Flow Run. Reagent A and reagent B's flow rates were set at 6 mL min^{-1}. The continuous flow setup was then run until steady state was established. After this time elapsed, 7 mL of the product reaction mixture was collected in a vial containing 10 mL of NaOAc buffer (0.1 M, pH 4.0). The mixture was then transferred into a beaker with additional deionized water washings (50 mL) and the precipitated solid was collected via filtration, washed further with deionized water (50 mL) and then dried *in vacuo*. See Table 4 for yields and Supporting Information for further details.

Multistep Continuous Flow Synthesis of 2,5-Dioxopyrrolidin-1-Yl (*E*)-4-(Methyl(4-((4-nitrophenyl)diazenyl)phenyl)amino)-butanoate (3a)

Continuous Flow Reactor Setup. Two Vapourtec SF-10 peristaltic reagent pumps with a coiled reactor of made of PFA tubing (1 mL volume, I.D. 1 mm) with temperature controlled by an water or ice bath. This was used in conjunction with a Vapourtec R-series flow reactor equipped with piston pumps, a coiled reactor made of PFA tubing (1 mL volume, I.D. 1 mm). The coiled reactor was temperature controlled by a water bath and delivery of the reactants to the coiled reactor was controlled by two piston pumps set at particular flow rates. All tubing used was made from PFA with an I.D. of 1 mm.

Preparation of Reagents. Step 1: Solution A: 4-nitroaniline (1 equiv) and boron trifluoride etherate (1.2 equiv) was dissolved in DMF (20 mL). The resulting reaction mixture was sonicated for 15 min, making a stock solution of 0.2257−1.168 M concentration of the 4-nitroaniline. This was placed into a Vapourtec reagent bottle, labeled A. Solution B: A 90% purity solution of *tert*-butylnitrite (1.1−1.2 equiv) was dissolved in DMF (20 mL). The resulting reaction mixture was sonicated for 15 min, making a stock solution of 0.2467−1.402 M concentration. This was placed into a Vapourtec reagent bottle, labeled B.

Step 2: Concentration of 4-nitrobenzenediazonium tetrafluoroborate (product of step 1) is 0.1129−0.584 M. Solution C (Coupler): 2,5-dioxopyrrolidin-1-yl 4-(methyl(phenyl)amino)butanoate (1.03−1.1 equiv) in DMF (20 mL) making a stock solution of 0.1168−0.6424 M concentration. This was placed into a Vapourtec reagent bottle, labeled C. DMF, 30 mL was used as the solvent reservoir bottle. See Table 5 for exact concentrations and conditions used for experiments 1−4.

Continuous Flow Run. Solution A, solution B, and solution C's flow rates were set corresponding to that of Table 5. The continuous flow setup was then run until steady state was established. After this time elapsed, 5−7 mL of the product reaction mixture was collected in a vial containing 10 mL of NaOAc buffer (0.1 M, pH 4.0). The mixture was then transferred into a beaker with additional deionized water washings (50−70 mL) and the precipitated solid was collected via filtration, washed further with deionized water (50−70 mL) and then dried in a vacuum oven overnight at 45 °C. This was repeated for each of the tested reaction conditions. The results of each experiment can be seen in Table 5. Further details can be found in the Supporting Information.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.oprd.5c00461.

Synthetic chemistry details; experimental; NMR spectral data; LCMS data; HRMS data; IR data; DOE details (PDF)

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Author Contributions

The manuscript was written with contributions from both authors. Both authors have given their approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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