CHAPTER FOURTEEN

# FlICk (fluorescent isoindole crosslinking) for peptide stapling

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## Contents



## Abstract

The rigidification of peptide secondary structure via stapling is an important and enduring goal in the development of functional peptides for biochemical and pharmaceutical applications. In addition, the incorporation of fluorophores and chromophores has been a sought-after application for creating peptidic probes of cellular function and localization. The combined application of peptide stapling and fluorescent-readout is featured by the reaction of *ortho*-phthalaldehydes to create isoindole staples, thus transforming inactive linear and monocyclic precursors into fluorescent or visibly colored monocyclic and bicyclic products with noted biological activity. Given its user-friendliness, we have termed this approach FlICk (fluorescent isoindole crosslink) chemistry and we have featured this application on an array of high-affinity macrocyclic α-MSH derivatives as well as for late-stage intra-annular isoindole stapling furnished a bicyclic peptide mimic of α-amanitin that is cytotoxic to CHO cells. The synthetic methods for preparing substituted *ortho*-phthalaldehydes along with subsequent applications to FlICk stapling are detailed herein.

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## 1. Introduction

The development of bio-orthogonal reactions for rapid, high-yielding, site-selective installation of molecular functionality has wide-ranging implications in peptide chemistry. Noteworthy goals include constraining peptide conformations and achieving structural rigidification, both of which can be achieved with stapling. In addition, there is enduring interest in grafting of reporter groups, most notably in the context of chromophores, for the construction of absorbent and fluorescent probes. Rarely, a reaction that affords stapling can be designed to provide an emissive or visible spectral signature that not only confirms successful stapling but through the installation of a chromophore, enables applications in screening and target identification. Indeed, the very development of such a reaction and its methodological implementation for use in peptide chemistry is the basis of this report.

On this subject, a classic analytical reaction used to quantify the concentration of a primary amine present in a sample involves the condensation of ortho-phthalaldehyde (OPA) with a primary amine and a thiol to produce a fluorescent 1-thio-2H-isoindole. The OPA assay has found broad utility in amino acid analysis (Roth, 1971) as well as in the food and beverage industries (Dukes & Butzke, 1998) (Fig. 1).

To date, for most applications, OPA-mediated isoindole formation finds its primary use in quantifying peptides that have at least one free amine (i.e., N-terminal, lys) (Go, Horikawa, Garcia, & Villarreal, 2008). Quantifying the exact amount of peptide ligand to a high degree of certainty is essential to accurately measure its $K_d/IC_{50}$ values in a dose-dependent assay. Such is particularly at issue in cases where the ligand, either produced or isolated in minute quantities, renders gravimetric quantification unreliable due to the inherent imprecision of weighing sub-milligram amounts of material as well as the unaccountable presence of contaminating trifluoroacetic acid (TFA) or formic acid (FA) molecules residually found in lyophilized samples.

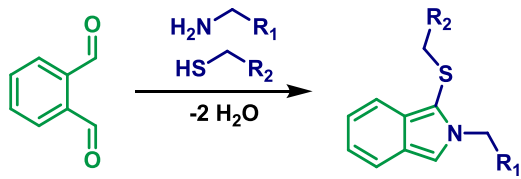



**Fig. 1** OPA condensation with a primary amine and a thiol to form a fluorescent isoindole.

In contrast, using OPA to quantify peptides and amino acids results in consistent extinction coefficients irrespective of amino acid identity (Hernández, Camañas, & Alvarez-Coque, 1990) leading to accurate quantification of primary amine containing peptides. With excess OPA and thiol (dissolved in aqueous buffer pH 8–10), isoindole formation is generally complete upon mixing enabling quantification of the amine of interest. Absorbance of the resulting isoindole is acquired at 335 nm (λ-max) and concentration is calculated using an extinction coefficient of 6800 $M^{-1}$ $cm^{-1}$, found to be generally constant irrespective of the side chain of the amino acid in question (Hernández et al., 1990). This method can be combined with HPLC analysis for even greater accuracy (Hernández et al., 1990). A variety of thiols can be employed in the OPA assay, with β-mercaptoethanol being employed early on (Roth, 1971; Simons & Johnson, 1977, 1978). However, use of this particular thiol has the drawback of producing an isoindole that autodegrades to an isoindolin-1-one on standing in the reaction media (Fig. 2A). The rate of degradation appears to be enhanced by super-stoichiometric OPA concentrations that are often used in analysis (Simons & Johnson, 1977, 1978; Stobaugh et al., 1983). This observation let to a proposed mechanism that involves the formation of an unusual episulfonium intermediate (Stobaugh et al., 1983) that presumably undergoes hydrolysis by a different pathway (Fig. 2B). Use of a

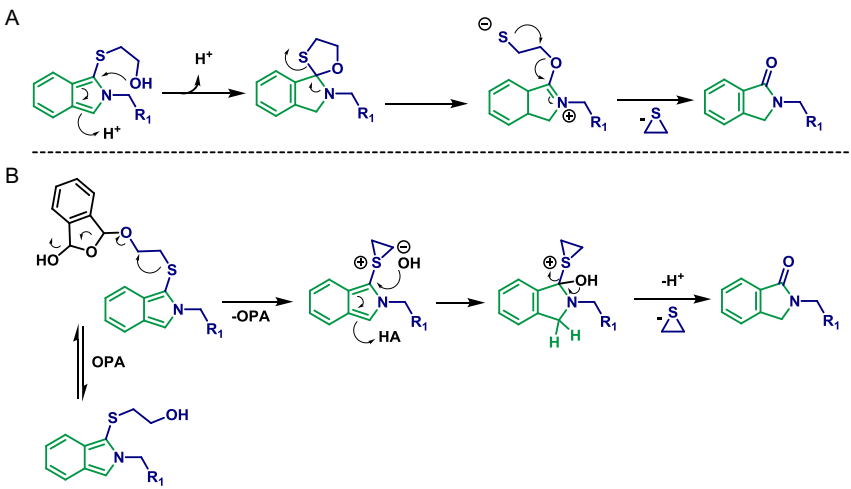



**Fig. 2** (A) Isoindole degradation mechanism with anchimeric assistance from 2-mercaptoethanol, originally proposed by Simons and Johnson (1977). (B) OPA-enhanced degradation proposed by Stobaugh, Repta, Sternson, and Garren (1983).

longer alkyl chain that separates the thiol from the hydroxyl group greatly reduces this problem (Stobaugh et al., 1983) as does the use of thiols lacking an alcohol, such as *N*-acetyl cysteine. However, Jacobs found that isoindole degradation was expedited by OPA even in cases where the thiol employed lacked a pendant alcohol and proposed a mechanism with nucleophilic attack on OPA by the isoindole (Leburg & Jacobs, 1986). A contemporary review found no general agreement on the mechanism of degradation (García Alvarez-Coque, Medina Hernández, Villanueva Camañas, & Mongay Fernández, 1989).

The rapidity of this reaction, along with the mild aqueous conditions under which it proceeds, makes it highly compatible with applications in biological chemistry and thus it has also found applications in enzymology. To wit, as lysines and cysteines often play key catalytic roles within the active site of enzymes, OPA naturally finds a role as an active site titrant. Employing OPA, others showed that yeast hexokinase could be deactivated, implicating a lysine and cysteine in catalysis (Puri, Bhatnagar, & Roskoski, 1988). In a related application, selective active site labeling was achieved through the synthesis of a "Reporter Affinity Label," an OPA-β-funaltrexamine conjugate that could react specifically with the opioid receptors to provide a fluorescent readout confirming their presence (Le Bourdonnec et al., 2000) (Fig. 3A). The idea of exploiting the unique reactivity of OPA for enzyme-selective bioconjugation was elegantly extended into the realm of kinases. By constructing an OPA-adenosine conjugate, others showed that kinases/substrate pairs could be covalently tethered only upon substrate recognition and the resulting isoindole once again serves as a fluorescent reporter (Maly, Allen, & Shokat, 2004) (Fig. 3B). The labeling was facilitated by preparing "mutant" peptide substrates where a cysteine is introduced in place of a serine or threonine residue, which would be naturally phosphorylated (Maly et al., 2004).

In light of increasing pharmaceutical interest in biologic drugs, demand for new peptide stapling methods has grown. Desirable attributes of these reactions include mild aqueous conditions, rapid kinetics, chemoselectivity, and access to readily available reagents. When considering these constraints, OPA condensation emerges as a viable candidate for investigation. Indeed, an extensive survey of the literature provided scant examples towards this goal; the sole exception being crosslinking of glutathione for the purpose of assessing the extinction coefficient of the resulting isoindole (Simons & Johnson, 1978).

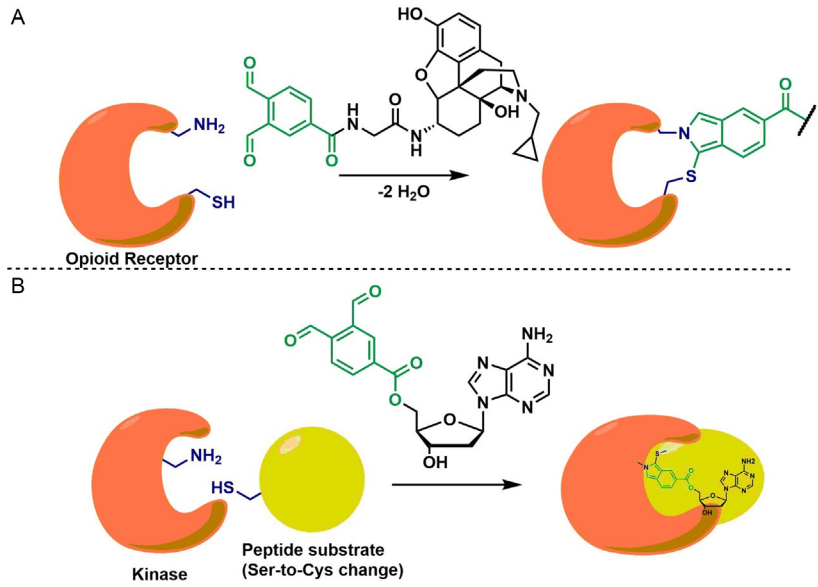



**Fig. 3** Enzymatic labeling reactions of OPA; (A) Reaction of an opioid receptor with an OPA-opioid conjugate; (B) three-component crosslinking of a kinase-substrate pair by an OPA-Adenosine conjugate.

Notwithstanding such precedent, it was surprising that isoindole formation had yet to be explored in the context of directed peptide stapling despite decades of work on both intermolecular variants of the reaction with amino acids and amine-containing peptides reacted with exogenous thiols as well as intramolecular variants with enzymes and receptors. Thus, we decided to explore this reaction and discovered the smooth application of OPA condensation to prepare peptide mono- and bicyclic peptides (Todorovic et al., 2019). Confident that this method would work rather generally across many peptides, we focused our efforts to develop this methodology directly on biologically relevant and potentially highly-active peptides, avoiding preliminary investigations on sequences with limited functionality or of non-evident pharmacological/biochemical significance. In so doing, we sought important and meaningful biological readouts to provide the community with confidence, not only in the robustness of the chemical reaction, but in its potential for imparting high biological activity to the resulting stapled peptides. In the course of this work, we synthesized new OPA analogs (vide infra), and in so doing, we termed this method: Fluorescent Isoindole Crosslinking, or "FlICking" for short.

Before discussing the application of FlICk staples in test peptides of biological relevance, we appreciated that the area of substituted OPAs is scantly explored. Hence, in broadening this application, we saw potential for tuning the photophysical properties of isoindoles through the synthesis of a series of substituted OPAs. To this end, we selectively reduced a small set of phthalic acids to their corresponding diols with Borane-THF complex, leaving nitro groups untouched where present (Fig. 4). The use of a Swern-type oxidation was critical to achieving the desired oxidation at both benzylic carbons, as in our hands, other oxidants which produced a discrete aldehyde prior to quenching led to complex mixtures (while oxidant was still present in the reaction pot), commonly including a lactone as a major product. The products of the Swern oxidations were isolated in the form of methylhemiacetals in case of electron-deficient OPAs. The condensation of these phthalaldehydes with hexylamine and N-Acetyl cysteine produced isoindoles in short order (<1 min) in all cases expect the nitro-containing OPAs, most notably, the 3-Nitro OPA which required ~20 min to reach completion.

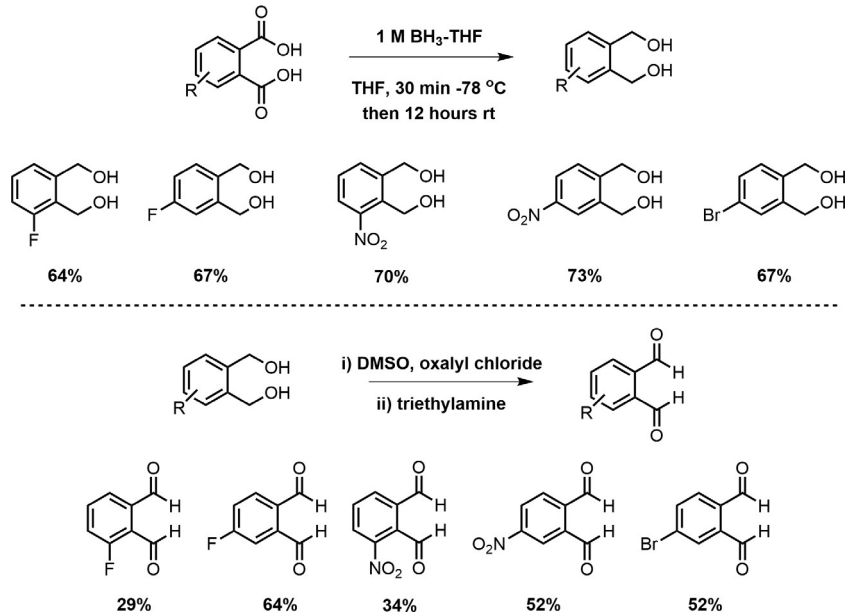


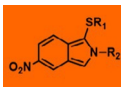
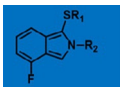
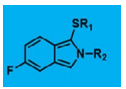
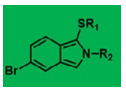
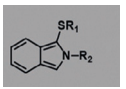

**Fig. 4** Synthesis of Substituted OPAs. Compounds were isolated as hemiacetals with methanol except in the case of the 4-Fluoro and 4-Bromo substituted OPAs.

In the case of the nitro-OPAs, the $\lambda_{max}$ was significantly red-shifted into the visible range, leading to orange-red products, which were unfortunately not fluorescent in aqueous media. Halogen substitution had little effect on the $\lambda_{max}$, but significantly affected the fluorescent output, with the 4-Fluoro OPA being the most fluorescent of the modified OPAs in our model system. Gratifyingly, when we measured the quantum yield of the peptides stapled with fluorogenic OPAs, we found that fluoro-substitution significantly increased fluorescent output (Table 1).

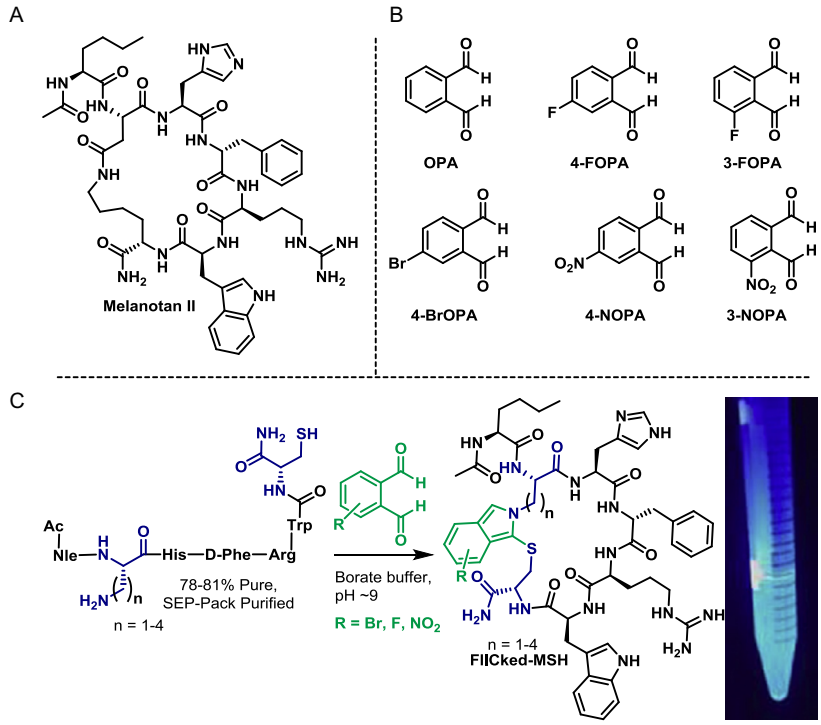
Hence, in applying FlICk synthesis to peptide stapling, we synthesized isoindole stapled macrocyclic analogues of a melanoma drug lead (Melanotan II) and of a bicyclic natural product peptide toxin (α-amanitin) to provide two important comparators for valuing the application of FlICks. Our choice for FlICking a heptapeptide MSH sequence was informed by the fact that MSH has been heavily investigated over the years and is known to be challenging in terms of delivering biological potency by standard stapling reactions, particularly in the context of a short heptapeptide that, in the linear format, shows no measurable biological activity. Nevertheless, at least one standard staple, an isopeptide linkage between lysine and glutamate, afforded Melanotan-II (MTII), a clinically-trialed peptide macrocycle that shows low nanomolar dissociation constant values for the melanocortin-1 (MRC-1) receptor. Although MTII ultimately failed as a treatment for melanoma, recently it was elaborated for $^{18}$F- and $^{68}$Ga-labeling and successfully used to PET-image melanoma xenografts in mice thus providing new radiochemical leads for imaging and targeted therapy (Zhang et al., 2018). As such, MTII represents a readily accessible peptide macrocycle that may serve as a benchmark against which new peptide stables can be evaluated in this context.

We arrayed this series of OPAs against four linear MSH precursors with varying lengths of the amine-bearing side chain to afford a focused library of 24 FlICked peptides (Fig. 5). Reaction in the context of this series of linear heptapeptides was rapid and generally complete in under a minute when performed in the presence of excess OPA (e.g., concentrations of 1–10 mM). The cyclization yields, as assessed by HPLC, ranged from moderate to roughly quantitative (as the linear precursors were ∼75%–80% pure). All FlICked MSH peptides bound MCR-1 in a competitive binding assay that employs a standard radio ligand. When diaminobutryic acid, or an amino acid with a longer amine-bearing sidechain was used, an order of magnitude of binding affinity was gained over the diaminopropionic acid

**Table 1** Photophysical properties of substituted isoindoles.

| |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| | **4/7-Nitroisoindole** | **5/6-Nitroisoindole** | **4/7-Fluoroisoindole** | **5/6-Fluoroisoindole** | **5/6-Bromoisoindole** | **Isoindole** |
| $\lambda_{ex}$ (nm) | 487 | 444 | 329 | 338 | 345 | 338 |
| $\lambda_{em}$ (nm) | [a] | [a] | 415 | 454 | 459 | 452 |
| Stokes Shift (nm) | – | – | 86 | 116 | 114 | 117 |
| Quantum Yield | – | – | 0.04 | 0.30 | <0.01 | 0.30–0.47 (Chen, Scott, & Trepman, 1979) |
| | | | 0.02[a] | 0.31[b] | | 0.13[c] |
| Ex. coefficient (ε) | 5600 | 5200 | 8200 | 7400 | 6700 | 6500–7000 (Hernández et al., 1990) |

[a]3-FOPA derived peptide.
[b]4-FOPA derived peptide.
[c]OPA derived peptide.
$R_1$ is N-Acetyl Cysteine and $R_2$ is hexlyamine expect where starred.




| | n=1 | n=2 | n=3 | n=4 |
|---|---|---|---|---|
| OPA | 76% | 61% | 59% | 55% |
| 3-FOPA | 73%* | 63%** | 75%* | 65%* |
| 4-FOPA | 72% | 55%* | 57% | 48% |
| 3-NOPA | 82% | 70% | 38% | 38% |
| 4-NOPA | 53% | 64%* | 39% | 23% |
| 4-BrOPA | 48% | 38%* | 36% | 28%** |

**Fig. 5** (A) Melanotan-II, (B) substituted OPAs employed in library synthesis; (C) FlICked MSH synthesis, with yields in the table below (measured by HPLC with absorbance at 280 nm); at right a sample of HPLC-peptide, directly eluted, as visualized by use of hand-held UV lamp.

variant. Ornithine-derived FlICk-MSH exhibited the highest affinity with a $K_i \sim 1$ nM, irrespective of isoindole substitution.

In order to prepare a bicyclic FlICk peptide macrocycle with the potential for biological activity, we turned our attention to alpha-amanitin, a potent natural bicyclic peptide toxin found in *A. phalloides*, which is under investigation as a payload for clinically emergent antibody-drug conjugates.

Amanitin is known to be highly rigid with a unique tryptathionine staple contributing significantly to its structural definition and inherent toxicity. Although there is limited structure activity relationship data due to limited synthetic accessibility (Matinkhoo, Pryyma, Todorovic, Patrick, & Perrin, 2018), the natural product is cytotoxic against CHO cells at 1–2 μM. As with MTII, we supposed that amanitin could provide an important control for evaluating the biological activity of FlICk staples in the context of an analogous cytotoxic bicyclic peptide. Notably, our choice of amanitin was also based on the appreciation that the tryptathionine staple itself represents a constitutional isomer of a FlICk staple thus making amanitin a highly relevant model for testing aspects of potential bio-isosterism (Fig. 6).

Given the circularly permutable nature of peptide macrocycles, we recognized that two regioisomeric FlICked-amanitins could be prepared. Molecular modeling of both (Fig. 6) led us to note not-so-subtle differences in the orientation of the isoindole with respect to peptide macrocycle as well as significant differences in terms of the orientation of the side chains on the macrocycle that are constrained by virtue of this topology of the FlICk. While it is known that the tryptathionine engages in both a pi-stacking and pi-cation interactions at the active site of RNA Polymerase II, our choice favored the FlICked amanitin that appeared to provide greater

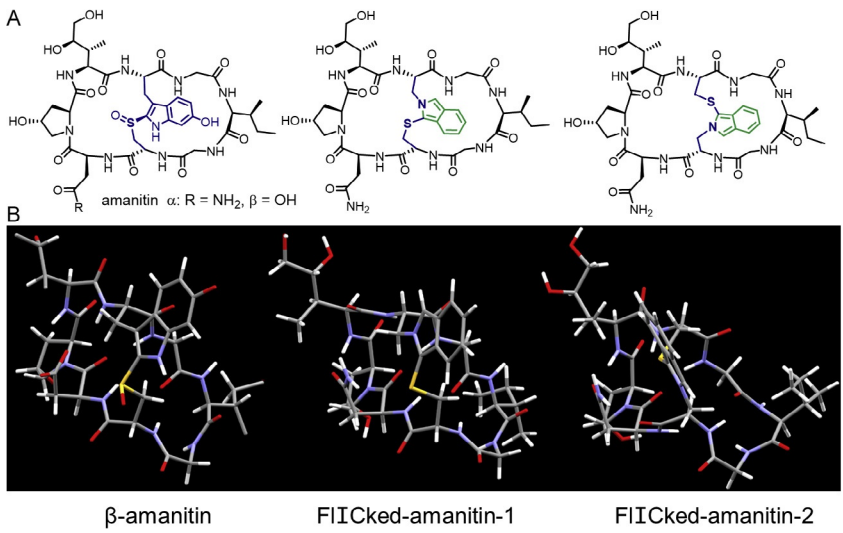



**Fig. 6** Modeling of FlICked amanitins. (A) The chemical structures of β-amanitin along with the two possible cyclic topologies of a FlICked amanitin. (B) The crystal structure of β-amanitin juxtaposed with energy-minimized molecular models of FlICked amanitins.

overall macrocyclic geometry compared to alpha-amanitin despite significant alteration in orientation of the isoindole.

In contrast to FlICk synthesis on a linear heptapeptides that was essentially complete on mixing, for the bicyclic amanitin-like structures, FlICking required up to 30 min at the same concentrations of OPA, likely reflecting inherent ring strain in such bicylic peptides. Remarkably, despite different orientations of the isoindole, FlICked-amanitin-1 exhibited an $IC_{50}$ on CHO cells of 70 μM. Notably, the macrocyclic precursor showed no inhibitory properties. While we are unable to assert that FlICked amanitin engaged RNA Pol II to promote cell death, were this not the case, then it is equally interesting to contemplate the eventuality of discovering a new target for cytotoxicity.

Finally, to demonstrate the generalizability of this method for other peptide sequences, we also sought a route to synthesizing FlICk peptides containing free amines. Hence, we found that we could FlICk peptides containing amines as long as they were protected with either Dde or Fmoc, which could then be subsequently removed with either hydrazine or DBU respectively. The model peptides studied in our published work showed that the reaction conditions were chemoselective and left other potentially reactive peptide side chains untouched (alcohols, imidazoles, indoles, guanidines, etc.). In additional unpublished work, we confirmed that the reaction is absolutely specific to primary amines, leaving secondary amines unmodified even when accomplished with excess OPA. Herein, we describe the details of all practical aspects of FlICk chemistry relevant to carrying out the reaction in the contexts we have so far explored. Given the interest in a rapid and robust peptide stapling method, herewith we wish to report on the specific practical methodology of this reaction.

## 2. Materials

All reagents and solvents were acquired from vendors at the highest purity specification available except where noted.

MilliQ Water: Tap water was purified using an EMD Millipore Milli-Q® IQ 7000 water purification system equipped with an EMD Millipore Progard® 2 cartridge and dispensed through a 0.22 μm Millipak filter.

Borate Buffer: Dissolve 6.2 g of boric acid and 2.83 g of NaOH in 1 L of milliQ water. Check the pH, it should be around 9–9.5, if not adjust accordingly.

0.1% formic acid $H_2O$: Add 1 mL of formic acid to milliQ $H_2O$ to make a 1 L solution. For use with HPLC, filter through a 0.2 μm PAL filter
0.1% formic acid MeCN: Add 4 mL formic acid to HPLC grade MeCN to make a 4 L solution. For use with HPLC no further manipulations were done
40 mM $NH_4OH_{(aq)}$ (pH ~ 8): Add 4.7 mL conc. Ammonium Hydroxide to milliQ $H_2O$ to make a 1 L solution. Add formic acid to reach a pH of ~8. For use with HPLC, filter through a 0.2 μm PAL filter.
Pierce™ Centrifuge Columns (89896) 2 mL, 5 mL or 10 mL variety.
SiliaFlash® Silica, 40–63 μm (230–400 mesh).
Merck Silica gel high-purity grade (9385), pore size 60 Å, 230–400 mesh particle size.
Gilman Pipettes were used to measure μL volumes.

## 3. Peptide preparation

1. Cleave the peptide from the resin by incubation with 95:2.5:2.5 TFA/TIS/$H_2O$ for a period of around 2 h at room temperature in a pierce centrifuge column.
2. Drain the contents of the column and collect the filtrate into a round bottom flask. Concentrate the solution under reduced pressure to afford a gummy residue.
3. Triturate the residue with $Et_2O$. Add ~5 mL of $Et_2O$ to the flask, and pipette the $Et_2O$ up and down and rinse the walls of the flask with a pasteur pipette. Collect the resulting residue, resembling flakes, at the bottom of the flask and transfer it to a 15 mL falcon tube along with the residual $Et_2O$. Spin the contents of the falcon tube spun down in a clinical centrifuge until a tight pellet is achieved. Pour out the supernatant and add 3 mL of fresh $Et_2O$ to the falcon tube. Vortex the tube and then spin it down as described above. Repeat this once again for a total of three triturations.
4. Take the cap off the tube and wrap a piece of filter paper around the top of the tube with holes poked in it. Allow the residue to dry overnight.
5. Once dry, dissolve the peptide residue in a minimal amount of 0.1% formic acid $H_2O$. Note: Even if the peptide is incompletely soluble, do not add MeCN at this stage as it may impair resolution on reverse phase silica.
6. Prepare a reverse phase silica column (SEP-PACK) by flushing the column with MeOH ~5–10 mL. Then load the column by adding the

crude peptide dissolved in 0.1% formic acid $H_2O$. The rate of elution can be aided by using a syringe with a rubber septum attached to the end. Run an additional ~5–10 mL 0.1% formic acid $H_2O$ through the column.

7. Collect successive fractions of ~10 mL of 9:1 (v/v) 0.1% formic acid $H_2O$/MeCN, 8:2 (v/v) 0.1% formic acid $H_2O$/MeCN, 7:3 (v/v) 0.1% formic acid $H_2O$/MeCN, 6:4 (v/v) 0.1% formic acid $H_2O$/MeCN. Check the fractions for the presence of product using either ESI Mass Spectrometry or analytical reverse phase HPLC. If using ESI, the unconcentrated elute can be directly analyzed.
8. Pool the fractions containing the desired product in a 50 mL Falcon tube, freeze and lyophilize them. If quantifying large amounts of peptide by gravimetry, pre-weigh the falcon tube.

## 4. FIICk reaction

1. Dissolve the quantified crude peptide in convenient amount of 0.1% formic acid $H_2O$ (often 1 mL). Note: It is very important to use an acidic solution to dissolve the peptide at this stage, as it will have a free thiol. The use of neutral $H_2O$ has been observed to result in significant disulfide formation in the course of a few days. Crude linear peptides dissolved in 0.1% formic acid $H_2O$ can be stored at 4 °C for a period of months and remain usable in FIICk reactions.
2. Pipette out a convenient amount of peptide into a 15 mL falcon tube (~0.5 μmole was often used) and freeze and lyophilize it.
3. Add 80 μL Borate Buffer (~1 M HEPES buffer to pH 8–9 with $K_3PO_{4(aq)}$ can be substituted and led to cleaner reaction in some cases) to the dry peptide in the falcon tube and vortex to dissolve. Add 20 μL 0.05 M XOPA dissolved in EtOH and vortex. Note: The reaction tended to proceed faster and cleaner if the OPA solution was made prior to immediate use. It can be stored at room temperature for weeks and remain usable, or for at least a year if stored at 4 °C. The reaction can be deemed complete when a fluorescent precipitate forms. For unmodified ortho-phthalaldehyde or the halo derivatives, this took only a few seconds. For the nitro-derivatives, 5–20 min was required. In general, most reactions were simply allowed to proceed for 20 min.
4. To redissolve the precipitate, add 20 μL DMF or 0.5 μL formic acid to the solution and vortex. If desired, ESI mass spectrometry can be used to check for reaction completion. If the acidic work up is done, this

solution can directly be analyzed, if not, adding formic acid to the sample to reach pH ~2.5–3 is ideal for observing a strong signal in positive ion mode.

5. Directly inject the entire reaction mixture onto reverse phase HPLC. In the case of our MSH analogue peptides we used a gradient elution with 0.1% formic acid $H_2O$ against 0.1% formic acid MeCN. 25%–35% over 21 min. In general, we found it best to use a more general method with a wide range of MeCN proportion and then make a focused method noting the MeCN proportion range at which the relevant peaks elute. This reduced the time per run and solvent use. The elution of the product was followed at 335 nm for unmodified isoindoles and at the respective $\lambda_{max}$ of the given modified isoindole. To evaluate the reaction progress and ensure the consumption of the starting material, channels at the $\lambda_{max}$ of useful sidechains (280 or 260 nm for Tryptophan or Tyrosine, respectively) and 230 nm for general amide bonds. Once the suspected product peak is collected, ESI mass spectrometry can be used to confirm the identity product by directly sampling the column eluent.
6. To ensure no byproduct formation, cool the product immediately after collecting the fraction. If possible, freeze and lyophilize the product immediately. However, most isoindole peptides we have made can be stored at −20 °C for days to weeks in the acidic solution that comes off the HPLC with minimal or no degradation. Note: storage at room temperature, in solution, in the direct path of sunlight leads to bleaching of the isoindole over time.
7. To accurately quantify the isolated peptides, dissolve the dry powder in $H_2O$ and read at 335 nm if it is an unmodified isoindole or at the respective $\lambda_{max}$ of the given modified isoindole. A molar extinction coefficient of 6800 was used for unmodified isoindoles or the extinction coefficient we measured for the modified isoindoles.

## 5. Modifications for bicyclic alpha amanitin analogue

1. Despite the use of 0.1% formic acid $H_2O$, we observed disulfide formation in the case of the monocyclic precursor in this case.
2. To enable smooth reaction progress use 50 mM TCEP Borate Buffer (pH ~8–9) in place of regular borate buffer if significant disulfide formation has occurred. Note: It is critical that pH of the buffer be basic

at this step, or the reaction rate will be very slow or the product not form at all. If TCEP·HCl is used to make the buffer, check the pH and add $NaOH_{(aq)}$ to reach pH 8–9. For the amanitin analogue, a reaction time of around 30 min was usually used, but the reaction is likely done in less than 10.

3. The amanitin analogue was found to be sensitive to acid, so DMF was used if necessary or nothing was added if no precipitate was observed.
4. In the case of the amanitin analogue, the use of acidic HPLC eluents resulted in no product isolation. So, the use of 40 mM $NH_4OH$ buffered to pH ~8 with formic acid was essential to the isolation of product. Use MeCN with no additive as the other solvent. We suspect that the increased sensitivity of the amanitin analogue was due to the high degree of ring strain and that these precautions may not be necessary for other bicyclic isoindole peptides.

## 6. Preparation of modified ortho-phthalaldehydes

### 6.1 Borane THF reduction

1. Flame dry a round bottom flask containing a stir bar. Alternatively, if the precursor is made in house and is a gel on the inside of the flask, dry the flask containing the precursor (and a stir bar) in a desiccator under vacuum against $P_2O_5$ (housed in a beaker) for 2–3 h. After this period, quickly take the flask out of the desiccator and flush with Argon 3 times after evacuating the flask of its previous atmosphere.
2. Add dry THF (freshly distilled) to the flask under Argon and stir until the precursor is dissolved. Alternatively, if a commercial phthalic acid or powder precursor is used, dissolve it in dry THF (also freshly distilled) and add it to the reaction flask under Argon. Cool the contents of the flask to −78 °C by placing the flask in an aluminum dewar containing a slurry of dry ice in acetone.
3. After allowing ample time for the flask to cool, add 10 equivalents of 1 M Borane-THF complex (solution in THF) dropwise to the stirring reaction mixture. Note: The use of fresh Borane-THF complex is critical to reaction success. Old and compromised bottles result in a complex mixture of partially reduced products in our experience. Note: Slow addition at low temperature is critical as the reaction is highly exothermic, but with appropriate precautions the reaction has been safely carried out at scale.

4. After adding the full volume of Borane-THF solution allow the reaction to stir at −78 °C for 30 min. Then, remove the flask from the dewar and allow the reaction to stir at room temperature overnight.
5. After overnight stirring cool the reaction mixture to 4 °C by placing the flask in an ice bath and ensure that the stirring continues. Quench the reaction by slowly adding ~2/3 reaction volume of 1:1 THF/$H_2O$ dropwise to the reaction flask via syringe.
6. After opening the flask to the atmosphere, transfer the solution to a separatory funnel and add solid $K_2CO_3$ to the funnel until two layers form. Collect the THF layer and add another portion of THF to the separatory funnel and extract the aqueous layer once more and pool the resultant THF with the first THF layer. Dry the combined THF layers over $MgSO_4$ and concentrate the solution under reduced pressure on rotovap to yield solids dispersed in a thick oil.

   Alternate workup: After quenching add ~2 total volume $H_2O$ and transfer to separatory funnel, then extract with three successive portions of EtOAc (~1/3 volume of total solution in funnel per portion). Pool the organic layers. Note: If some product remains in the aqueous layer can be acidified to pH ~1 with conc. $HCl_{(aq)}$ and extracted with successive portions of EtOAc (~1/5 volume of total solution in funnel per portion). Check the new layers by TLC to ensure no product degradation upon acidification, if clean, combine the organic layers and dry over $MgSO_4$ and concentrate the solution under reduced pressure on rotovap to yield solids dispersed in a thick oil.
7. Purify the crude product by flash chromatography on silica. Silicycle 40–63 μm silica can be used, Merck grade silica is unnecessary. Set up the column by first adding sand to the glass tube then adding some of the loading solvent. At this point carefully add the silicycle as a slurry in the loading solvent at first, once a base of silica is established the rest can be more quickly. Load the product (dissolved in a minimum amount of 98:2 (v/v) DCM/EtOH) with 98:2 (v/v) DCM/EtOH and elute with 96:4 DCM/EtOH. Note: Change the eluent composition if the rf of the given substituted phenyl dimethanol deviates significantly from this report. Evaluate the purity of each fraction by TLC with 92:8 (v/v) DCM/EtOH. Pool the pure fractions and concentrate the solution under reduced pressure on rotovap to a white or colored solid. Note: MeOH can be used in place of EtOH.

## 7. Swern oxidation

1. Dissolve the starting material in dry DCM if possible. If it is insoluble (as is the case with several substituted phenyl dimethanols) use a minimal amount of dry THF or dry DMSO.
2. Flame dry a round bottom flask containing a stirbar. After allowing it to cool add the appropriate volume of DCM (final concentration of the reaction should be ~0.19 M). Cool the reaction to -78 °C and carefully add Oxalyl chloride (2.2 eq) dropwise, to the stirring reaction mixture.
3. Slowly add DMSO (3.4 eq.) dropwise to the stirring reaction mixture. Note: This process is highly exothermic, take precautions to minimize the evolution of an exotherm. Allow the reaction to stir for 15 min at −78 °C.
4. Add the dissolved starting material slowly to the stirring reaction mixture. Stir the reaction at −78 °C for 2 h.
5. Add Triethylamine (18 eq) dropwise to the stirring reaction mixture and allow it to proceed for 10 min at −78 °C. Then allow it to slowly warm up to room temperature removing the dry ice/acetone bath. Allow the reaction to stir at room temperature for 30 min.
6. To quench the reaction there are two options:
   **(a)** If the substituent on the phthalaldehyde is highly electron withdrawing (in our experience, the 3-fluoro, 3-nito, and 4-nitro) quench the reaction with approximately the same volume of MeOH. Stir for 10 min at room temperature and then concentrate under reduced pressure.
   **(b)** If the substituent on the phthalaldehyde is not highly electron withdrawing (in our experience, the 4-fluoro and 4-bromo) quench the reaction with $H_2O$ approximately the same volume as the DCM used in the reaction. Collect the organic layer and concentrate under reduced pressure.
7. **(a)** Purify the crude product by flash chromatography on silica. High-purity grade Silica gel, 60 Å pore size is necessary for adequate product recovery in our hands. Set up the column by first adding sand to the glass tube then adding some of the loading solvent. At this point carefully add the Merck grade silica as a slurry in the loading solvent at first, once a base of silica is established the rest can be more

quickly. Load the product (dissolved in a minimum amount of 1:39:60 (v/v/v) MeOH/EtOAc/Hexanes) with 1:39:60 (v/v/v) MeOH/EtOAc/Hexanes and elute isocratically. Note: Change the eluent composition if the rf of the given substituted phenyl dimethanol deviates significantly from this report. Evaluate the purity of each fraction by TLC with 1:39:60 (v/v/v) MeOH/EtOAc/Hexanes. Pool the pure fractions and concentrate the solution under reduced pressure on rotovap to a white or colored solid. Note: As modified phthalaldehyde methyl hemiacetals elute as a mixture of regio and stereoisomers, multiple spots will be the desired product and when isolated will equilibrate back to the mixture. All regioisomers are useful for FlICk reactions and inconsequential for the outcome of reaction.

**(b)** Purify the crude product by flash chromatography on silica. High-purity grade Silica gel, 60 Å pore size is recommended for adequate product recovery. Set up the column by first adding sand to the glass tube then adding some of the loading solvent. At this point carefully add the Merck grade silica as a slurry in the loading solvent at first, once a base of silica is established the rest can be more quickly. Load the product (dissolved in a minimum amount of 4:6 (v/v) EtOAc/Hexanes) with 4:6 (v/v) EtOAc/Hexanes and elute isocratically. Note: Change the eluent composition if the rf of the given substituted phenyl dimethanol deviates significantly from this report. Evaluate the purity of each fraction by TLC with 4:6 (v/v) EtOAc/Hexanes. Pool the pure fractions and concentrate the solution under reduced pressure on rotovap to a white or colored solid.

## 8. Conclusions

The development of any new chemical methodology should be driven by unmet needs reflected in the desire to advance the state of the art in a significant way. To that end, the method described herein satisfies the initially stated motivation: to develop a useful bio-orthogonal stapling reaction with the anticipation that beyond any novelty, it could readily be anticipated to provide biological activity. Indeed, in two test cases, precursors lacking any measureable biological activity were converted to FlICked macrocycles and bicycles that showed biological activity. When applied to a linear peptide highly decorated with potentially competitive side chains, FlICking is selective for a primary amine and a thiol and ignores

other potential nucleophiles. The reaction proceeds smoothly under biotic conditions and has shown to be compatible with proteins in the literature. Additionally, it adds useful functionality in the chemical moiety that is incorporated into the macrocycle in the form of nascent fluorescence. In a further testament to the robustness of this method, isoindole peptide stapling was independently demonstrated simultaneously to our report (Zhang, Zhang, Wong, & Li, 2019).

In concert with its demonstrable utility, FlICk resonates with the concept of "click" chemistry as articulated by Sharpless (Kolb, Finn, & Sharpless, 2001), namely: a reaction that ideally employs mild reagents at room temperature which produces benign byproducts and incorporates desired functionality into the starting materials in an atom-economical manner. The FlICk reaction herein described neatly fits into this schema: it employs commercially available OPA, runs at room temperature in water, requires no workup, produces only water as a byproduct and is complete on mixing. We hope that the operational ease of conducting these reactions, especially given the detail with which they have been described here, will motivate other researchers to employ this method when cross-linking peptides. Current limitations of the method include a lack of regioselectivity in FlICk reactions with some of the substituted OPAs. Preliminarily, we believe this work demonstrates the fertility of isoindole chemistry in the context of peptide stapling, particularly in the context of combinatorial peptide arrays that can be converted into fluorescent peptide macrocycles. We anticipate great potential in exploring this chemical space and suggest that its operational simplicity will entice additional investigation by others.

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