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Chemoselective, Regioselective, and Positionally Selective Fluorogenic Stapling of Unprotected Peptides for Cellular Uptake and Direct Cell Imaging

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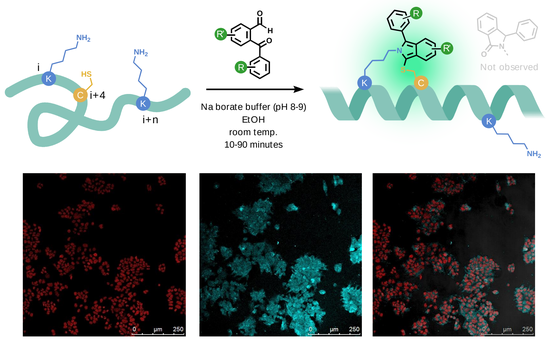
Abstract

In light of a growing need for regio- and positionally selective stapling methods involving natural amino acid residues in their unprotected states, we report a rapid, mild, and highly chemoselective three-component stapling reaction using a class of molecular linchpins based on 2-arylketobenzaldehydes (ArKBCHOs) that create a fluorescent staple, hereafter referred to as a Fluorescent Isoindole Crosslink (FIICk). This methodology offers:

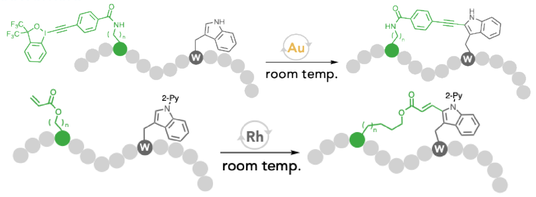
- Positional selectivity favoring *i,i + 4* helical staples comprising a lysine and cysteine, in the presence of competing nucleophiles on unprotected peptides.
- Excellent chemoselectivity, where isoindolanone side product was suppressed.
- Regioselective fluorescent isoindole staple
- Enhanced photophysical properties. Red-shifted excitation maxima and improved quantum yield. The resulting isoindole staple allows for direct cellular imaging in the qualitative assessment of peptide cellular uptake

Lastly, in our efforts to further validate this chemistry, we have successfully shown in vitro cytotoxicity of a FIICk-ed peptide (IC_{50} = 5.10 ± 1.27 mM), equipotent to an olefin-stapled congener, thus bridging therapeutic potential with cytological probe development.



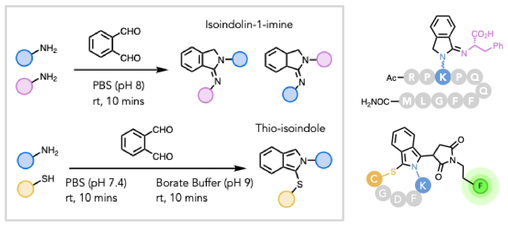
Introduction

Advances in fluorogenic peptide stapling: *Waser[1], Zhu[2], and coworkers*

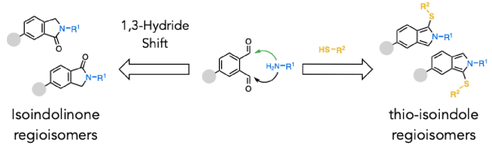


Metal catalyzed, involving unnatural amino acids

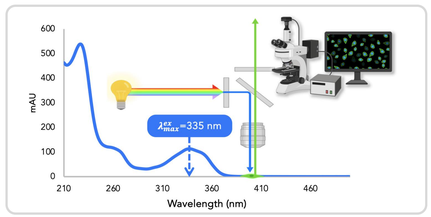
Advances in ortho-phthalaldehyde chemistry: *Li[3], Chen[4], Perrin[5], and coworkers*

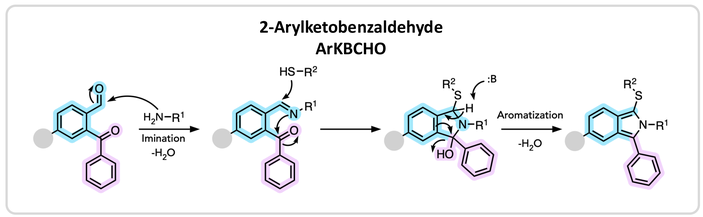


- **Issues:** Regioisomers, excess thiol, weak fluorescence and low excitation wavelength
- Incompatible with common cellular imaging techniques



Project Aim – Regioselective FIICk for Direct Cellular Imaging



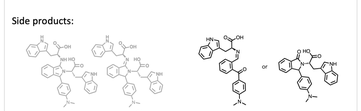


- Enhance the photophysical properties of thio-isoindole by **red-shifting its excitation wavelength and improving its quantum yield**
- Regioselective isoindole achieved with **2-Arylketobenzaldehyde as a molecular linchpin**. Aldehyde is replaced with a ketone

Results

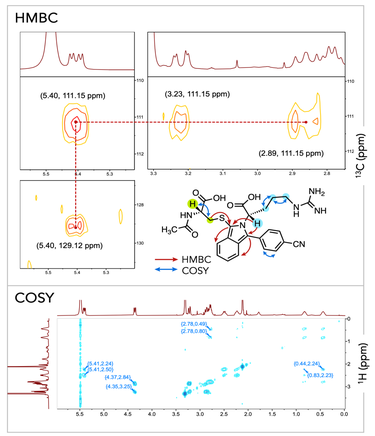
Optimization and Characterization of regioselective thio-isoindole

Table 1. Screening of conditions for the conversion of **6aa** to isoindole **7aa**



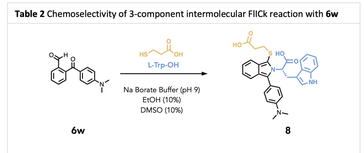
Entry	NAc-Cys	Additive : Solvent	% Conv. to **7aa** [d]
1[a]	1 eq. [b]	Na borate buffer pH 9	0%
2	3 eq. [b]	Na borate buffer pH 9	0%
3	1 eq. [b]	CH_3CN : Na borate buffer pH 9	0%
4	1 eq. [c]	DMSO : Na borate buffer pH 9	39%
5	1 eq. [c]	EtOH : Na borate buffer pH 9	66%

[a] Baseline run was obtained by injecting the crude reaction mixture immediately after all reagents were added, amounting to < 2 minutes of reaction time. [b] Prepared as 50mM solution in H_2O. [c] Prepared as 50mM solution in H_2O (0.1% formic acid). [d] % conversion was determined by HPLC peak integration of **7aa** relative to peaks observed in the baseline run (entry 1).



Chemoselectivity study

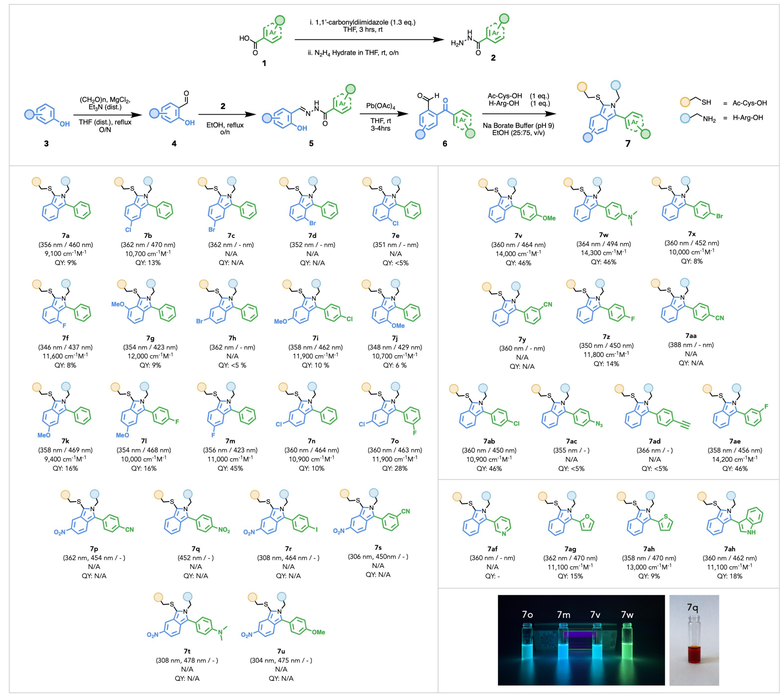
Table 2 Chemoselectivity of 3-component intermolecular FIICk reaction with **6w**



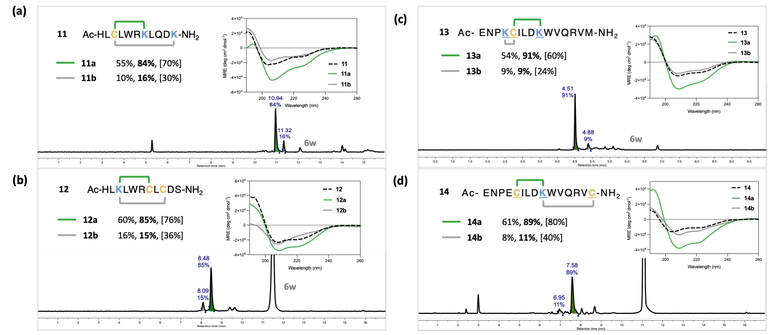
Entry	Equiv. Trp	Equiv. Thiol	Reaction time	Conversion (%) [a] 8	9a	9b	10a/10b
1	2.0	-	1 hour	0	0	0	0
2	5.0	-	5 hours	0	0	0	16
3	2.0	1.3	5 min	62	0	0	0
4	2.0	1.3	10 min	81	0	0	0
5	2.0	1.3	1 hour	90	0	0	0

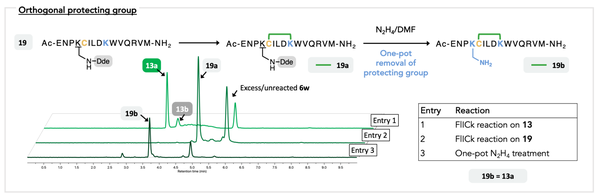
Chemoselectivity of 3-component intermolecular FIICk reaction with **6w**. [b]Percent conversion is calculated as the area under the curve of the chromatogram observed at 230nm relative to 1 equivalent of 6w.

Synthesis, scope, and photophysical properties of regioselective thio-isoindoles



Peptide Stapling showing positional selectivity





Entry	Reaction
1	FIICk reaction on **13**
2	FIICk reaction on **19**
3	One-pot N_2H_4 treatment

- ✓ **Chemoselective**
- ✓ **Regioselective**
- ✓ **Positional selective**

Biological Results

Jurkat Cell Viability comparable to RCM-stapled BIMBH3[6]

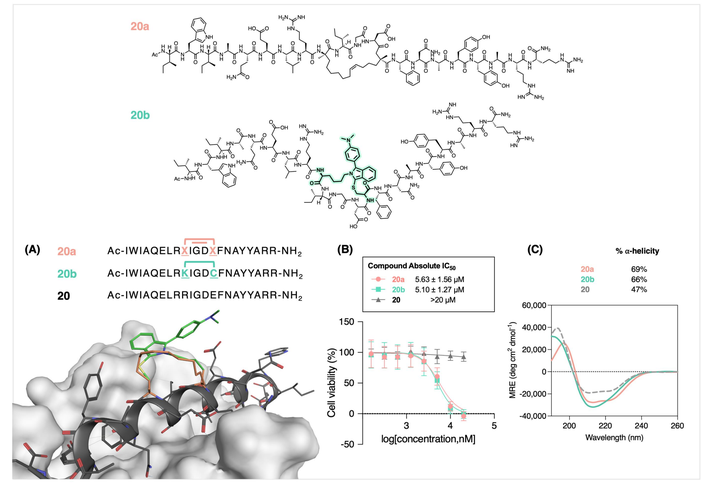


Figure 5: (A) Modelling **20a** with respect to **20b** binding to a target. **(B)** Comparative cell viability of Jurkat cells upon treatment of **20b** and positive control **20a** after a 24-hour dosing period. **(C)** Comparative CD. Samples were prepared as 50μM solutions in 2:8 TFE/H_2O.

Direct imaging of DLD-1 cells with FIICk-stapled Axin mimic[7]

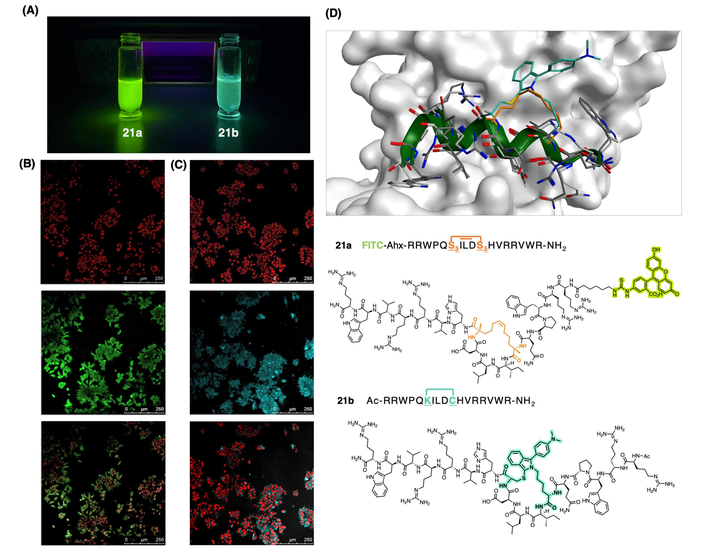


Figure 6 Solution of **21a** and **21b** in PBS Buffer (pH 7.4), 10% EtOH. **(B)** Imaging of cellular uptake (DLD1 cells) of **21a** FITC-labeled peptide (7.5μM, 1hr) and **(C) 21b** FIICk-peptide (5μM, 1hr) by confocal fluorescence microscopy. Overlaid images, in red: nuclear TO-PRO-3 Iodide, in green: FITC-labeled **21a** positive control, in blue: **21b** FIICk peptide. TO-PRO-3 was excited at 663nm, FITC at 488nm, and FIICk peptide at 405nm. **(D)** Molecular modelling of **21a** and **21b** (FITC not shown in the model). Orange bonds represent the olefin staple found in **21a**, and teal bonds represent the FIICk staple found in **21b**.

Future Directions

1. Exploring the potential for using thio-isoindole in **fluorescence polarization assay**
2. Post-FIICk reactions using **readily accessible conjugation handles** (e.g. COOH, CuAAc reaction with R–N_3, cross-coupling, etc.)
3. Investigating **potential on/off switch** found in our ArKBCHO library between EDG-EWG pairs: Reducing –NO_2 (**7q**) to –NH_2 may have turn-on fluorescence properties

References

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