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Oxidative Modification of Tryptophan-Containing Peptides

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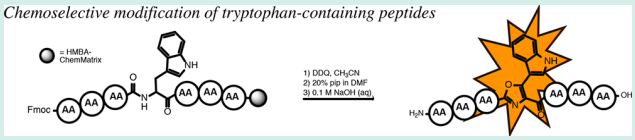
Supporting Information

ABSTRACT: We herein present a broadly useful method for the chemoselective modification of a wide range of tryptophan-containing peptides. Exposing a tryptophan-containing peptide to 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) resulted in a selective cyclodehydration between the peptide backbone and the indole side chain of tryptophan to form a fully conjugated indolyl-oxazole moiety. The modified peptides show a characteristic and significant emission maximum at 425 nm, thus making the method a useful strategy for fluorescence labeling.



KEYWORDS: *solid-phase peptide synthesis, fluorescent labeling, tryptophan, site-selective protein modification*

INTRODUCTION

Fluorescence labeling of proteins and peptides is fundamental for the study of biological systems, as it can provide detailed visualization of complex cellular processes.[1] The visualization of biological processes has been crucial for our understanding of molecular dynamics and the development of new potent drugs. Nowadays, the most common approach to fluorescence labeling of proteins comprises the introduction of fluorescent small molecules to the nucleophilic side chain of lysine, serine, threonine, or cysteine residues in a peptide or protein of interest.[2] However, such strategies often suffer from poor site selectivity, where multiple residues are modified. Though less established, chemoselective functionalization of other residues, such as methionine,[3] glutamine,[4] arginine,[5] *N*-terminal serine/threonine,[6] tyrosine,[7] and tryptophan,[8] has been described. Among these residues, tryptophan is particularly interesting because of its scarce abundance in proteins. With a natural abundance of only 1.09%,[9] many proteins of interest will contain only a single or few tryptophan residues accessible for functionalization, thus enabling high control of the position for modification. Furthermore, the relative large size of organic dyes, including undesired physiochemical properties may give rise to several challenges, that compromise the biologically activity of the labeled target. Therefore, labeling strategies that introduce minimal structural perturbation to the peptide of interest is of high importance.

Herein, we describe our efforts toward the oxidative modification of small peptides containing tryptophan. The conjugated nature of the generated indolyl-oxazole moiety emits blue-fluorescence,[10] which may advantageously be utilized for spectroscopic studies of biological systems. For instance, the indolyl-oxazole moiety of diazonamide A derivatives has been utilized as intrinsic fluorophores for in vitro cellular uptake studies.[11] In addition, the indolyl-oxazole scaffold is present in a variety of naturally occurring biologically active compounds including those shown in Figure 1,[12−15] as well as cyclic derivatives such as the diazonamides.[11]

RESULTS AND DISCUSSION

Using standard reagents for solid-phase peptide synthesis, the HMBA linker was easily immobilized and synthetically elaborated on an amino-functionalized ChemMatrix resin (Table 1). Initially, a range of conditions for the oxidative cyclodehydration of model compound **6** (Scheme 1) was examined.[16] Oxidation of the α-carbon of indoles has been performed with the dehydrogenating agent DDQ to form the keto-indole derivative.[16] Therefore, it was expected that DDQ could be a suitable reagent for the oxidative cyclodehydration of tryptophan. Furthermore, cyclodehydration of keto-indoles has been carried out with a mixture of triphenylphosphine, metallic iodine, and triethylamine in CH_2Cl_2, to form a conjugated indolyl-oxazole moiety.[17] Various solvents were screened (entries A−G), and it was disclosed that exposure of a tryptophan-containing oligopeptide to DDQ (4 equiv) in MeCN led to near-quantitative conversion into the desired indolyl-oxazole product **9** (Scheme 1). Interestingly, the peptide was fully converted to the desired indolyl-oxazole derivative with only two equivalents of DDQ (entry J). The reaction most likely occurs via the α,β-unsaturated imine **7** (Scheme 1). However, in reactions where partial formation of the ketone product **8** was initially observed, it was noted that prolonged reaction times resulted in full conversion to the desired cyclo-dehydrated product **9**.

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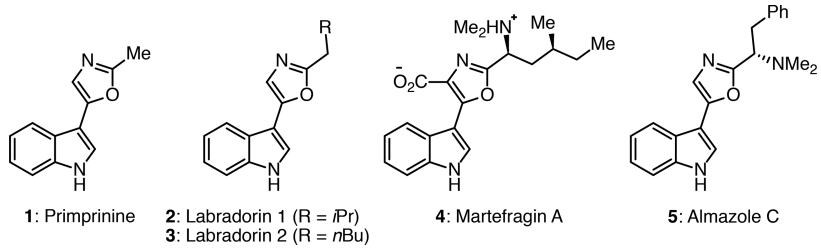
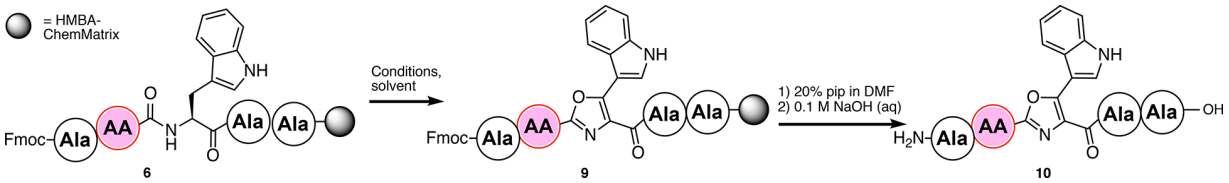


Figure 1. Biologically active indolyl-oxazole natural products.

Table 1. Chemical Data for the Indolyl-oxazoles 10A−AQ



entry	AA	conditions	solvent	purity[a] (%)	yield[b] (%)	entry	AA	conditions	solvent	purity[a] (%)	yield[b] (%)
A	Ala	DDQ (4 equiv)	toluene	20		W	Asp(OBzl)	DDQ (2 equiv)	CH_3CN	48	
B	Ala	DDQ (4 equiv)	THF	40		X	Asp(O*t*Bu)	DDQ (2 equiv)	CH_3CN	81	30
C	Ala	DDQ (4 equiv)	CH_2Cl_2	60		Y	Glu	DDQ (2 equiv)	CH_3CN	64	23
D	Ala	DDQ (4 equiv)	DMF	5		Z	Glu(O*t*Bu)	DDQ (2 equiv)	CH_3CN		14
E	Ala	DDQ (4 equiv)	CH_3CN	90		AA	Cys(*t*Bu)	DDQ (2 equiv)	CH_3CN	66	17
F	Ala	DDQ (4 equiv)	H_2O	0		AB	Cys(S*t*Bu)	DDQ (2 equiv)	CH_3CN	34	
G	Ala	DDQ (4 equiv)	MeOH	5		AC	Ser	DDQ (2 equiv)	CH_3CN	81	15
H	Ala	tetrachloro-1,4-benzoquinone (4 equiv)	CH_3CN	0		AD	Ser(OBn)	DDQ (2 equiv)	CH_3CN	77	19
I	Ala	DDQ (3 equiv)	CH_3CN	89		AE	Gln	DDQ (2 equiv)	CH_3CN	91	12
J	Ala	DDQ (2 equiv)	CH_3CN	88	25	AF	Gln(Trt)	DDQ (2 equiv)	CH_3CN	>95	
K	Ala	DDQ (1 equiiv)	CH_3CN	65		AG	Lys	DDQ (2 equiv)	CH_3CN	0	
L	Gly	DDQ (2 equiv)	CH_3CN	86	31	AH	Lys(Boc)	DDQ (2 equiv)	CH_3CN	>95	13
M	Val	DDQ (2 equiv)	CH_3CN	75	15	AI	His	DDQ (2 equiv)	CH_3CN	80	10
N	Leu	DDQ (2 equiv)	CH_3CN	77	20	AJ	His(Boc)	DDQ (2 equiv)	CH_3CN	53	
O	Ile	DDQ (2 equiv)	CH_3CN	75	17	AK	Thr	DDQ (2 equiv)	CH_3CN	62	12
P	Pro	DDQ (2 equiv)	CH_3CN	77	31	AL	Thr(O*t*Bu	DDQ (2 equiv)	CH_3CN	69	13
Q	Phe	DDQ (2 equiv)	CH_3CN	63	24	AM	Asn	DDQ (2 equiv)	CH_3CN	>95	
R	Met	DDQ (2 equiv)	CH_3CN	76	40	AN	Asn(Trt)	DDQ (2 equiv)	CH_3CN	62	25
S	Tyr	DDQ (2 equiv)	CH_3CN	81	25	AO	Arg(Pmc)	DDQ (2 equiv)	CH_3CN	62	18
T	Tyr(All)	DDQ (2 equiv)	CH_3CN	38	16	AP	Ala-Trp-Gly-Pro-Trp-Leu	DDQ (2 equiv)	CH_3CN	82	
U	Tyr(OMe)	DDQ (2 equiv)	CH_3CN	92	18	AQ	Ala-Trp-Val-Trp-Ile-Trp-Phe	DDQ (3 equiv)	CH_3CN	75	
V	Asp	DDQ (2 equiv)	CH_3CN	45	15						

[a]Crude purities. [b]All compounds were purified by prepHPLC before yield determination and NMR analysis.

The sequence tolerance of the site-selective tryptophan oxidation protocol was investigated through the synthesis of a combinatorial library of natural amino acids and common protective groups (entry L-AO). Gratifyingly, the developed

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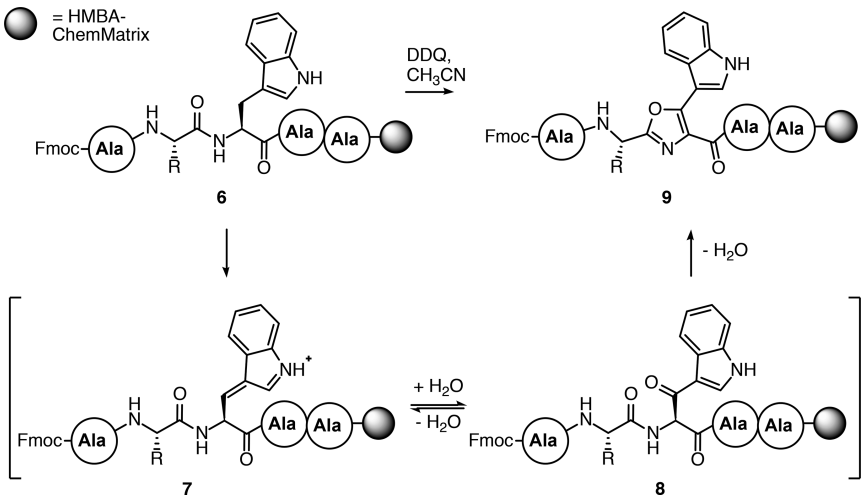
ACS Comb. Sci. XXXX, XXX, XXX−XXX

Scheme 1. Synthesis of Indolyl-oxazole-Functionalized Peptide 9



Scheme 2. Synthesis of Peptides 11(J,L-AO)

protocol showed compatibility with a wide range of peptides and generally only the desired product was observed by UPLCMS (see Supporting Information). Unfortunately, nonprotected lysine residues were not tolerated (entry AG). Here a range of byproducts was observed by UPLC, including a nucleophilic addition of the lysine side-chain amino group to the conjugated imine (7) as well as a Michael reaction between the amino group and DDQ.

The isolated yields of indolyl oxazole peptides are in the range typically observed for solid-phase synthesis followed by preparative HPLC purification. From our results, we cannot identify a correlation between purity and isolated yields neither is there apparent structure-yield correlation.

Furthermore, the methodology was investigated for peptides containing more than one tryptophan residue (entry AP−AQ). Using two and three equivalents of DDQ, respectively, peptides containing two or three indolyl-oxazole moieties were obtained (see Supporting Information).

In order to investigate the potential of the technique for fluorescent labeling, the fluorescence properties of the indolyl-oxazole containing peptide **10(J,L-AO)** was measured and compared to the emission spectrum of the corresponding nonoxidized peptides **11(J,L-AO)**, Scheme 2.

As shown in Figure 2, the indolyl-oxazole containing peptides show a remarkably change in fluorescence with a distinct band now appearing at 425 nm. Importantly, this absorption is not affected by the presence of aromatic side chain functionalities in naturally occurring amino acids.

Having identified conditions allowing for oxidative cyclodehydration of tryptophan in various peptides, we sought to demonstrate the use of this methodology in the labeling of biological relevant peptides. GLP-1 is a 30 amino acid-containing peptide hormone that possesses several pharmacological properties, making it a subject of intensive investigation. Gratifyingly, when exposing GLP-1(**12**), to the DDQ conditions the desired fluorescence labeled indolyl-oxazole analog **13** was formed (Figure 2) with a satisfactory conversion of 85%, as confirmed by HPLC.

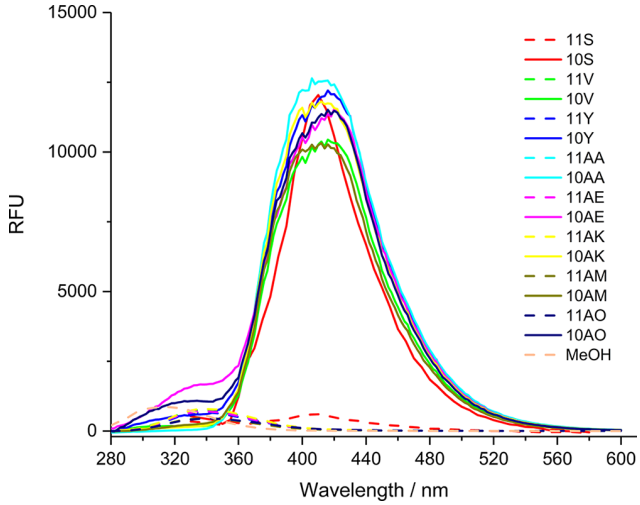


Figure 2. Fluorescence measurement of compound **10J,L-AO** and **11J,L-AO**.

Currently, the methodology has only been demonstrated for immobilized peptides that tolerate acetonitrile. It would be desirable to adapt this chemistry to aqueous conditions, thereby allowing indolyl-oxazole formation in proteins. This would

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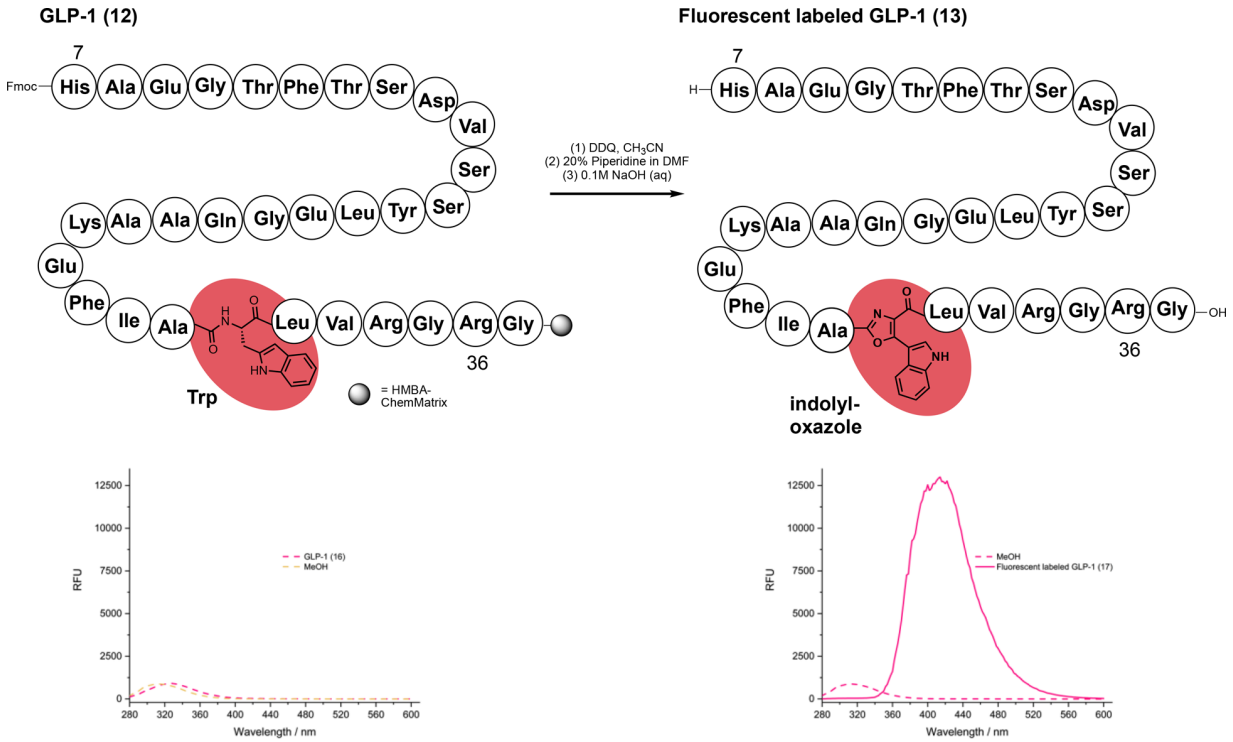

Figure 3. Fluorescent labeling of GLP-1.

require the development of a more stable dehydrogenation reagent, which resists hydrolysis in aqueous solutions.

CONCLUSIONS

In summary, we report a method that allows for the chemoselective labeling of tryptophan-containing peptide residues. DDQ-mediated oxidative cyclization leads to the installation of an indolyl-oxazole moiety with unique fluorescence properties. We further demonstrate that the indolyl-oxazole moiety selectively may be installed in a pharmaceutically relevant peptide, thereby emphasizing the important potential of the methodology to illuminate biological mechanism of relevance to drug discovery.

EXPERIMENTAL SECTION

General Methods. All reagents and materials used were purchased from ordinary chemical suppliers and were used without purification. The solvents used were of standard HPLC grade. Solid-phase synthesis was carried out using plastic-syringe techniques. Flat-bottomed PE-syringes were fitted with PP-filters and situated in Teflon valves equipped with Teflon tubing allowing for a moderate vacuum to be applied to the syringes.

Yields of solid-phase synthesis protocols are corrected for salt contents and given as percentage of product mass recovery to the theoretical product loading mass, calculated from the resin loading (4 mmol/g) as specified by the supplier.

Products were analyzed on a Waters Alliance reverse-phase HPLC system consisting of a Waters 2695 Separations Module equipped with a Symmetry C18 column (3.5 μm, 4.6 × 75 mm, column temp 25 °C, flow rate 1 mL/min) and a Waters Photodiode Array Detector (detecting at 215 nm). Elution was carried out in a linear reversed phase gradient fashion (gradient A: 0% organic for 0.2 min, 0% organic to 100% organic in 10 min, hold for 1 min, 100% organic to 0% organic in 0.3 min, hold for 1.5 min, gradient B: 0% organic for 0.2 min, 0% organic to 40% organic in 10 min, 40% organic to 100% organic in 0.8 min, hold for 1 min, 100% organic to 0% organic in 0.3 min, hold for 1.5 min) combining water and acetonitrile (buffered with 0.1% (v/v) TFA).

Preparative RP-HPLC was carried out on a Waters Alliance reverse-phase HPLC system consisting of a Waters 2545 Binary Gradient Module equipped with an xBridge TM.

Prep BEH130 C18 column OBDTM (5 μm, 19 × 100 mm, column temp 25 °C, flow rate 20 mL/min), a Waters Photodiode Array Detector (detecting at 210−600 nm), a Waters UV Fraction Manager and a Waters 2767 Sample Manager. Elution was carried out in a linear reversed phase gradient fashion combining water and acetonitrile (buffered with 0.2% (v/v) TFA).

1D and 2D NMR spectra were recorded using a Varian Unity Inova-500 MHz, a Varian Mercury-300 MHz instrument, a Bruker Ascend-400 MHz instrument equipped with a 5 mm Prodigy cryoprobe or a Bruker Avance-800 MHz instrument, equipped with a 5 mm cryoprobe TCI, in DMSO-d_6 or $CDCl_3$ using the residual DMSO or $CHCl_3$ solvent peaks, respectively, as the internal standard. All ^{13}C NMR spectra were proton decoupled. DQF-COSY, HSQC, HMBC, and 2D NOESY spectra were acquired using standard pulse sequences.

LC-DAD-HRMS was performed on an Agilent 1100 LC system equipped with a Agilent Technologies Diode Array Detector and a Luna C18 column (3 μm, 50 mm × 2 mm, column temp 40 °C, flow rate 400 μL/min). Separation was achieved using a linear reversed phase gradient (20% to 100% organic in 8 min, hold for 2 min, 100% to 20% organic in 1 min, hold for 4 min) again combining water and acetonitrile (buffered with 20 mM HCO_2H). The LC was coupled to a Micromass LCT orthogonal time-of-flight mass spectrometer,

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ACS Comb. Sci. XXXX, XXX, XXX−XXX

equipped with Lock Mass probe and operated in positive electrospray mode.

General Solid-Phase Procedures. The commercial available amino functionalized ChemMatrix (0.4 mmol/g) was washed with DMF. Coupling of the first amino acid building block to the resin was carried out as follows. Dry resin was weighed in a syringe, equipped with a PP-filter. The amino acid (3 equiv) was weighed, dissolved in DMF (0.02 mL/mg resin) and *N*-ethylmorpholine (4 equiv) was added using a microliter pipet. *N*-[(1*H*-Benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide (TBTU, 2.9 equiv) was weighed and likewise added. The solution was transferred to the resin, the swelled resin stirred gently with a spatula and allowed to react for 2 h. The resin was filtered, washed with DMF (×6) and CH_2Cl_2 (×6) and lyophilized. The Fmoc-group was removed by swelling the resin in a solution of piperidine (20% v/v) in DMF for 2 min, filtering and then swelling the resin again in a fresh solution of piperidine (20% v/v) in DMF for 18 min. The resin was washed with DMF (×6) and CH_2Cl_2 (×6) and lyophilized.

The oxidatively modified peptides were liberated from the HMBA-functionalized ChemMatrix resin by addition of 4 mL of 0.1 N aqueous NaOH. The syringes were left overnight under vigorous shaking followed by neutralization with 0.1 N HCl (aq). The aqueous solutions containing the peptides were collected by filtration and the resins were washed with water (×5) and MeCN (×5). The purity of the crude reaction mixture was monitored by UPLC-MS. The MeCN and water was removed by evaporation and freeze-drying. The residue was redissolved in 3 mL of DMF, filtrated and purified by preparative RP-HPLC. The solvent was removed from the product-containing fractions by evaporation and freeze-drying before NMR data collection and measurement of the fluorescence properties using a Tecan microplate reader.

Evaluation of Spectroscopic Properties. The fluorescence experiments were conducted by soluting each of the peptides in methanol. The fluorescence of the peptides was monitored using a Tecan microplate reader, which first records the absorbance properties to identify the required wavelength for excitation of the compound. The fluorescence was then measured in the arbitrary unit 'Relative Fluorescence Units' (RFU) and plotted against their respective wavelengths. The measurements were acquired setting the gain to 70.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscombsci.8b00014.

Analytical data (^{1}H and ^{13}C NMR spectra and LC-MS chromatograms) of all compounds synthesized (PDF)

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Notes

The authors declare no competing financial interest.

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