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1 **Oxidative Modification of Tryptophan-Containing Peptides**

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7 **S** Supporting Information

8 **ABSTRACT:** We herein present a broadly useful method for
 9 the chemoselective modification of a wide range of
 10 tryptophan-containing peptides. Exposing a tryptophan-
 11 containing peptide to 2,3-dichloro-5,6-dicyano-1,4-benzoqui-
 12 none (DDQ) resulted in a selective cyclodehydration between
 13 the peptide backbone and the indole side chain of tryptophan
 14 to form a fully conjugated indolyl-oxazole moiety. The modified peptides show a characteristic and significant emission maximum
 15 at 425 nm, thus making the method a useful strategy for fluorescence labeling.

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

Chemoselective modification of tryptophan-containing peptides

17 **INTRODUCTION**

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

43 Herein, we describe our efforts toward the oxidative
 44 modification of small peptides containing tryptophan. The
 45 conjugated nature of the generated indolyl-oxazole moiety
 46 emits blue-fluorescence,¹⁰ which may advantageously be
 47 utilized for spectroscopic studies of biological systems. For
 48 instance, the indolyl-oxazole moiety of diazonamide A
 49 derivatives has been utilized as intrinsic fluorophores for in
 50 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

21 **RESULTS AND DISCUSSION**

22 Using standard reagents for solid-phase peptide synthesis, the
 23 HMBA linker was easily immobilized and synthetically
 24 elaborated on an amino-functionalized ChemMatrix resin
 25 (Table 1). Initially, a range of conditions for the oxidative
 26 cyclodehydration of model compound 6 (Scheme 1) was
 27 examined.¹⁶ Oxidation of the α -carbon of indoles has been
 28 performed with the dehydrogenating agent DDQ to form the
 29 keto-indole derivative.¹⁶ Therefore, it was expected that DDQ
 30 could be a suitable reagent for the oxidative cyclodehydration of
 31 tryptophan. Furthermore, cyclodehydration of keto-indoles has
 32 been carried out with a mixture of triphenylphosphine, metallic
 33 iodine, and triethylamine in CH_2Cl_2 , to form a conjugated
 34 indolyl-oxazole moiety.¹⁷ Various solvents were screened
 35 (entries A–G), and it was disclosed that exposure of a
 36 tryptophan-containing oligopeptide to DDQ (4 equiv) in
 37 MeCN led to near-quantitative conversion into the desired
 38 indolyl-oxazole product 9 (Scheme 1). Interestingly, the
 39 peptide was fully converted to the desired indolyl-oxazole
 40 derivative with only two equivalents of DDQ (entry J). The
 41 reaction most likely occurs via the α,β -unsaturated imine 7
 42 (Scheme 1). However, in reactions where partial formation of
 43 the ketone product 8 was initially observed, it was noted that
 44 prolonged reaction times resulted in full conversion to the
 45 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

● = HMBA-ChemMatrix

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

^aCrude purities. ^bAll compounds were purified by prepHPLC before yield determination and NMR analysis.

79 The sequence tolerance of the site-selective tryptophan
80 oxidation protocol was investigated through the synthesis of a

combinatorial library of natural amino acids and common 81
protective groups (entry L-AO). Gratifyingly, the developed 82

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

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

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

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.

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

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 μ m, 4.6 $\times$ 75 mm, column temp 25 $^{\circ}$ 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 μ m, 19 $\times$ 100 mm, column temp 25 $^{\circ}$ 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 CDCl₃ using the residual DMSO or CHCl₃ solvent peaks, respectively, as the internal standard. All ¹³C 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 μ m, 50 mm $\times$ 2 mm, column temp 40 $^{\circ}$ C, flow rate 400 μ L/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 HCO₂H). The LC was coupled to a Micromass LCT orthogonal time-of-flight mass spectrometer, 190

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 CH₂Cl₂ (×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 CH₂Cl₂ (×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 (¹H and ¹³C 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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