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Communication

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Mild and Selective Mono-Iodination of Unprotected Peptides as Initial Step for the Synthesis of Bioimaging Probes

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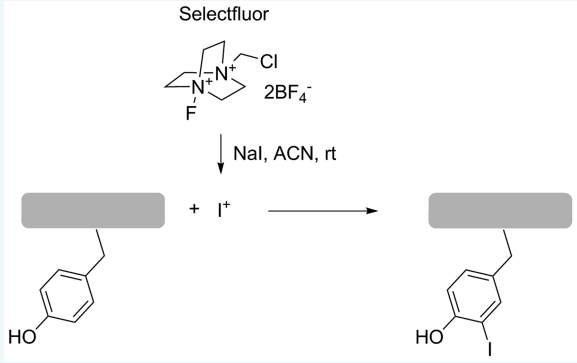
Supporting Information

ABSTRACT: Chemoselective functionalization of peptides and proteins to selectively introduce residues for detection, capture, or specific derivatization is of high interest to the synthetic community. Here we report a new method for the mild and effective mono-iodination of tyrosine residues in fully unprotected peptides. This method is highly chemoselective and compatible with a wide variety of functional groups. The introduced iodine can subsequently serve as a handle for further functionalization such as introduction of fluorescent dyes and thus be used for chemoselective labeling of isolated peptides.



INTRODUCTION

While incorporation of conjugation sites into small peptides can be easily achieved at will by selection of appropriately functionalized amino acid building blocks for solid phase peptide synthesis, selective functionalization of isolated peptides and proteins not containing protecting groups still remains a challenging task. Among the 20 naturally occurring amino acids, the most relevant ones for bioconjugation purposes are cysteine and lysine, as they can act as potent nucleophiles.[1] However, the high abundance of lysine on protein surfaces makes specific acylation challenging, and nonoxidized cysteines are less frequently displayed on protein surfaces. As they are most often involved in disulfide bridges in their natural environment, reduction of the target disulfide is a prerequisite for labeling, which may also alter the overall structure of the target protein. Therefore, the development of additional modification techniques targeting alternative amino acids remains of high significance.

Tyrosine offers a wide range of reactivities: the phenolate ring may be O-alkylated;[2] it can undergo electrophilic addition using diazonium derivatives[3] or can be involved in Mannich-type condensations[4] and reactions with ene-type electrophiles.[5] Owing to the electron-rich aromatic ring, tyrosine residues can also be a target for halogenation. Notably, iodination is of particular importance due to its utility for further transformations. It has been used to label biomolecules for a wide variety of purposes, such as mass spectrometry,[6] to help in the elucidation of foldamer structures[7] or to improve the self-assembly properties of peptides.[8] Moreover, tyrosine iodination with ^{125}I for the radiolabeling of compounds of medical and biological interest is the method of choice owing to its high specific radioactivity and convenience in counting γ-emissions.[9]

Iodinations are carried out either enzymatically, for example, by the enzyme thyroid peroxidase[10] or by direct electrophilic aromatic substitution using an iodinating agent such as N-iodosuccinimide[11] or Barluenga's reagent.[12] Other alternatives involve the combination of sodium iodide with strong oxidizing agents like Iodogen[13,14] or Chloramin-T.[13,15] However, existing methods display limitations as iodination frequently results in a mixture of unreacted starting material, mono- and di-iodinated peptides,[9,11,13−20] which reduce yields and may also impose challenges in product purification. Formation of oxidized peptides[9,16,19] and histidine labeling[9,20,21] have also been described as limiting factors. For most applications a mono-iodinated version of the compound of interest would be highly desirable in order to minimize the negative influence of the added substituents on the activity of the target biomolecule. Indeed, examples were reported where the di-iodinated peptide exhibited a 3- to 10-fold lower receptor binding activity whereas the binding potency of the mono-iodinated was maintained.[17,18] Accordingly, further investigations toward a selective iodination method yielding selectively a mono-iodinated tyrosine are of high interest. Here we present a

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new and efficient iodinating agent allowing highly controlled mono-iodination reactions and demonstrate its utility in the preparation of a wide range of fully unprotected and structurally diverse biologically active peptides.

RESULTS AND DISCUSSION

We explored the combination of sodium iodide and Selectfluor in the search of a mild and selective iodinating reagent for tyrosine (Figure 1, Figure S1). We first examined the iodination

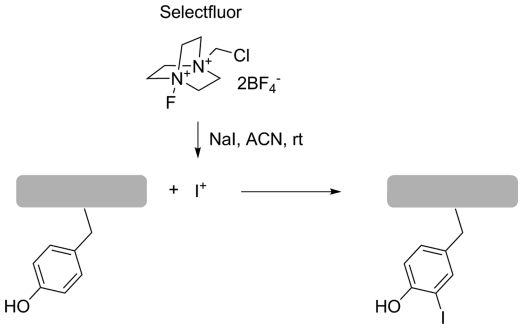
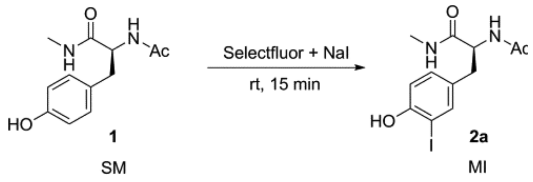


Figure 1. Iodination of tyrosine with sodium iodide activated by Selectfluor.

reaction in different solvents on the protected tyrosine Ac-Tyr-NH-Me **1** as a model system to mimic the reactivity of a tyrosine incorporated in a peptide sequence. The reaction employing a slight excess (1.1 equiv) of Selectfluor and NaI proceeded instantly at room temperature, affording a mixture of unreacted starting material **1** (SM), mono-iodinated product (**2a**, MI), and di-iodinated product (**2b**, DI). Extending the reaction time to 120 min did not have an impact on the conversion (Figure S2). A strong influence of the solvent and pH was observed (Table 1). Aprotic polar solvents (entries 1, 2) or aqueous systems (entries 3 to 6) favored the formation of the di-iodinated product **2b**. Dichloromethane supplemented with TFA (entries 7 to 11) proved to be the optimal conditions to achieve a controlled mono-iodination. Addition of TFA allowed proper dissolution of the starting material **1** but, more importantly, positively modulated the iodination reactivity. Besides, most peptides are soluble in mixtures of 1−20% TFA/DCM; these conditions are commonly used for cleavage of resins with hyperacid labile linker systems like SASRIN, HAL, or chlorotrityl.

Table 1. Solvent Screening for the Mono-Iodination of 1



entry	solvent	TFA	SM **1**[a] (%)	MI **2a** (%)	DI **2b** (%)
1	DMSO	-	49	7	44
2	DMF	-	45	24	31
3	tBuOH/H_2O (5/1)	-	17	57	26
4	MeOH/DCM	-	23	54	23
5	H_2O/ACN (1/1)	-	22	44	33
6	H_2O/ACN (1/1)	10% TFA	27	57	16
7	DCM	1% TFA	46	48	6
8	DCM	5% TFA	46	53	1
9	DCM	10% TFA	47	53	-
10	DCM	20% TFA	54	46	-
11[b]	DCM	20% TFA	6	86	8

[a]SM = Starting material, MI = Mono-iodinated product, DI = Di-iodinated product. Relative ratio dertermined by LC/MS at 215 nm. [b]After extra addition of 2 × 0.25 equiv of iodinating reagent.

We assume that, after conversion of the iodide anion to an electrophilic iodonium species I^+ by Selecfluor, the reaction proceeds via an electrophilic substitution mechanism as previously postulated by Syvret et al.[22] We further applied the iodination reaction to other amino acids containing aromatic residues, phenylalanine, tryptophane, and histidine, that are also prone to undergo electrophilic aromatic substitutions. However, under these conditions no iodination of these amino acids was observed (Figure S3). We then investigated the optimized iodination conditions on multifunctional, fully unprotected peptides. A total of eight biologically relevant peptides, either commercially available or prepared in our laboratory, were surveyed: Leucine-Enkephalinamide (agonist of μ and δ opioid receptors), Angiotensin III (agonist of AT_1 and AT_2 receptors), Cyclo(RGDyK) (high affinity $\alpha v\beta 3$ integrin ligand), ACP fragment (65−74) (fragment of the acyl carrier protein), Goserelin (superagonist of LH-releasing hormone), Tocinoic acid (agonist of the oxytocin receptor), AcMeYVAD-CHO (reversible inhibitor of caspase-1), and [Tyr^0]-Bradykinin (ligand of the kinin B_1 and B_2 receptors) (Table 2, entries 1−8). The peptides studied were structurally diverse and collectively included (i) different aromatic amino acids, (ii) a free acid at the C-terminus, (iii) a disulfide bridge between two cysteines, (iv) cyclic structures, (v) non-natural amino acids, and (vi) an aldehyde function. Since the typical protein absorbance at 280 nm can shift to a maximum around 315 nm due to the iodination of the tyrosine phenolate ring,[23] we decided to carry out the analysis via LC-MS at 215 nm absorbance to quantify the relative amounts of starting material (SM), mono-iodinated product (MI) and di-iodinated product (DI). In all cases, the mono-iodinated product was the predominant species with a relative ratio of 88% to 97% as outlined in Table 2. The position of the tyrosine in the sequence did not seem to have an influence on the mono-iodination efficacy. An example is shown in Figure 2, the mild mono-iodination reaction proceeded cleanly in the presence of a tryptophane residue, an aza-peptide moiety, and a *tert*-butyl protected serine which remained untouched. Interestingly, this method delivered superior results compared to a previously reported approach using *N*-iodosuccinimide in the case of Cyclo(RGDyK).[11]

Next, we applied our method to a peptide containing a methionine residue: [Tyr^8]-Substance P (ligand of the neurokinin-1 receptor, entry 9). The ability of sulfur to react with halonium in acidic conditions is known and widely exploited, e.g., in glycosylation chemistry where thioglycosides can serve as glycoside donors. As expected, when reacting the peptide with the first equivalent of iodination reagent, we observed oxidation at the methionine residue, but also formation of the desired mono-iodinated product (around 25%). However, total consumption of both starting material and oxidized starting material was reached by careful addition of excess iodination reagent (2 × 0.35 equiv) to afford an almost exclusive mixture of the mono-iodinated product and its oxidized methionine analog (Figure 3, Figure S4). We searched

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Table 2. Mono-Iodination of Multifunctional, Fully Unprotected Peptides with Selecfluor and NaI

Entry	Peptide	Sequence	Results[a]	Yield[b]
1	AcMeYVAD-CHO	Ac-(NMe)**Tyr**-Val-Ala-Asp-CHO	SM: 8.3% / **MI: 91.7%** / DI: - %	[c]
2	Tocinoic acid	H-Cys-**Tyr**-Ile-Gln-Asn-Cys-OH	SM: 5.5% / **MI: 93.1%** / DI: 1.4%	[c]
3	Goserelin	Glp-His-Trp-Ser-**Tyr**-Ser(tBu)-Leu-Arg-Pro-azaGly-NH_2	SM: - % / **MI: 97.4%** / DI: 2.6%	[c]
4	Leu-Enkephalinamide	H-**Tyr**-Gly-Gly-Phe-Leu-NH_2	SM: 1.0% / **MI: 88.2%** / DI: 10.8%	62%
5	Angiotensin III	H-Arg-Val-**Tyr**-Ile-His-Pro-Phe-NH_2	SM: 6.2% / **MI: 91.3%** / DI: 2.5%	74%
6	ACP fragment (65-74)	H-Val-Gln-Ala-Ala-Ile-Asp-**Tyr**-Ile-Asn-Gly-NH_2	SM: 2.3% / **MI: 93.7%** / DI: 4.0%	63%
7	Cyclo(RGDyK)	Cyclo(Arg-Gly-Asp-D-**Tyr**-Lys)	SM: 5.5% / **MI: 88.3%** / DI: 6.2%	77%
8	[Tyr^0]-Bradykinin	H-**Tyr**-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	SM: 1.9% / **MI: 92.7%** / DI: 5.4%	72%
9	[Tyr^8]-Substance P	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-**Tyr**-Gly-Leu-Met-NH_2 [d]	SM: 5.9% / **MI: 93.6%** / DI: 0.5%	72%
10	GLP-1 (7-37)	H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-**Tyr**-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Gly-NH_2	SM: 4.5% / **MI: 91.3%** / DI: 4.1%	52%

[a]Reactions were monitored using LC-MS and the relative amounts of starting material (SM), mono-iodinated product (MI), and the diiodination product (DI) were quantified using area under the curve integration (absorbance at 215 nm). [b]Isolated yields after HPLC purification. [c]Not isolated. [d]Methionine oxidation was detected as described in Figure S4.

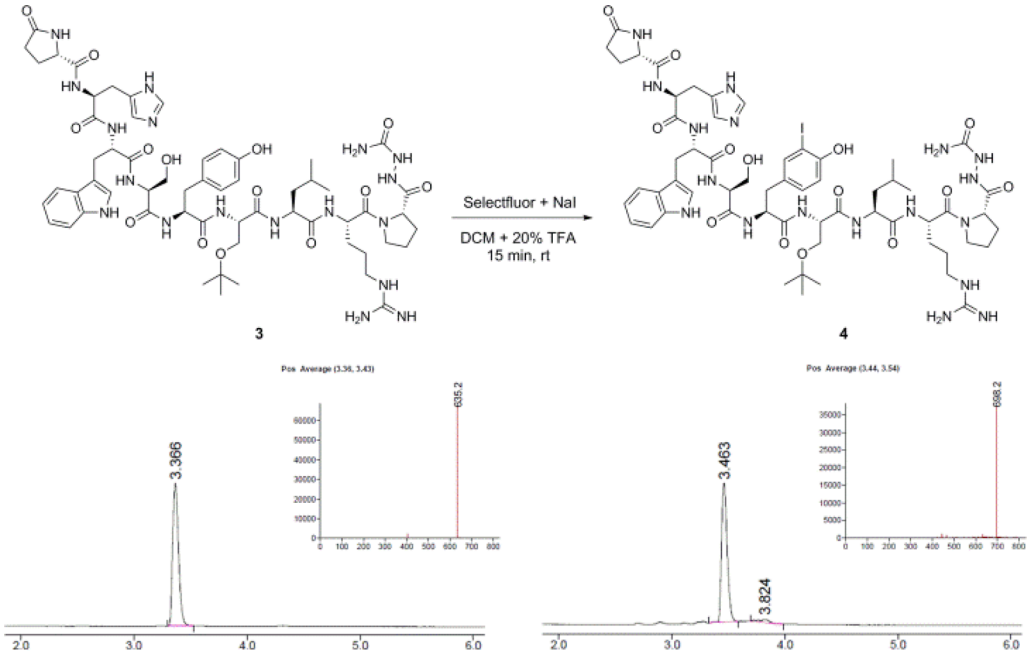

Figure 2. Mono-iodination of Goserelin with Selectfluor and NaI.

for a strategy to effectively reduce the oxidized mono-iodinated product, while keeping the reduced version intact. Several reagents have been reported to reduce methionine sulfoxide residue in peptides, e.g., trimethylsilyl bromide with 1,2-ethanedithiol, *N*-methylmercaptoacetamide, tetrabutyl ammonium bromide, or ammonium iodide with dimethylsulfide. Treatment of the peptide reaction mixture with either Bu_4NBr or NH_4I−Me_2S in TFA was successful in reducing the oxidized methionine but led to formation of byproducts, rendering these conditions inefficient. Finally, the use of potassium iodide and ascorbic acid in TFA[24] offered a very clean conversion of the methionine sulfoxide to the desired reduced peptide (Figure 3, Figure S4). Nicolas et al.[25] reports this reduction to proceed via nucleophilic iodide attack on the protonated sulfoxide leading to the methionine sulfide and elemental iodine. The latter directly reacts with the ascorbic acid, with the consumption of the generated iodine driving further the reduction forward.[24]

To verify the relevance of the method, mono-iodinations of Cyclo(RGDyK), Leucin-Enkephalinamide, [Tyr^0]-Bradykinin, Angiotensin III, ACP-fragment 65−74 and [Tyr^8]-Substance P were performed on different scales varying from 1 mg up to 35 mg (1 to 65 μmol). After isolation by HPLC, yields between 62% and 77% were obtained. Characterization by UPLC, HRMS, and ^1H−^{13}C NMR confirmed the identity of the desired mono-iodinated products, including the desired introduction of the iodine atom in ortho position of the phenol, as expected. Additionally, no trace of iodination was observed on Phe, or most importantly, on His—the second

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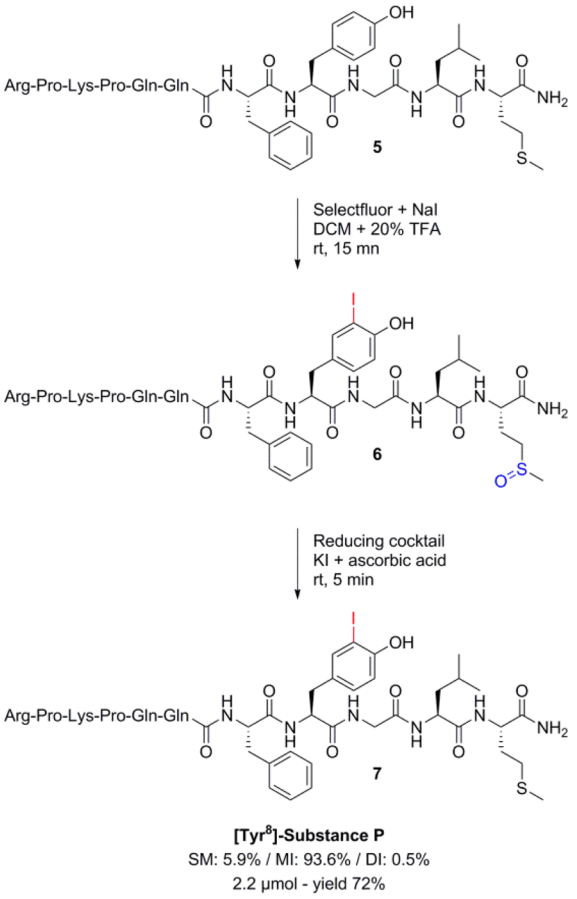

Figure 3. Mono-iodination strategy for a methionine-containing peptide [Tyr^8]-Substance P.

most reactive amino acid toward iodination[26]—confirming the high chemoselectivity of the reagent for Tyr. Furthermore, careful UPLC and NMR analysis did not reveal any evidence of racemization.

A recent peptide drug class for the treatment of type 2 diabetes is the group of glucagon-like peptide-1 (GLP-1) agonists which includes GLP-1 itself, but also modified analogues such as Victoza (Liraglutide) and others. We therefore sought to demonstrate the wide and general applicability of the strategy by examining the mono-iodination of GLP-1 (7−37), a native hormone which binds to the GLP-1 receptor and triggers insulin secretion glucose dependently. Single incorporation of an iodine atom on the tyrosine residue of the human GLP-1 (7−37) was achieved with high selectivity (ratio of mono-iodinated compound >91%), and the desired mono-iodinated peptide was isolated with 52% yield after HPLC purification, confirming the success of the approach on a challenging 31-mer peptide (Table 2, entry 10).

Taking profit of the high reactivity of the newly formed aryl-iodo peptides, we applied a Suzuki-Miyaura cross-coupling as a final step in the preparation of bioimaging probes, as examplified in Figure 3. Carboxylphenyl boronic acid pinacol ester functionnalized with a dansyl fluorophore **9** was directly attached to [mono-Iodo]-Leucin Enkephalinamide **8** using a water-soluble complex of $Pd(OAc)_2$ with the dihydroxypyrimidine ligand developed by Chalker et al.[27] To circumvent prior chemical modification of expensive fluorophores, the cross coupling with a boronic acid pinacol ester functionalized with an azide offers an alternative which enables subsequent copper-catalyzed azide−alkyne cycloaddition (CuAAC) to connect the peptide to the dye—most of them being commercially available as alkyne derivatives (Figure 4B). In both cases, cross-couplings proceeded smoothly at 38 °C in 12 h and addition of glycerol was found to improve solubility of the coupling partners and to increase yields as previously reported.[28]

CONCLUSION

In summary, we have developed a new method enabling the efficient, specific, and highly controlled mono-iodination of tyrosine within fully unprotected peptides. Successful application of the approach was demonstrated on peptides with various sizes and complexity, including peptides containing

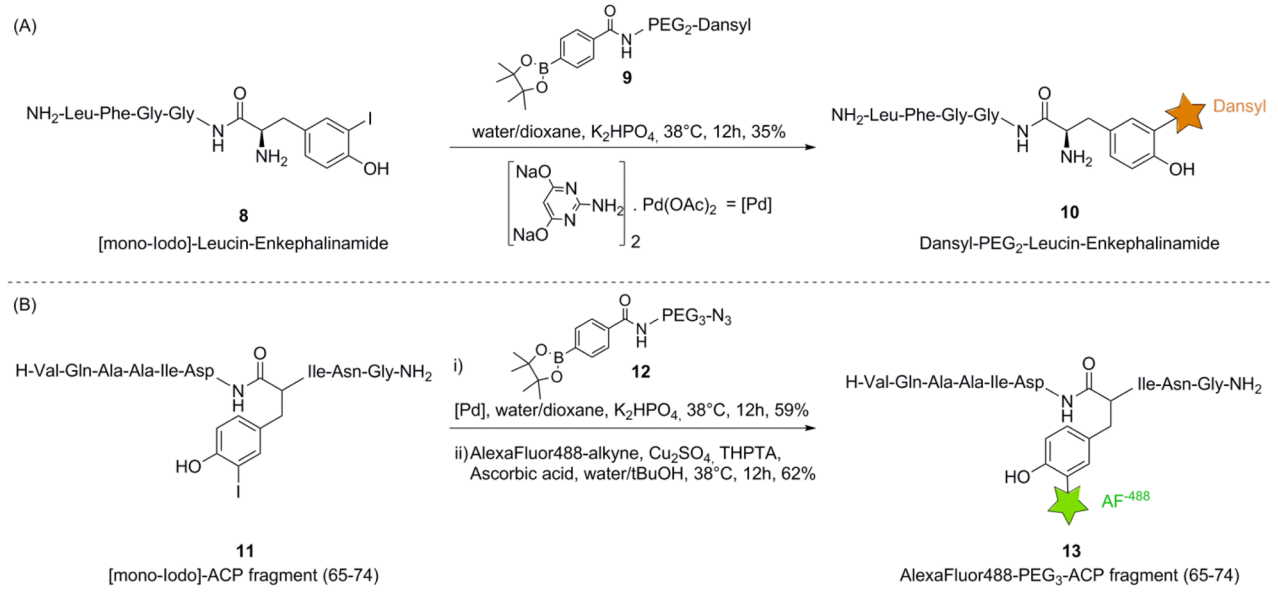

Figure 4. Conjugation of fluorophores to mono-iodinated peptides Leucin-Enkephalinamide and ACP fragment (65−74).

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other aromatic amino acids such as tryptophane and histidine, but also sulfur containing amino acids like methionine or cysteine. By exploiting the reactivity of the mono-iodo peptides we performed conjugation with fluorescent building blocks via Suzuki-Miyaura cross-coupling, as an example of bioimaging probe synthesis. We believe our findings will prove to be a useful additional tool in the arsenal of bioconjugation chemistry, notably for peptides obtained by isolation from natural sources in which incorporation of bioorthogonal conjugation sites is not possible and lysine or cysteine residues are not available for specifc labeling.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00461.

Detailed description of the chemical syntheses, including figures (PDF)

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Notes

The authors declare no competing financial interest.

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