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An optimized strategy for the mild and efficient solution phase iodination of tyrosine residues in bioactive peptides

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Usually, the accessibility of 3-iodo-Tyr-containing peptides relies on the time-consuming de novo solid phase peptide synthesis. In this study, methods for the direct (mono)iodination of unprotected peptides were evaluated. The use of *N*-iodosuccinimide (NIS) in acetonitrile/water proved to be a particularly mild, fast ($\leqslant$5 min) and efficient method with broad applicability to structurally diverse peptides. NIS iodination therefore represents a very practicable tool for the generation of iodinated peptides on a small (<1 mg) to medium (1–20 mg) scale in reasonable isolated yields (28 ± 8%), providing easy and straightforward access to iodinated reference compounds, for example, for in vitro evaluation.

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The development of peptide receptor targeted radiopharmaceuticals for in vivo receptor imaging and, eventually, of the corresponding endoradiotherapeutic agents, represents an unchangedly active and productive field of research. Although most developments ultimately aim toward designing chelator-functionalized receptor ligands allowing radiometal labeling (e.g., ^{68}Ga and ^{64}Cu for Positron Emission Tomography or ^{177}Lu/^{90}Y for radionuclide therapy), radioiodination oftentimes represents a first and crucial step in the preclinical evaluation of new peptide radiopharmaceuticals and their corresponding lead structures.

First, radioiodination represents a straightforward and structurally "minimally invasive" strategy to obtain a first receptor targeted probe for initial proof-of-concept studies with respect to, for example, expression and regulation of the target receptor or for affinity studies. Secondly, due to the ease of separation of the ^{125}I-labeled compound and the unreacted precursor peptide via HPLC, radioiodination invariably results in radiolabeled probes with high specific activities, allowing to work with very low ligand concentrations and thus avoiding unwanted saturation of the target receptor, especially when expression levels are low. Finally, due to the long half-life of iodine-125 (60 d), ^{125}I-labeled peptides may be stored for several weeks without significant decomposition, making them practical and robust tools for in vitro evaluations. Despite a certain inherent susceptibility for in vivo deiodination,[1] radioiodinated peptides are also valuable assets for the initial assessment of in vivo targeting efficiency and overall pharmacokinetics of novel targeted probes.

Mostly, due to the small structural changes introduced by radioiodination of one tyrosine residue in the peptide sequence, it may be safely assumed that receptor affinity of a given ligand is unbiased by radioiodination. This has been confirmed for e.g. radioiodinated FC131,[2,3] a cyclic pentapeptide with high affinity for the chemokine receptor CXCR4. Interestingly, however, there are also cases, where (radio)iodination even leads to a significant improvement of receptor affinity, for example, in the case of the somatostatin receptor ligands DOTA-Tyr^{3}-octreotide[4] and DOTA-Tyr^{3}-octreotate.[5,6]

For a detailed in vitro characterization of these compounds, in particular for determination of receptor affinity in a standardized IC_{50} assay, however, the respective non-radioactive 3-iodo-Tyr analogs are indispensable. Unfortunately, this usually requires the tedious and time-consuming de novo synthesis of the respective peptide ligands, incorporating 3-iodo-Tyr instead of Tyr. In most cases, this approach is further complicated by the fact, that Fmoc-3-iodo-Tyr is only commercially available in its side-chain unprotected form, giving rise to a variety of side products during solid phase peptide synthesis and subsequent reaction steps and thus very poor yields. Especially in cases, where 3-iodo-D-Tyr needs to be reacted with *N*-methylated amino acids (as in *cyclo*(yorn'(AMBS,DOTA)RNalG),[7,8] Fig. 1; AMBS = 4-aminomethylbenzoyl), coupling yields are additionally very poor, further challenging the success of this synthetic route.

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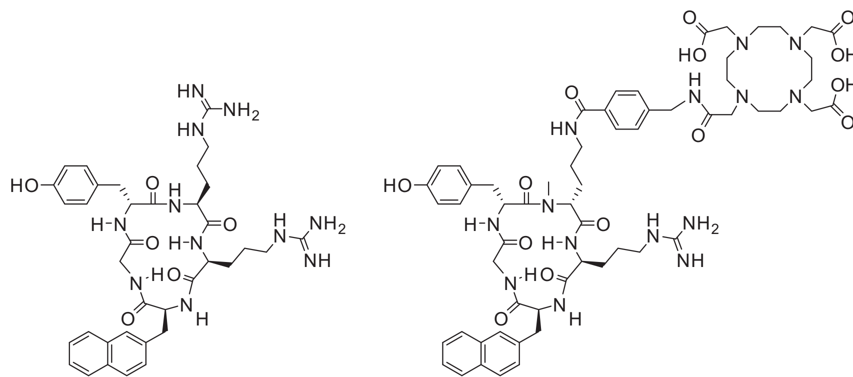

Figure 1. Structures of FC-131 (left) and *cyclo*(yorn′(AMBS,DOTA)RNalG) (right).

To date, there is only one report in the literature describing direct iodination of unprotected tyrosine-containing peptides.[9] Several somatostatin analogs were iodinated in reasonable yields (⩽35% for the respective mono-iodinated products) using chloramine-T (in solution and immobilized on IodoBeads®) or IodoGen® as oxidizing agents. However, the peptide amount used for the iodination reactions was very low (⩽70 nmol), and a preparative scale-up for the isolation of the iodinated peptides in the mg-range was not attempted. It was therefore the major objective of the present study to provide an easy, general method for the small to medium-scale (ca. 20 mg) direct synthesis of 3-iodo-Tyr-peptides from non-iodinated unprotected precursor peptides. Although the corresponding 3,5-diiodo-Tyr-analogs represent interesting intermediates for e.g. subsequent arylation of Tyr side chains,[10] di-iodination represents the major unwanted side reaction during iodination of unprotected peptides. Therefore, particular focus in the comparative evaluation of alternative direct iodination methods was directed toward optimizing the selectivity and yield of mono-iodination.

The use of *N*-iodo-succinimide (NIS) in the presence of *p*-toluenesulfonic acid (PTSA) has been shown to allow rapid and high-yield synthesis of iodinated phenols under mild conditions.[11] Both iodination efficiency, that is, turnover of starting material into the mono- and multiply iodinated species, and regioselectivity were greatly enhanced by the presence of PTSA in the reaction mixture, and both showed strong solvent dependence. Since unprotected peptides are insoluble in most of the organic solvents used in the original study, initial experiments were focused on an adaptation of the solvent system to peptide chemistry. Results for the iodination of the cyclic CXCR4-ligand FC-131 (Fig. 1) as a model compound using NIS in different solvent systems[12] are summarized in Table 1.

Table 1
Dependence of the iodination efficiency and selectivity for mono-iodination of FC-131 on the reaction conditions[a]

Solvent	Additive	NIS (equiv)	SM (%)	MIP (%)	DIP (%)
CH_3CN/H_2O (1:1)	PTSA (1 equiv)	1	1	27	72
CH_3CN/H_2O (1:1)	–	1	12	12	76
CH_3CN/H_2O (1:1)	–	0.5	37	30	33
MeOH	–	0.5	44	15	41
DMF	–	0.5	60	27	13

[a] Reactions were carried out at room temperature, and final concentration of FC-131 in the reaction mixture was kept constant at 9 mM. Reaction control was performed using HPLC, and the relative amounts of starting material (**SM**), mono-iodinated product (**MIP**) and di-iodinated product (**DIP**) were quantified using manual integration (absorbance at 220 nm). For an exemplary analytical HPLC profile see Supporting information.

Since UV absorption maxima of tyrosine, mono-iodo-, and di-iodo-tyrosine only differ marginally,[13] and since other aromatic residues in the peptide as well as the peptide backbone itself significantly contribute to the molar extinction coefficient of the peptides investigated in this study,[14] UV absorption at 220 nm was assumed to be only marginally affected by iodination, justifying the quantification of reaction yields via integration of the respective HPLC chromatograms.

Based on the excellent solubility of peptides of various size and amino acid composition in acetonitrile/water solvent systems, a 1:1 (v/v) mixture of acetonitrile and water was used for the initial transfer of the NIS/PTSA-iodination methodology to the direct iodination of FC-131. Interestingly, PTSA-addition did enhance overall iodination efficiency, that is, conversion of the starting peptide into the mono- and di-iodinated species. However, as opposed to previous data,[11] PTSA-addition did not lead to the desired effect of repressing formation of the di-iodinated side-product in favor of mono-iodination. One potential reason for this finding is the fact that FC-131 (as all other peptides used as starting material in the present study), had been purified via preparative gradient HPLC using solvents containing 0.1% TFA. Hence, the corresponding TFA salt was used for all iodination reactions, leading to the inherent presence of 2 mol equiv of TFA in the reaction mixture. Even catalytic amounts of TFA have been shown to greatly enhance the iodination yields of deactivated aromatic compounds using NIS due to the in situ formation of highly electrophilic trifluoroacetyl hypoiodite.[15] It is therefore not surprising that the further addition of PTSA (Table 1) only had a small effect on the result of the iodination reaction, and thus PTSA addition was omitted in all subsequent iodination reactions.

As shown in Table 1, the presence of equimolar amounts of NIS and peptide in the reaction mixture leads to considerable conversion of the mono- into the (unwanted) di-iodinated species. This is the result of an increased electron density in iodo-tyrosine as compared to the non-iodinated phenolic system and thus an enhanced activation for electrophilic substitution. To improve the selectivity for mono-iodination, the molar ratio of NIS to precursor peptide was therefore reduced to 0.5, leading to significantly improved yields for the mono-iodinated product.

Interestingly, the choice of the solvent has strong impact on the selectivity for mono-iodination under these conditions. While di-iodination of FC-131 is strongly promoted by the use of methanol (as compared to the water/acetonitrile solvent system), it is suppressed in DMF, all the while affording the mono-iodinated product in comparable yields to the acetonitrile/water solvent system. This represents a major advantage in cases, where efficient recovery of the non-iodinated starting peptide represents an important issue. If this, however, is not crucial, the greater ease

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