iEDDA Conjugation Reaction in Radiometal Labeling of Peptides with $^{68}$Ga and $^{64}$Cu: Unexpected Findings

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ABSTRACT: The inverse electron demand Diels–Alder conjugation reaction has gained increasing importance over the past few years for efficient in vivo and ex vivo radiometal labeling of antibodies. However, the application of this very fast reaction type has not been studied for radiolabeling of peptides so far. We show here the synthesis of 3-benzyl-1,2,4,5-tetrazine-comprising ([(1,4,7,10-tetraazacyclododecane-4,7,10-triy]-triacetic acid-1-glutaric acid) (DOTA−GA) and [(1,4,7-triazacyclononane-4,7-diyl)-diacetic acid-1-glutaric acid] (NODA−GA) chelators and their radiometal labeling with $^{68}$Ga$^3+$ and $^{64}$Cu$^{2+}$. The secondary labeling precursors $^{68}$Ga−DOTA−GA−Tz, $^{68}$Ga−NODA−GA−Tz, and $^{64}$Cu−DOTA−GA−Tz were obtained in high radiochemical yields (RCYs) and purities as well as molar activities for further labeling of trans-cyclooctene (TCO)-modified peptides. However, the following reactions of the radiometal-labeled tetrazines with different TCO-comprising model peptide analogs unexpectedly resulted in the formation of a considerable amount of side products (20−55%) which limits the overall achievable RCYs and purities as well as molar activities of the target radiopeptides. Under otherwise identical, nonradioactive reaction conditions, this effect could however not be observed. In contrast, the corresponding one-step radiolabeling protocols provided the target $^{68}$Ga-labeled radiopeptides in exceptionally high RCYs and purities of ≥99% and molar activities of 68−72 GBq/μmol starting from activities of 340−358 MBq of $^{68}$Ga. Thus, the usefulness of the two-step labeling of TCO-modified peptides with radiometal-labeled chelator-tetrazines seems to be limited.

INTRODUCTION

Chemoselective and highly efficient conjugation reactions play an important role in radiochemistry, as the modification of biologically active substances in an ideally defined position of the molecule has to be possible within a reasonable time frame compared to the half-life of the respective radionuclide.

Among the available so-called click chemistry reactions, the inverse electron demand Diels–Alder (iEDDA) reaction has emerged as one of the most important biomolecule ligation reactions over the past few years. This reaction type not only proceeds chemoselectively without requiring any catalyst at physiological pH and ambient temperature, but also exhibits exceptionally fast reaction kinetics even at very low reactant concentrations, rendering the iEDDA reaction an extremely powerful ligation technique in radiochemistry.1

Over the past few years, the iEDDA reaction has been shown to be a versatile click chemistry approach for the labeling of small molecules, peptides, and proteins with $^{18}$F, but also for radiometal labeling with $^{68}$Ga, $^{64}$Cu, $^{99}$Zr, $^{99m}$Tc, and $^{177}$Lu.1

In the case of radiometal labeling, the iEDDA reaction is usually used for in vivo labeling of antibodies or antibody fragments via the so-called pretargeting approach. Thereby, a dienophile-modified protein (in general, trans-cyclooctene (TCO) is used) is applied to the animal and the antibody is given time to accumulate in the target lesion (usually a tumor) which takes about 1−3 days. After this time, a clearing agent can—but not necessarily has to—be used to remove residual antibody from the circulation. Subsequently, the radiometal-labeled tetrazine is applied, reacting with the protein in vivo and by this visualizes the antibody distribution and the tumor target. This approach enables a very fast and clear visualization of the target structure only a few hours after injection of the radiolabeled tetrazine,1−4 resulting in a much faster imaging in diagnostic settings and reduces the dose applied to healthy organs and tissues in therapeutic settings compared to the use of directly labeled antibodies.5,6

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For the common direct labeling of antibodies, $^{89}$Zr is a very favorable radionuclide as it exhibits a long half-life of 3.27 days and emits positrons of a low mean energy of 0.389 MeV enabling positron emission tomography (PET) images of high resolution. Due to these favorable properties, $^{89}$Zr is also clinically applied for tumor imaging by positron emission tomography (PET) using $^{89}$Zr-labeled antibodies. A limitation for the use of such $^{89}$Zr-labeled antibodies is, however, the stable complexation of the radiometal. The currently clinically used chelating agent for $^{89}$Zr-introduction is desferrioxamine B (DFO) which is, however, not able to stably encapsulate the radiometal so that it gets released from the complex under in vivo imaging conditions. This results in a considerable background activity and, more importantly, the liberated $^{89}$Zr accumulates in mineral bone, depositing a significant dose in the bone marrow. Thus, several groups have been working on the development of new chelating agents that are able to stably complex $^{89}$Zr over the past few years with some of them having shown very favorable results regarding an increased stability of the formed $^{89}$Zr-complexes.

Among these, ((1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) (DOTA) was described to form stable complexes with $^{89}$Zr in an initial in vivo evaluation setting. But so far, only the stability of the unconjugated $^{89}$Zr–DOTA-complex has been studied under in vivo conditions which, however, does not allow a proper judgment of the $^{89}$Zr–DOTA stability when used for antibody labeling as the unconjugated complex gets eliminated from the circulation and the organism relatively fast. In contrast, $^{89}$Zr-labeled antibodies exhibit much slower pharmacokinetics, and thus, a much longer residence time in the organism which increases the probability of complex challenge, and thus, $^{89}$Zr liberation. To be able to obtain a correct assessment of the stability of the $^{89}$Zr–DOTA complex compared to $^{89}$Zr–DFO, the in vivo pharmacokinetics of antibody conjugates of both complexes have to be determined under identical conditions.

For this purpose, a chelating agent based on the DOTA chelator core has to be developed which can efficiently be conjugated to antibodies or other biologically active molecules such as peptides. As a functional group for biomolecule conjugation, the iEDDA click chemistry reaction is especially suitable as it enables—due to the aforementioned advantages—the fast and chemoselective introduction of the radiometal-containing complex into sensitive biomolecules.

Furthermore, tetrazines are expected to be of significantly higher thermal stability than, for e.g., isothiocyanates or active esters and due to this they are much better suited for radiometal incorporation of respectively functionalized chelators at elevated temperatures as they are often required for radiometal labeling.

Although different DOTA–tetrazines have been developed over the past few years, the $^{89}$Zr$^{4+}$ cation requires 8 donor atoms and a complete encapsulation of the central ion to achieve a high complex stability. Acid amides, however (resulting from the conjugation of one of the carboxylates of DOTA to a tetrazine, and thus, perspectively to a biomolecule), do not offer the same coordinative strength than carboxylates, potentially decreasing the resulting complex stability. Thus, a DOTA–tetrazine can be assumed to not form complexes as stable as, for e.g., a backbone-modified DOTA or a ((1,4,7,10-tetraazacyclododecane-4,7,10-triyl)-triacetic acid-1-glutaric acid) (DOTA–GA)-based chelator with $^{89}$Zr.

Thus, we intended to develop here a chelating agent based on DOTA–GA and a 3-benzyl-1,2,4,5-tetrazine scaffold (the latter exhibiting a significantly higher reactivity than its methylated analog 3-benzyl-6-methyl-1,2,4,5-tetrazine which can be efficiently introduced into biomolecules of different kinds and complexities by the iEDDA reaction. For this purpose, the chelator should be modified with a tetrazine instead of a TCO functional group as the latter was described to be susceptible to thermally induced isomerization to its $^{105}$-fold less reactive cis-isomer reaction conditions which are however required for efficient radiometal complexation by DOTA derivatives. Using this DOTA-GA-tetrazine derivative, the $^{89}$Zr-labeling of even susceptible biomolecules such as antibodies would become possible by a two-step radiolabeling protocol (Figure 1).
In the following, we intended to assess the suitability of the developed chelating agent for radiometal labeling of biomolecules on a model biomolecule—radiometal nuclide system which enables an efficient and detailed analysis of the obtained products (in this regard, peptides are better suited than proteins due to their limited structural complexity) using a radiometal of widespread availability ($^{68}$Ga$^{3+}$ instead of $^{89}$Zr$^{4+}$ as DOTA–GA should be a well-suited chelating agent for both radionuclides with regard to complex formation and stability).

Furthermore, since no labeling of smaller bioactive molecules than antibodies has been shown using radiometal-labeled tetrazines so far, we not only intend to synthesize a DOTA–GA–3-benzyl-1,2,4,5-tetrazine and show its radiometal labeling using $^{68}$Ga as model nuclide, but further intended to study the introduction of the radiometal-labeled DOTA–GA–tetrazine into TCO-modified peptides of different sizes and complexities to assess the applicability of the approach for biomolecule—radiometal labeling in general. In particular, we aimed to determine if there are any differences in terms of labeling yields, molar activities, or reaction kinetics in peptide labeling depending on the molecular complexity of the peptide to be labeled or between direct, one-step and two-step radiometal labeling (Figure 1).

The obtained results should be directly transferrable to the radiometal labeling of other biologically active molecules, such as antibodies.

## RESULTS AND DISCUSSION

As mentioned before, the iEDDA reaction has emerged as one of the most important biomolecule ligation techniques over the past few years being of special interest for the radiolabeling of biomolecules due to its high reaction efficiency. In contrast to other reaction types often used in radiolabeling such as the formation of acid amides or thioureas using active esters or isothiocyanates of radionuclide-comprising secondary labeling precursors, tetrazines exhibit the significant advantage of reacting chemoselectively, thus, only reacting with dienophiles but not with other functional groups usually present in biomolecules such as amino acid side chain functionalities or N- or C-termini of peptides or proteins. Furthermore, the radiolabel has to be introduced into the secondary labeling precursor molecule, a reaction for which elevated temperatures are often required which are, however, incompatible with thermally susceptible reactive groups such as active esters and isothiocyanates. Compared to other, also chemoselective click chemistry reactions, the iEDDA reaction requires no catalyst, proceeds at physiological pH and ambient temperature, and most importantly exhibits exceptionally fast reaction kinetics even at very low reactant concentrations as usually applied in radiolabeling, rendering the iEDDA reaction an almost ideal ligation technique in radiochemistry.

Thus, it should also be ideally suited to introduce radiometal nuclides into biomolecules such as proteins but also peptides by tetrazine-modified chelators, and we intended to develop a DOTA–GA-based tetrazine and assess its applicability to efficiently introduce $^{68}$Ga into TCO-modified peptidic ligands of varying complexity.

**Synthesis of DOTA–GA–Tz, TCO-Modified Peptide Analogos and Their Conjugation Products.** As the first step, a 3-benzyl-1,2,4,5-tetrazine-modified analog of DOTA–GA (DOTA–GA–Tz, 3) was synthesized by the reaction of (4-(1,2,4,5-tetrazine-3-yl)phenyl)methylamine formate (2) with DOTA–GA–anhydride (1) (Scheme 1) which opened—as expected$^{27}$—quantitatively to give the target chelator 3.

**Scheme 1. Schematic Depiction of the Synthesis Pathway Yielding DOTA–GA–Tz (3)**

![Scheme 1](image)

“Reaction conditions: 1 (47.8 μmol), 2 (47.8 μmol), N,N-diisopropylethylamine (DIPEA) (239 μmol), dimethylformamide (DMF) (1 mL), ambient temperature (RT), 10 min, yield: 44%.”

An alternative approach towards 3, reacting tetra-$^{3}$Bu-DOTA–GA with 2 and deprotecting the obtained intermediate tetra-$^{3}$Bu-DOTA–GA–tetrazine with neat trifluoroacetic acid (TFA) to the target chelator did not yield the product but only resulted in a very fast and quantitative fragmentation of the tetrazine.

As we assumed that the reaction efficiency of the iEDDA reaction might depend on the molecular complexity of the TCO-functionalized peptide scaffold under radiolabeling conditions, we further synthesized different TCO-derivatized peptidic structures as model compounds for direct comparison of the labeling conditions and efficiencies following one- and two-step labeling approaches.

For this purpose, we synthesized two TCO-modified peptide monomers of differing complexity as well as a TCO-modified peptide tetramer. As peptide monomers, c(RGDfK) (4), binding to integrin $\alpha$β3, being, for e.g., overexpressed during angiogenesis in tumor progression$^{28}$ and PEG$_2$-bombesin$_{1-14}$ (PESIN), 5, being a peptide agonist binding to the gastrin-releasing peptide receptor (GRPR) which is overexpressed in different malignancies such as prostate cancer$^{29}$ were synthesized by standard solid-phase peptide synthesis (SPPS) methods$^{22,30}$ and further modified with TCO in solution by reacting them with (E)-cyclcooct-4-ene p-nitrophenyl ester 6 to give RGD–TCO (7) and PESIN–TCO (8) (Scheme 2).$^{18}$

Furthermore, we synthesized a TCO-modified peptide tetramer (neurotensin analog-tetramer (NT$_\text{analog}$–TCO (12)) as a structurally more complex model reaction partner for 3. 12 was obtained by tetramerization of a neurotensin receptor-specific peptide (NT$_\text{analog}$–PEG–Cys, 9, Scheme 3) on a dendritic, TCO-functionalized maleimide tetramer scaffold (11, Scheme 4) (Scheme 5).

The neurotensin peptide sequence chosen for multimerization, peptide NT$_\text{analog}$ being a modified and stabilized variant of the endogenous neurotensin peptide with the sequence (Pip)Gly–Pro–(PipAm)Gly–Arg–Pro–Tyr–Tle–Leu, was shown before to efficiently bind to the neurotensin receptor type 1 which is overexpressed on different tumors and involved in tumor growth, survival, and metastatic spread.$^{31,32}$ As 4 and 5, 9 was synthesized by standard SPPS methods (Scheme 3).

The dendritic, TCO-functionalized maleimide tetramer scaffold (11) was obtained by a combination of solid and liquid phase syntheses (Scheme 4), first building the dendritic

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maleimide tetramer 10 on the solid phase and reacting it with TCO-active ester 6 at the lysine \( N_\varepsilon \) amino functionality to the TCO-modified maleimide tetramer 11.

The TCO-modified maleimide tetramer 11 was further reacted with the thiol-comprising neurotensin analog 9 under mild conditions via the Michael addition reaction, giving the TCO-functionalized neurotensin analog-tetramer NT\(_{\text{analag}}\) (12) in a moderate yield of 38% (Scheme 5).

In addition to the TCO-modified peptides, also their conjugation products with DOTA−GA−tetrazine 3 were synthesized via the iEDDA reaction within 10 min to 2 h at ambient temperature. Interestingly, the iEDDA reactions did
not proceed comparably fast in every case but the reaction time strongly depended on molecular complexity. During the synthesis of 13, an excess of only 1.5 equiv of 3 over 7 was applied but the reaction was nevertheless complete within 10
min, whereas in the case of reacting 3 with 12 to 15, an excess of 7 equiv was applied but the reaction still required 2 h until completion. Nevertheless, no formation of any side products was observed during these reactions, giving only the respective conjugation products in high yields of 67−95% (Scheme 6).

**Radiochemistry.** With the synthesized tetrazine-modified DOTA−GA chelator 3, the TCO-modified peptides 7, 8, and 12 as well as the respective DOTA−GA−peptide-conjugates 13−15, we proceeded in studying the reaction characteristics of the iEDDA reaction in radiometal labeling of peptides, directly comparing one- and two-step labeling (Figure 1). In the one-step labeling reaction, the chelator−peptide conjugate is directly labeled with the radiometal. In the two-step approach (which could find application in radiometal labeling of susceptible biomolecules such as antibodies), the radiometal is first incorporated into the chelator at elevated temperatures and the formed radiometal complex, acting as the secondary labeling precursor, is in the following reacted with the biomolecule.

The first important question to be answered for the two-step radiolabeling reaction was if DOTA−GA−Tz (3) shows a significant decomposition under radiolabeling conditions—especially regarding the elevated temperatures necessary for radiometal incorporation into the chelator. To determine the thermal stability of DOTA−GA−Tz, we reacted 3 with 68Ga as model radionuclide using standard reaction conditions of 99 °C in slightly acidic, aqueous, sodium acetate-buffered solution at pH 3.5−4.0 for 10 min. Under these conditions, only a slight decomposition of the tetrazine of about 6% could be observed over a time span of 45 min (Figure S1), a time frame which was described to be sufficient for quantitative 89Zr-incorporation into DOTA.15 This slight thermal instability is, however, not critical for subsequent biomolecule labeling. Thus, 3 can be considered as sufficiently stable for radiometal complexation even at high reaction temperatures and for two-step radiometal labeling of peptides and other, more susceptible biomolecules.

In the following, we studied the radiolabeling of the model peptides RGD−TCO, PESIN−TCO, and NT analog−TCO...
with $^{68}$Ga via the two-step labeling reaction. For this purpose, 5 nmol of DOTA−GA−Tz (3) were first reacted with $^{68}$Ga as described before within 10 min at 99 °C and $^{68}$Ga3 was obtained in ≥97% radiochemical yield (RCY) and purity (RCP) in molar activities of 68−76 GBq/μmol starting from activities of 339−380 MBq of $^{68}$Ga. $^{68}$Ga3 was reacted via the iEDDA conjugation reaction with the TCO-modified peptides 7, 8, and 12 in equimolar amounts.

The results obtained in the iEDDA conjugation reactions were very similar for the different peptides and besides the very fast formation of the respective target peptide radioligands, the formation of three different side products was also observed in analytical radio-HPLC (high performance liquid chromatography). None of these side products was identical to $^{68}$Ga3 despite the similar retention times (Figure 2A).

Interestingly, the three side products formed were identical, irrespective of which TCO−peptide, 7, 8, or 12, was used as the reaction partner for $^{68}$Ga3 and were also formed by reacting $^{68}$Ga3 with TCO−alcohol (Figure 2A). Thus, the formed side products were not the result of, for e.g., fragmented conjugation products as otherwise, they would exhibit strongly differing retention times depending on the TCO-modified peptide used.

In comparison, the direct, one-step labeling of the DOTA−GA-modified peptides 13−15 yielded the radiolabeled peptides in pure form (Figure 2B) without giving any of the before mentioned side products. This confirms that these side products observed in the two-step process were not the result of product degradation or isomerization. The products $^{68}$Ga13 and $^{68}$Ga15 could be obtained by a one-step labeling approach within 10 min at 99 °C using 5 nmol of precursor in ≥99% RCP and purity and molar activities of 68−72 GBq/μmol starting from activities of 340−358 MBq of $^{68}$Ga. $^{68}$Ga14 also showed a complete incorporation of the radiometal under the same conditions (≥97%) but as well a significant thermal decomposition of the radioligand (Figure 2B), being absent in the two-step labeling protocol where 8 was radiolabeled at ambient temperature with $^{68}$Ga3 to $^{68}$Ga14 (Figure 2A). This effect of thermal lability of the BBN$_{14}$ peptide sequence is in accordance with own previous observations.

Taken together, the one-step protocol unexpectedly yielded the target radioligands in much higher RCP and purity than the two-step protocol as in the two-step radiolabeling process, the formed side products decreased RCYs and would necessitate further significant purification steps of the target radiopeptides such as radio-HPLC purification of the products and subsequent reformulation.

In the following, we intended to determine the reason for the formation of the mentioned side products of which the occurrence has not been described before. The reason for this might lie in the application, radiometal-labeled tetrazines are generally used for. Usually, these secondary labeling precursors are used for in vivo or ex vivo antibody modification which is not directly comparable to the labeling of peptides. The labeling reaction of antibodies in vivo, for e.g., cannot be analyzed with regard to chemical processes and formed products and in the case of ex vivo labeling of antibodies, the reaction mixtures are rather complex compared to the labeling of peptides, potentially masking the formation of low molecular weight side products, especially, if—as usually applied in antibody labeling—size-exclusion HPLC systems are used for analysis of the reaction mixtures.

To determine the reasons for the formation of the observed side products—which were interestingly not detected during the cold syntheses of the DOTA−GA−peptide conjugation products 13, 14, and 15—we first studied different reaction volumes and pH values of the labeling solutions to determine if these parameters influence the formation of the side products, which was however not the case.

Assuming that the side products might be formed as a result of the ionizing conditions during the conjugation reactions with $^{68}$Ga3, we added a 100-fold excess of ascorbic acid or ethanol (acting as radical scavengers) to the reaction mixtures which, however, also did not suppress or reduce the side product formation.

As we so far used equimolar amounts of $^{68}$Ga3 and the peptidic precursors 7, 8, and 12 for the two-step labeling reactions, we tested if an excess of TCO−peptide is able to suppress or reduce the formation of the side products and found this assumption confirmed. The formation of side products was less pronounced, the higher the excess of TCO-
modified peptide was detected during the iEDDA reaction (53.5, 39.2, and 30.3% using 1, 5, and 10 equiv of 12, respectively) (Figure S2).

This indicates that the formation of the side products is a kinetically driven process which can be reduced by offering a higher number of reaction partners to \([^{68}\text{Ga}]\)3+. Although the formation of unintended products can be reduced by this procedure, it cannot be completely suppressed, and furthermore, this approach considerably decreases the achievable molar activity of the radiolabeled peptides.

As the formation of the side products did not occur under nonradioactive conditions during the syntheses of the DOTA–\([^{68}\text{Ga}]\)GA peptide precursors 13–15 but was only observed if \([^{68}\text{Ga}]\)3 was reacted with a TCO-comprising compound (and thus, could also not be observed in radiolabeling solution under iEDDA conjugation conditions when no TCO-modified reaction partner was present), we intended to eliminate the eventuality of the Ga3+ ion being the reason for the formation of the side products. For this purpose, we first synthesized the natGa–DOTA–GA–Tz complex (16, Scheme S1) and reacted it with the TCO-modified peptide derivatives 7, 8, and 12 to 17–19 (Scheme S1) to study if the analogous side products as observed during the radioactive experiments could be detected.

However, no formation of the corresponding side products as observed in the radioactive experiments using \([^{68}\text{Ga}]\)3 could be detected using the “cold” natGa-complex 16 for iEDDA-based conjugation to the TCO-modified peptides 7, 8, and 12 (Figure S3).

Thus, the radiometal ion in the \([^{68}\text{Ga}]\)3 complex was not the cause for the formation of the observed side products during the radiosyntheses of \([^{68}\text{Ga}]\)13–15.

In the following, we tested further different reaction parameters regarding their influence on the formation of the side products. At first, we determined if the presence of certain amino acids can promote the side product formation. For this purpose, we incubated \([^{68}\text{Ga}]\)3 with the endogenous GRPR-binding peptide bombesin (Pyr−QRLGNQWAVGHLMD−NH2) under the afore described iEDDA reaction conditions used and detected—as assumed, as the iEDDA reaction is highly chemoselective—no reaction between both molecules, excluding the possibility that the amino acids of the peptides interfere with the formation of the intended products under radiolabeling conditions.

As the side products were only formed during the radioactive experiments, we further tested if uncomplexed \(^{68}\text{Ga}^{3+}\) is able to interfere with product formation. For this purpose, we exemplarily incubated \(^{68}\text{Ga}^{3+}\) with TCO−peptide 12 and tetrazine-amine 2. As expected, no chemical reaction, and thus, also no formation of the side products could be observed.

In the following, we studied if the \(^{68}\text{Ga}^{−}\)DOTA-complex itself (without an attached tetrazine moiety) is able to interfere with any molecule part present during the labeling reactions. We thus radiolabeled DOTA with \(^{68}\text{Ga}^{3+}\) under the same conditions as used for the synthesis of \([^{68}\text{Ga}]\)3 and incubated the \(^{68}\text{Ga}^{−}\)DOTA complex with 12, 2 and both molecules (12 and 2) together. As expected, no reaction of \(^{68}\text{Ga}^{−}\)DOTA with any of the substances present in the mixture or the formation of radioactive side products could be found.

In a further attempt, we repeated the experiments of radiometal labeling of 3 and subsequent iEDDA-based conjugation reaction to 7, 8, and 12 with the radiometal \(^{64}\text{Cu}^{2+}\) instead of \(^{68}\text{Ga}^{3+}\) to determine if the same side products are formed and thus, if the radiometal used makes any difference regarding side product formation. For this purpose, 3 was first radiolabeled with \(^{64}\text{Cu}^{2+}\) under similar conditions as used for \(^{68}\text{Ga}\)-complexation (sodium acetate-buffered solution, pH 8.0, 99 °C, 10 min). \(^{64}\text{Cu}^{3+}\) was obtained in non-optimized molar activities of 4–5 GBq/μmol (due to the low amounts of starting activity of 19–25 MBq of \(^{64}\text{Cu}^{2+}\) used), high RCY and RCP of ≥95% and found to be stable in solution at ambient temperature for at least 20 h. \(^{64}\text{Cu}^{3+}\) was further reacted at first with equimolar amounts of 7, 8, and 12 for 5 min at ambient temperature. The results obtained from these experiments are shown in Figure 3 and also demonstrate the formation of side products as observed during \(^{68}\text{Ga}\)-radiolabeling experiments.

Figure 3. Analytical radio-HPLC signals of the reaction mixtures between \(^{64}\text{Cu}^{3+}\) and the TCO-bearing peptide derivatives 7, 8, and 12 (equimolar amounts). The identical side products formed during all radiolabeling reactions (Rt of 1.56 and 1.69 min) are accentuated by the red box.

However, only two side products were formed during \(^{64}\text{Cu}\)-labeling—instead of three as in the case of \(^{68}\text{Ga}\)-labeling. As expected, the retention times of these side products (Rt of 1.56 and 1.69 min) differed from those found during \(^{68}\text{Ga}\)-labeling (Rt of 1.39, 1.45, and 1.55 min). Confirming the results obtained during the \(^{68}\text{Ga}\)-radiolabeling experiments, the amount of the obtained side products could be reduced by applying a higher excess of the respective TCO-modified peptide (Figure S4).

Finally, we intended to exclude the eventuality that the chelator DOTA–GA–Tz itself or the fact that it possesses more carboxylic functionalities than necessary for stable complex formation with \(^{68}\text{Ga}^{3+}\) (requiring only two of the four carboxylic groups present in the chelator) and \(^{64}\text{Cu}^{2+}\) (also requiring only two of the four carboxylic groups for complex formation) resulted in the appearance of the observed side products.

For this purpose, we further synthesized a NODA–GA-based tetrazine (NODA–GA–Tz, 20) by reacting NODA–GA–NHS ester with tetrazine-amine 2. NODA–GA–Tz was radiolabeled with \(^{68}\text{Ga}^{3+}\) using standard reaction conditions of 45 °C in slightly acidic, aqueous, sodium acetate-buffered solution at pH 3.5–4.0 for 10 min, leaving no redundant carboxylic functionality in the formed complex. \([^{68}\text{Ga}]20\) could
be obtained in ≥95% RCY and purity in molar activities of 63−72 GBq/μmol starting from activities of 313−359 MBq of $^{68}$Ga.

$[^{68}\text{Ga}]$20 was reacted via the iEDDA conjugation reaction with the TCO-modified peptides 7, 8, and 12 in equimolar amounts and the results of these conjugation reactions can be found in Figure 4. As can be seen, also in this experimental setup, side products of different retention times as in the preceding experiments were formed which were also identical for every TCO-modified peptide used, being in accordance with the results obtained for $[^{68}\text{Ga}]$3 and $[^{64}\text{Cu}]$3. However, using this combination of radionuclide and chelator, four different side products were formed compared to three, using $[^{68}\text{Ga}]$3 or two, using $[^{64}\text{Cu}]$3. Taken together, the obtained results suggest that the radiometal labeling of 3-benzyl-1,2,4,5-tetrazine-comprising DOTA−GA and NODA−GA chelators can efficiently be achieved using $[^{68}\text{Ga}]^{3+}$ and $[^{64}\text{Cu}]^{2+}$ and that the tetrazine scaffold exhibits a sufficient stability even if high temperatures are required for radiometal introduction.

Regarding the iEDDA-based reaction of the tetrazine-comprising radiometal complexes with TCO-modified peptides, some unexpected effects were found: (i) Side products were formed which were specific for the used radiometal nuclide and also for the used chelating agent. (ii) The side products were however independent of the TCO-modified peptide (with each TCO−peptide, the same side products were formed in a similar proportion) and thus, cannot result from degradation, isomerization, or rearrangement of the iEDDA conjugation products. (iii) The formation seems to be a kinetically driven process as the presence of an excess of the TCO-comprising reaction partners was able to diminish—but not to completely suppress—the formation of the side products. (iv) The observed side product formation could only be found during the radiolabeling experiments, and not in the case of identical, nonradioactive experiments; the radioactivity itself was, however, not the elicitor of side product formation or were the amino acids of the peptides involved. (v) The formation of the side products was independent of the specific TCO-modified substance used but only started if a TCO-comprising compound was present in the mixture.

Determination of the chemical identity of the formed side products by conventional chemical analysis methods is unfortunately not possible as their formation only occurred during the radiolabeling experiments, and thus, in extremely low quantities, not even producing UV signals in analytical HPLC. Thus, we can only speculate about what is the reason for the observed effects.

One of the observed side products seems to be identical to the thermal degradation product which was— as described before—formed to a low extent of about 2−5% upon radiometal incorporation by the chelating agent (by heating to 99 °C for 10 min) (Figure S1).

Regarding the other one to three side products observed (depending on radiometal nuclide and chelating agent used), an explanation is not that easily possible. Although it was described before that MeCN reacts with tetrazines via the iEDDA reaction, this cannot be the reason for the formation of the side products found here as otherwise, we would have observed the formation of different side products, depending on if MeCN was present in the reaction mixture or not. This was, however, not the case although some reaction mixtures contained MeCN whereas others did not, depending on the solubility of the respective TCO−peptide used.

Also, it was described that the conjugation products formed by the iEDDA reaction can in some cases undergo oxidation to an aromatic system (Figure S5A). This can, however, not be the reason for our observed side products as the retention times of these aromatized systems in analytical HPLC were then depending on the respective TCO-comprising reaction partner of the tetrazines applied, which was not the case.

A more likely explanation for one of the other side products formed could be the formation of 1,4-dihydro-tetrazines from the starting tetrazines as it was described before (Figure S8B). This assumption also fits to the observation made that all of the detected side products were not able to react with TCO-modified peptides. Furthermore, radiometal-labeled 1,4-dihydro-tetrazines can be expected to exhibit a similar retention time in analytical radio-HPLC compared to their nonreduced tetrazine counterparts, which is also in accordance with the observations made.

Furthermore, a contamination of the 4-TCO-modified compounds with the respective 2-TCO-analogs might account for one of the side products observed as these 2-TCO-analogs could react with the radiometal-labeled tetrazines by the iEDDA reaction and release the respective peptide amine by a so-called click-and-release reaction (Figure S5C). This theory is, for e.g., able to explain why the retention time of the respective side product is independent of the TCO−peptide used (as this peptidic part of the molecule is cleaved during the release reaction) or why it is not able to react with the radiometal-labeled tetrazine chelator and shows a similar retention time than the latter. However, it is not able to explain why the side products were not observed under nonradioactive conditions (as the ratio between 2-TCO and 4-TCO should be the same during the radioactive and the nonradioactive experiments) or why different numbers of side products were formed when using different chelators and radiometals.

Thus, the origin of three of the found side products might be explainable. The identity of the other side product (one more formed during the iEDDA reaction between $[^{68}\text{Ga}]$20 with 7, 8, and 12) can only be speculated about but might be a result.
of radical formation of the formed 1,4-dihydro-tetrazines under the ionizing reaction conditions present in the radiolabeling reactions.

There is, however, no logical explanation why always the same number of side products should be formed using a certain chelator−radiometal-pair which, however, differs from that of another chelator−radiometal-pair used if in principle the same side reactions can occur during all reactions.

The presented work, thus, shows limitations of the iEDDA click chemistry reaction in the two-step synthesis of radio-peptides, thus being important for others working in the field of radiopeptide development. The iEDDA reaction can be—as shown here—as a valuable tool to generate peptidic precursor molecules intended for direct, one-step radiometal labeling but seems to be only of limited usefulness for a two-step labeling approach using radiometal-labeled, tetrazine-comprising secondary labeling precursors for peptide labeling due to the considerable amount of side products formed, limiting RCYs and purities as well as molar activities of the target radiopeptides.

CONCLUSIONS

We showed here that 3-benzyl-1,2,4,5-tetrazine-comprising DOTA−GA and NODA−GA chelators can efficiently be radiolabeled with $^{68}$Ga$^{3+}$ and $^{64}$Cu$^{2+}$. The resulting secondary labeling precursors could be obtained in high RCYs and purities as well as molar activities for further labeling of TCO-modified peptides and exhibit a sufficient stability even if high temperatures are required for radiometal incorporation. However, the following reaction of the radiometal-labeled tetrazines with TCO-modified peptides unexpectedly resulted in the formation of a significant amount of side products which limits the overall achievable RCYs and purities as well as molar activities of the target radiopeptides. In contrast, the corresponding one-step radiolabeling protocol provided the target radioligands in exceptionally high RCYs, purities, and molar activities.

These findings provide important information regarding chemical and radiochemical study design in the development of peptide-based radiotracers using the iEDDA conjugation reaction.

MATERIALS AND METHODS

General. All commercially available chemicals were of analytical grade and were used without further purification. Resins for solid phase-based syntheses, Fmoc-protected standard $N_\alpha$ amino acids, and Fmoc-$N_\alpha$-Bu-Gly-OH were purchased from Novabiochem. Fmoc-$N_\alpha$-Gly-$4$-Pip$[N$-amidino$(Pmc)]$-OH and Fmoc-$N_\alpha$-Gly-$4$-Pip(Boc)-OH were obtained from RSP amino acids. (O-(Benzotriazol-1-yl)-$N$,$N$,$N'$,$N'$-tetramethylyuronium hexafluorophosphate) (HBTU), $N$,$N$-bis-$(N'$-Fmoc-3-aminopropyl)-glycine potassium hemisulfate, and...
Tracepur was purchased from Carl Roth, PolyPeptide, and VWR, respectively. NODA–GA–NHS ester, DOTA–GA anhydride, and DOTA were obtained from CheMatech. (E)-Cyclooct-4-ene p-nitrophenyl ester and (+1,2,4,5-tetrazine-3-yl)phenyl)methanamine formate were purchased from Sirius Fine Chemicals.

c(RGDk) (4), c(RGDk(TCO)) (7), and bombesin were synthesized as previously described.18,36

Unless otherwise stated, the coupling reactions during solid phase-based syntheses were usually carried out in DMF for 30 min using 4 equiv of acid, 3.9 equiv of HBTU as the coupling reagent and 4 equiv of DIPEA (N,N-diisopropylethylamine) as the base. Fmoc protecting groups were removed using 50% (v/v) piperidine in DMF.

For analytical and semi-preparative HPLC chromatography, Dionex UltiMate 3000 systems equipped with a Chromolith Performance (RP-18e, 100 μm, Merck) and a Chromolith SemiPrep (RP-18e, 100–4.6 mm, Merck) column were used, equipped with a Chromolith Performance (RP-18e, 100–4.6 mm, Merck) column and a GabiStar radioactivity detector (Raytest) was used and operated with a flow rate of 4 mL/min and H2O + 0.1% TFA as eluents. For radio-analytical HPLC chromatography, a Dionex UltiMate 3000 system equipped with a Chromolith Performance (RP-18e, 100–4.6 mm, Merck) column and a GABIStar radioactivity detector (Raytest) was used and operated with a flow rate of 4 mL/min and H2O + 0.1% TFA and MeCN + 0.1% TFA as eluents. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) spectra were obtained with a Bruker Daltonics Microflex spectrometer or a Finnigan MAT95Q spectrometer, respectively.

**Synthesis of DOTA–GA–Tz (3).** To a suspension of DOTA–GA anhydride (25 mg, 47.8 μmol) in DMF (600 μL) was added a suspension of (+1,2,4,5-tetrazine-3-yl)phenyl)methanamine formate (11.1 mg, 47.8 μmol) in DMF (400 μL) followed by DIPEA (40.8 μL, 239 μmol). After 10 min, the reaction was complete, the solid was removed by centrifugation, the mixture was acidified using HCl (1 M, 200 μL) and the product was purified by semi-preparative HPLC using a gradient of 5–20% MeCN + 0.1% TFA in 5 min (Rf = 4.72 min) and isolated as a white solid after lyophilization in yields of 44% (13.5 mg, 20.9 μmol). 1H NMR (500 MHz, D2O, 25 °C): δ = 10.36 (s, 1H), 8.36 (d, 2H, J(H,H) = 8.6 Hz), 7.55 (d, 2H, J(H,H) = 8.2 Hz), 4.49 (s, 2H), 3.94–3.60 (m, 7H), 3.51–3.03 (m, 16H), 2.71–2.59 (m, 2H), 2.12–2.03 (m, 2H). APT-13C NMR (125 MHz, D2O, 25 °C): δ = 176.39, 175.12, 166.19, −157.24, 143.61, 130.08, −128.43, −128.15, −126.88, 42.83, 33.10, 24.57. MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 1187.62 (1187.62); [M + Na]+ (calculated): 1209.62 (1209.61); [M + K]+ (calculated): 1225.58 (1225.58). MALDI-MS (m/z) using 2,5-di-hydroxybenzoic acid as a matrix substance for [M + H]+ (calculated): 1187.30 (1187.62); [M + Na]+ (calculated): 1209.33 (1209.61); [M + K]+ (calculated): 1225.34 (1225.58).

**Synthesis of TCO–PEG3–BBN7−14 (5).** To a solution of PEG3−BBN7−14 (5) (10.0 mg, 8.42 μmol) in DMF (250 μL) was first added a solution of (E)-cyclooct-4-ene p-nitrophenyl ester (3.7 mg, 12.6 μmol) in DMF (3.7 μL) followed by DIPEA (3.6 μL, 21.1 μmol). After 2.5 h, the reaction was stopped by the addition of HCl (1 M, 100 μL), and the product was purified by semi-preparative HPLC using a gradient of 20–60% MeCN + 0.1% TFA in 5 min (Rf = 4.63 min). The product was isolated as a white solid after lyophilization in yields of 72% (7.9 mg; 5.9 μmol). MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 1339.96 (1339.70); [M + Na]+ (calculated): 1362.03 (1361.69); [M + K]+ (calculated): 1378.00 (1377.66). MALDI-MS (m/z) using 2,5-di-hydroxybenzoic acid as a matrix substance for [M + H]+ (calculated): 1339.74 (1339.70); [M + Na]+ (calculated): 1361.74 (1361.69); [M + K]+ (calculated): 1377.68 (1377.66). HR-ESI-MS (m/z) for [M + H]+ (calculated): 1339.71 (1339.70).

**Synthesis of NT analog–PEG3–Cys (9).** The peptide was synthesized on a solid support by standard Fmoc solid-phase peptide synthesis using a commercially available leucine-preloaded Wang resin (loading: 0.52 mmol/g), HBTU as the coupling reagent, standard Nε,Fmoc-amino acids, Fmoc-Nε-Bu-Gly-OH, Fmoc-Phe-Tyr-Phe-5-ami-nido(Pmc)-OH, Fmoc-γ-lysine-Prop(Boc)-OH, and Fmoc-PEG3-CH2OH. The crude N-terminally, cysteine-modified peptide was cleaved from the solid support using a mixture of trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H2O (95:2.5:2.5 (v/v)) for 30 min, suspended in diethyl ether and purified by semi-preparative HPLC using a gradient of 0–40% MeCN + 0.1% TFA in 6 min (Rf = 3.59 min) and isolated as a white solid after lyophilization in yields of 56% (39.8 mg; 27.8 μmol). HR-ESI-MS (m/z) for [M − H]− (calculated): 1428.80 (1428.81).

**Synthesis of Dendritic Amino-Modified Maleimide Tetramer (10).** The dendritic amino-modified maleimide tetramer was synthesized similarly to published procedures,22,37 using a low-loading rink amide resin (loading: 0.23 mmol/g), Fmoc-Nε-Lys(Mtt)-OH, Fmoc-PEG3-CH2OH, N,N,N-bis(N′-Fmoc-3-aminopropyl)-glycine potassium hemisulfate, and maleimido hexanoic acid as synthons. Compared to the standard reaction protocol, the conjugation times were prolonged to 45 min for the linear and 1.5 h or 3 h for the branched amino acids, respectively. The crude product was cleaved from the solid support using a mixture of TFA/TIS (95:5 (v/v)) for 30 min, the volatile materials were evaporated, the oily residue was dissolved in H2O/MeCN 1:1 (v/v) and purified by semi-preparative HPLC using a gradient of 20–40% MeCN + 0.1% TFA in 5 min (Rf = 3.27 min). The product was isolated as colorless, hardening oil after lyophilization in yields of 31% (9.0 mg; 5.1 μmol). MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 1767.81 (1768.11); [M + Na]+ (calculated): 1789.85 (1790.10); [M + K]+ (calculated): 1805.83 (1806.21). MALDI-MS (m/z) using 2,5-di-hydroxybenzoic acid as a matrix substance for [M + H]+ (calculated):
1767.90 (1768.11); [M + Na]+ (calculated): 1789.99 (1790.10); [M + K]+ (calculated): 1806.01 (1806.21).

Synthesis of Dendritic TCO-Modified Maleimide Tetramer (11). To a solution of amino-modified maleimide tetramer 10 (9.0 mg, 5.1 μmol) in DMF (250 μL) was first added a solution of (E)-cyclooct-4-ene p-nitrophenyl ester (1.9 mg, 6.5 μmol) in DMF (1.9 μL) followed by DIPEA (2.2 μL, 12.9 μmol). After 2 h, the reaction was stopped by the addition of HCl (1 M, 75 μL) and the product was purified by semipreparative HPLC using a gradient of 20–60% MeCN + 0.1% TFA in 5 min (Rf = 4.18 min). The product was isolated as colorless, hardening oil after lyophilization in yields of 51% (5.0 mg; 2.6 μmol). MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 1918.61 (1919.11); [M + Na]+ (calculated): 1939.64 (1940.11); [M + K]+ (calculated): 1959.67 (1960.21). MALDI-MS (m/z) using 2,5-dihydroxybenzoic acid as a matrix substance for [M + H]+ (calculated): 1918.57 (1919.11); [M + Na]+ (calculated): 1939.59 (1940.11); [M + K]+ (calculated): 1959.64 (1960.21). MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 1918.57 (1919.11); [M + Na]+ (calculated): 1939.59 (1940.11); [M + K]+ (calculated): 1959.64 (1960.21).

Synthesis of TCO-Modified NTanalog Tetramer (12). To a solution of TCO-modified maleimide tetramer 11 (5.0 mg, 2.6 μmol) in phosphate buffer (0.1 M, pH 6.0, 500 μL) was added a solution of NT−PEG3−Cys (9, CW4130) (22.4 mg, 15.6 μmol) as a solid and the pH of the reaction mixture was adjusted to 6.9 by the addition of phosphate buffer (0.1 M, pH 7.2, 200 μL). After 10 min, the reaction was complete and the product was purified by semipreparative HPLC using a gradient of 10–40% MeCN + 0.1% TFA in 5 min (Rf = 4.95 min). The product was isolated as a white solid after lyophilization in yields of 41% (2.7 mg, 3.78 μmol). MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 1767.89 (1768.35); [M + Na]+ (calculated): 1788.87 (1789.38); [M + K]+ (calculated): 1809.89 (1810.40). MALDI-MS (m/z) using 2,5-dihydroxybenzoic acid as a matrix substance for [M + H]+ (calculated): 1767.89 (1768.35); [M + Na]+ (calculated): 1788.87 (1789.38); [M + K]+ (calculated): 1809.89 (1810.40).

Synthesis of DOTA−GA-Modified c(RGDfK) (13). To a solution of TCO−c(RGDfK) 7 (0.6 mg, 790 nmol) in H2O/McCN 1:1 (v/v) + 0.1% TFA (250 μL) was added a solution of DOTA−GA−Tz (3) (0.78 mg, 1.19 μmol) in the same solvent (150 μL). After 10 min, the reaction was complete and the product was purified by semipreparative HPLC using a gradient of 10–40% MeCN + 0.1% TFA in 5 min (Rf = 5.19 min). The product was isolated as a white solid after lyophilization in yields of 67% (1.1 mg, 0.80 μmol). MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 1372.49 (1373.68); [M + Na]+ (calculated): 1394.50 (1395.67); [M + K]+ (calculated): 1410.46 (1411.64). MALDI-MS (m/z) using 2,5-dihydroxybenzoic acid as a matrix substance for [M + H]+ (calculated): 1373.85 (1373.88); [M + Na]+ (calculated): 1395.89 (1396.77); [M + K]+ (calculated): 1411.35 (1411.64).

Synthesis of DOTA−GA-Modified PEG−BBN7−14 (14). To a solution of TCO−PEG−BBN7−14 8 (3.1 mg, 2.3 μmol) in H2O/McCN 1:1 (v/v) + 0.1% TFA (250 μL) was added a solution of DOTA−GA−Tz (3) (1.8 mg, 2.8 μmol) in the same solvent (150 μL). After 1 h, the reaction was complete and the product was purified by semipreparative HPLC using a gradient of 10–50% MeCN + 0.1% TFA in 5 min (Rf = 5.32 min). The product was isolated as a white solid after lyophilization in yields of 94% (4.2 mg, 2.2 μmol). MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 1956.28 (1956.98); [M + Na]+ (calculated): 1978.27 (1978.97); [M + K]+ (calculated): 1994.23 (1994.94). MALDI-MS (m/z) using 2,5-dihydroxybenzoic acid as a matrix substance for [M + H]+ (calculated): 1956.24 (1956.98); [M + Na]+ (calculated): 1978.22 (1978.97); [M + K]+ (calculated): 1994.11 (1994.94).

Synthesis of DOTA−GA-Modified NT Analog Tetramer (15). To a solution of TCO-modified NTanalog tetramer 12 (2.5 mg, 0.33 μmol) in H2O/McCN 1:1 (v/v) + 0.1% TFA (250 μL) was added a solution of DOTA−GA−Tz (3) (1.5 mg, 2.3 μmol) in the same solvent (150 μL). After 2 h, the reaction was complete and the product was purified by semipreparative HPLC using a gradient of 10–40% MeCN + 0.1% TFA in 5 min (Rf = 4.69 min). The product was isolated as a white solid after lyophilization in yields of 95% (2.6 mg; 0.32 μmol). MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]+ (calculated): 8252.14 (8255.63). MALDI-MS (m/z) using 2,5-dihydroxybenzoic acid as a matrix substance for [M + H]+ (calculated): 8252.14 (8255.63).
2023.51 (2023.89); [M + Na]⁺ (calculated): 2045.61 (2045.88); [M + K]⁺ (calculated): 2061.44 (2061.85).

Synthesis of [68Ga]⁵⁻­DOTA—DGA-MODIFIED NT Analog Tetramer (19). To a solution of TCO-modified NT tetramer (12) (1.3 mg, 156 nmol) in H₂O/MeCN 3:1 (v/v) + 0.1% TFA (500 μL) was added a solution of [68Ga]⁵⁻­DOTA—DGA—Tz (16) (0.2 mg, 280 nmol) in H₂O (v/v) + 0.1% TFA (20 μL). After 10 min, the reaction was complete. The product was not purified but only characterized by MALDI mass spectrometry. MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]⁺ (calculated): 8323.00 (8322.54). MALDI-MS (m/z) using 2,5-dihydroxybenzoic acid as a matrix substance for [M + H]⁺ (calculated): 8324.36 (8322.54).

Synthesis of NODA—GA—Tz (20). To a solution of NODA—GA—NHS ester (20 mg, 27.3 μmol) in DMF (200 μL) was added (4+1,2,4,5-tetrazine-3-y)phenyl)methanamine formate (6.4 mg, 27.3 μmol) as a solid, followed by DIPEA (70.4 μL, 41 μmol). After 90 min, the reaction was complete. The solid was removed by centrifugation, the mixture was acidified using HCl (1 M, 50 μL) and the product was purified by semipreparative HPLC using a gradient of 5–25% MeCN + 0.1% TFA in 5 min (Rₑ = 4.55 min) and isolated as a pink solid after lyophilization in yields of 80% (11.9 mg, 21.9 μmol). ¹H NMR (500 MHz, D₂O, 25 °C): δ = 10.37 (s, 1H), 9.42 (m, 0.3H), 8.41–8.38 (m, 2H), 7.58 (dd, 2H, J(H,H) = 8.2 Hz, J(3J(H,H)) = 2.7 Hz), 4.53–4.46 (m, 2H), 3.87–3.79 (m, 4H), 3.49 (dd, 1H, J(H,H) = 8.2 Hz, J(3J(H,H)) = 6.0 Hz), 3.24–3.19 (m, 4H), 3.15–3.02 (m, 8H), 2.56 (t, 2H, J(3J(H,H)) = 7.3 Hz), 2.23–2.03 (m, 2H). APT.¹³C NMR (125 MHz, D₂O, 25 °C): δ = 175.22, 175.15, 167.57, 166.19, –165.96, 143.83, 130.18, –128.46, –128.26, –63.29, 55.27, 50.61, 48.98, 45.55, 42.77, 32.52, 24.46. MALDI-MS (m/z) using α-cyano-4-hydroxycinnamic acid as a matrix substance for [M + H]⁺ (calculated): 544.97 (545.24); [M + Na]⁺ (calculated): 567.03 (567.23); [M + K]⁺ (calculated): 583.00 (583.20). MALDI-MS (m/z) using 2,5-dihydroxybenzoic acid as a matrix substance for [M + H]⁺ (calculated): 544.71 (545.24); [M + Na]⁺ (calculated): 566.65 (567.23); [M + K]⁺ (calculated): 583.65 (583.20).

⁶⁸Ga-Radiolabeling of 3 and Subsequent Reaction with TCO-Modified Peptides 7, 8, and 12 (to[⁶⁸Ga]₁₃−­[⁶⁸Ga]₁₅) as well as TCO—Alcohol (Two-Step Radiolabeling). A solution of 3 (5 nmol) in Tracepur water (5 μL) was added to 339–358 MBq of [⁶⁸Ga]³⁺ in a solution obtained by fractioned elution of a Ge⁶⁸/Ga⁶⁸ generator (IGG100, Eckert & Ziegler, Berlin, Germany) with HCl (0.1 M, 1.4 mL) and subsequent titration to pH 3.5–4.0 by the addition of sodium acetate solution (1.25 M, 90–95 μL). After the reaction for 10 min at 99 °C, the reaction mixtures were analyzed by analytical radio-HPLC. The radiolabeled peptides [⁶⁸Ga]₁₃ and [⁶⁸Ga]₁₅ were obtained in ≥99% RCY and purity in molar activities of 68–72 GBq/μmol whereas [⁶⁸Ga]₁₄ showed a significant thermal decomposition, diminishing RCYs and purities.

⁶⁴Cu-Radiolabeling of 3 and Subsequent Reaction with TCO-Modified Peptides 7, 8, and 12 to [⁶⁴Cu]₁₃−­[⁶⁴Cu]₁₅ (Two-Step Radiolabeling). A solution of 3 (5 nmol) in Tracepur water (5 μL) was added to a sodium acetate solution (0.25 M, pH 8.0, 125 μL). To this solution were added 19–25 MBq of Cu²⁺ in a 0.1 M HCl solution (15–20 μL). After the reaction for 10 min at 99 °C, the reaction mixtures were analyzed by analytical radio-HPLC. The radiolabeled product [⁶⁴Cu]₃ was obtained in ≥95% RCY and purity in nonoptimized molar activities of 4–5 GBq/μmol.

To a solution of [⁶⁴Cu]₃ (1 nmol) obtained as described before was added a solution of 7, 8, or 12 (different excesses) in Tracepur water (7 and 8) or Tracepur water/MeCN 1:1 (12) and reacted for 1–10 min at ambient temperature. The reaction mixtures were analyzed by analytical radio-HPLC.

⁶⁸Ga-Radiolabeling of 20 and Subsequent Reaction with TCO-Modified Peptides 7, 8, and 12 (Two-Step Radiolabeling). A solution of 20 (5 nmol) in Tracepur water (5 μL) was added to 313–359 MBq of [⁶⁸Ga]³⁺ in a solution obtained by fractioned elution of a Ge⁶⁸/Ga⁶⁸ generator (IGG100, Eckert & Ziegler, Berlin, Germany) with HCl (0.1 M, 1.4 mL) and subsequent titration to pH 3.5–4.0 by the addition of sodium acetate solution (1.25 M, 90–95 μL). After the reaction for 10 min at 45 °C, the reaction mixtures were analyzed by analytical radio-HPLC. The radiolabeled product [⁶⁸Ga]₂₀ was obtained in ≥95% RCY and purity in molar activities of 63–72 GBq/μmol.

To a solution of [⁶⁸Ga]₂₀ (1 nmol) obtained as described before was added a solution of 7, 8, or 12 (1 nmol) in Tracepur water (7 and 8) or Tracepur water/MeCN 1:1 (12) and reacted for 5–25 min at ambient temperature. The reaction mixtures were analyzed by analytical radio-HPLC.

ASSOCIATED CONTENT
3 Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01926.

Analytical HPLC chromatograms and reaction scheme (PDF)

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