# Conjugation of DOTA-like chelating agents to peptides and radiolabeling with trivalent metallic isotopes

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**Peptides can be labeled with various trivalent radiometals for imaging or targeted radionuclide-therapy applications. The peptide is first conjugated to a chelating agent that is able to form stable complexes with the radionuclide of interest. This conjugation step can be carried out as part of the solid-phase peptide synthesis, or it can be undertaken in the solution phase after synthesis and purification of the peptide. The latter route, described here, involves reacting a molar excess of the activated tri-*tert*-butyl ester-derivatized chelator with a designated free amino group of a peptide analog, in which all other reactive amines are protected, in the presence of a coupling agent. The conjugate molecule is then purified prior to deprotection and further purification by HPLC. The product can be radiolabeled by addition of a suitable metal salt, followed, if necessary, by removal of the unchelated metal. The entire process of conjugation, purification and radiolabeling should take approximately 12.5 h.**

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## INTRODUCTION

A number of trivalent metal radionuclides have physical properties suitable for radioisotope imaging (indium-111 ($^{111}$In), gallium-67/68 ($^{67/68}$Ga) and yttrium-86 ($^{86}$Y)) or for targeted radionuclide therapy ($^{90}$Y and lutetium-177 ($^{177}$Lu)). These metal radionuclides can be combined with a targeting biomolecule (such as a peptide or antibody) in order to diagnose, monitor or treat disease. To obtain a radiolabeled biomolecule with the required stability, the peptide or protein must first be conjugated to a suitable chelator in order to complex the metal. The requirements of chelators for trivalent metals (such as In, Y, Ga and Lu) for labeling peptides are generally the same as those for labeling proteins[1]. The complexes should be stable in biological systems and their chelating ability should not be impaired by reaction with the peptide. Most often, diethylenetriaminepentaacetic acid (DTPA) and 1,4,7,10-tetra-azacyclododecanetetraacetic acid (DOTA) are used. Of the metals mentioned, the DOTA complexes are more thermodynamically and kinetically stable than the DTPA complexes. The drawback of using DOTA complexes is that they require a degree of heating to aid their formation, whereas the less-stable DTPA complexes can form at room temperature (19–25 °C). However, peptides (unlike proteins) are generally stable to heating; therefore, for peptide-labeling applications, DOTA is preferred. As DOTA is a tetra acid, it has four potential sites for conjugation, which can lead to a mixture of cross-linked conjugates. Fortunately, active-ester tri-*t*-butyl-protected DOTA compounds are commercially available along with other useful bifunctional chelators. For this protocol, we have described the use of 1,4,7,10-tetraazacyclododecane-1,4,7-tris (*t*-butyl acetate)-10-acetic acid (DOTA-(*t*Bu)$_3$; **Fig. 1**) a compound that requires activation of its free carboxyl group for the conjugation to occur. The low aqueous solubility of this compound and the need for deprotection after conjugation make it unsuitable for chelation with large water-soluble antibodies or proteins, but it is ideal for reacting peptides in non-aqueous solvents.

It is necessary to ensure that the conjugation of a chelator to the peptide does not substantially lower the peptide's receptor-binding affinity. It is advantageous to use peptides in which the active conjugation can be directed away from this receptor binding site. If necessary, spacers can be used to increase the distance between the conjugation site and the receptor binding site. Usually, the peptide is synthesized in such a way that the receptor binding site is at the carboxy terminus and the chelator is conjugated to the amino-terminal group. This can be done either during fluorenyl-methoxycarbonyl (Fmoc) solid-phase peptide synthesis[2–5] or in the solution phase after synthesis and purification of the peptide[6,7]. In the latter scenario, all free amino groups (such as those on lysine residues) other than the desired conjugation site should be tert-butyloxycarbonyl (BOC) protected, in order to prevent any undesirable side reactions. Chelators can also be conjugated to the free amino group of a lysine residue within the peptide sequence. As Fmoc solid-phase peptide synthesis is amply covered elsewhere[3], this

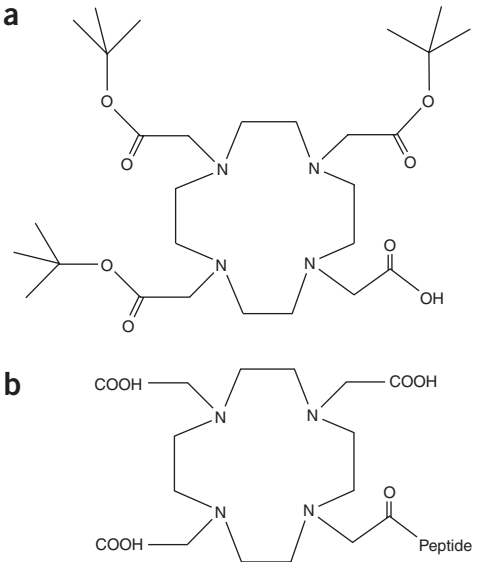


**Figure 1** | Chemical structures. (**a**) DOTA-tri-*t*-butyl ester. (**b**) The conjugated

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protocol provides a method for small-scale solution-phase conjugation of the DOTA-tri-*t*-butyl ester to a peptide amino group.

The conjugation method for peptides is quicker than that for proteins[1], as the solubility of peptides in non-aqueous solvents (such as *N*,*N*-dimethylformamide (DMF) or *N*-methyl-2-pyrrolidinone (NMP)) allows them to be reacted and purified more rapidly. In this protocol, a DOTA-tri-*t*-butyl ester is pre-incubated in NMP with equimolar amounts of the coupling agents, *O*-(7-azabenzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine (DIPEA), the last of which is an organic base. The peptide is added to this mixture in a peptide:chelator ratio of 1:3 to 1:4, which is suitable for small-scale reactions (using 1–5 mg peptide), such as those carried out early in the radiopharmaceutical development process. For reactions using a large amount of peptide, it is possible to use ratios close to 1:1 (ref. 7). After stirring for 4 h at room temperature, the product is purified by solid-phase extraction (or extracted into ethylacetate (EtOAc)) and the solvent is evaporated off under vacuum. The crude product is dissolved in a 94:4:2 (vol/vol) mixture of trifluoroacetic acid (TFA):thioanisole:water to remove the BOC-protecting groups. After 4 h, the residue is washed with ether, redissoved in acetonitrile (ACN)/water and purified by reversed phase (RP)-HPLC. The HPLC fractions are evaporated to dryness (overall yield, 60%). The final peptide product can then be dissolved in water or aqueous buffer (depending on the peptide) for radiolabeling.

In the subsequent radiolabeling step, the metal chloride salt (dissolved in dilute HCl) is added to a reaction vial, followed by acetate buffer at pH 4–6. This pH range is chosen to ensure that the metal remains in solution during the subsequent labeling procedure. At pH >6, many metals form insoluble hydroxide complexes with water and become unavailable for binding to the chelator. The presence of weakly chelating ions in the buffer (such as acetate or citrate) will also reduce the likelihood of hydrolysis. Some antioxidants (such as ascorbic or gentisic acid) might also be added to prevent radiolysis of the peptide. The peptide is added to this weak metal acetate chelate solution and the vial is heated (usually in a heating block). The general reaction variables are pH (4–6), temperature (37–100 °C) and reaction time (10–60 min). The reaction kinetics vary depending on the metal[8,9], and the peptide residues adjacent to the chelator can also have an effect. Therefore, the reaction conditions should be optimized for each peptide/chelator/metal combination.

The amounts of the radioactive metal added will depend on the required specific activity (MBq nmol$^{-1}$) of the product. For receptor imaging and therapy studies, it is usually the case that a high specific activity is required to prevent both saturation of the receptor and possible pharmacological side effects. Usually the peptide conjugate is in excess in comparison with the radionuclide, and the labeling will be highly efficient with almost 100% of the metal being complexed. If higher specific activity is required, then rigorous elimination of metal impurities (e.g., by performing the radiolabeling reaction in polypropylene instead of glass, using polypropylene weighing spatulas, and treating vials, pipette tips and so on with acid[10]) might become necessary, as well as some adjustments to the reaction conditions. As far as the reagents are concerned, it is important to start with high-grade materials with the lowest levels of metal impurities (for this reason, ammonium acetate buffer is used as opposed to sodium acetate). In addition, the buffers and solutions can be pretreated with Chelex chelating ion-exchange resin[11] to remove trace multivalent metals. However, care should be taken with this approach if high radiolabeling efficiencies are required. It has been reported that treatment of radiolabeling reaction solutions with Chelex resin, while potentially increasing the specific activity of the labeled product, can also reduce the labeling efficiency. This is probably due to the presence of chelating material residue in the solvents, which has leached from the resin. To obtain radiolabeled products with the highest possible specific activities, the labeling efficiency might therefore have to be compromised and a post-labeling purification step (such as a Sep-Pak purification) might need to be introduced.

This protocol describes the method required to conjugate a peptide to the DOTA chelator, and to label the conjugate with a radionuclide (such as $^{111}$In or $^{90}$Y) for use in radionuclide imaging or targeted therapy. In total, the entire process should take about 12.5 h.

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## MATERIALS

### REAGENTS

- Peptide (typically 1–5 mg depending on scale; e.g., piCHEM, Bachem, BioSynthema)
- DOTA-(*t*Bu)3 (Macrocyclics, cat. no. B260)
- NMP (Sigma-Aldrich)
- HATU (Sigma-Aldrich)
- DIPEA (Sigma-Aldrich)
- Metal-free water (typical resistance, 18 mΩ cm$^{-1}$; from an ELGA water-purification system)
- TFA (Spectrophotometric grade; Sigma-Aldrich)
- Thioanisole (Sigma-Aldrich)
- ACN (HPLC grade; Sigma-Aldrich)
- EDTA (Sigma-Aldrich)
- Ammonium acetate (choose the grade with the lowest possible metal impurities; Sigma-Aldrich)
- Glacial acetic acid (Sigma-Aldrich) ! **CAUTION** Highly corrosive.
- Ammonia solution (35%; Sigma-Aldrich) ! **CAUTION** Vapors are irritating to respiratory system; corrosive.
- Methanol (HPLC grade; Sigma-Aldrich)
- Chelex-100 analytical grade resin, 100–200 mesh (Bio-Rad Laboratories)
- Radionuclide as chloride salt in dilute hydrochloric acid
- $^{111}$In chloride (e.g., GE Healthcare, Tyco Healthcare, MDS Nordion)
- $^{90}$Y chloride (e.g., MDS Nordion, Perkin Elmer)
- $^{177}$Lu chloride (e.g., Perkin Elmer, IDB) ! **CAUTION** Radioactive; use appropriate radiation safety measures as laid out in local rules; standard shielding and radionuclide handling procedures should be employed; direct exposure to the radioactive dose should be kept to a minimum; individuals working with the material should monitor their radiation exposure with appropriate devices.
- HPLC Solvent A: 0.1% TFA (vol/vol) in water (Sigma-Aldrich)
- HPLC Solvent B: 0.1% TFA (vol/vol) in ACN (Sigma-Aldrich)

### EQUIPMENT

- Calibrated pH meter
- 1 ml Reacti-Vial with Reacti-Vial magnetic stirrer (Pierce and Warriner, cat. nos. 13221 and 16010)
- Magnetic stirrer plate
- C-18-Sep-Pak-Classic cartridge (Waters)
- Metal-free round-bottomed polypropylene tubes, 2 ml (e.g., Nunc Cryo-tubes, Corning Inc.)
- Centrifugal evaporator
- RP-HPLC gradient system with UV and radiochemical detectors (Beckman GmbH)

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- Jupiter C18 HPLC column, 5 μm, 300 Å, 250 × 4.6 mm internal diameter (i.d.) or similar (Phenomenex, cat. no. 00G-4053-E0)
- Chromatographic support material, silica-gel-impregnated glass-fiber (ITLC-SG, Pall Corp.) cut into strips approximately 2 × 10 cm in size with a faint pencil mark 1.5 cm from one end
- Gamma counter (e.g., Perkin Elmer)

**REAGENT SETUP**

**0.1 M ammonium acetate buffer (pH 6)** Dissolve 7.708 g ammonium acetate in 1 l water, add 300 μl acetic acid. Mix well and check that the pH is 5.5–6. ! CAUTION Acetic acid is highly corrosive.

**0.1 M ammonium acetate containing 50 mM EDTA** Dissolve 185 mg EDTA in 10 ml of 0.1 M ammonium acetate buffer (pH 6).

**1 M ammonium acetate buffer (pH 5.5–6)** Prepare a 1-M solution of ammonium acetate by dissolving 3.854 g in 50 ml water. Prepare a 1-M solution of acetic acid by making 3 ml acetic acid up to 50 ml. Add one part 1 M acetic acid to eight parts 1 M ammonium acetate. Mix well and check that the pH is 5.5–6. If an extremely high specific activity radiolabeled product is required, this buffer can be treated with Chelex-100 resin prior to use (see INTRODUCTION for a discussion of the merits of this approach). ! CAUTION Acetic acid is highly corrosive.

**1:1 mixture of 3.5% (vol/vol) ammonia solution and methanol** To prepare 100 ml, using an appropriate measuring cylinder, measure 45 ml water and make up to 50 ml using 35% (vol/vol) ammonia solution. Add this to 50 ml methanol and mix. ▲ CRITICAL This solution must be prepared fresh on the day of analysis to avoid evaporation of the solvents.

**EQUIPMENT SETUP**

**Preparation of metal-free vials and disposables** For preparation of an extremely high specific activity radiolabeled peptide, place vials, tubes, pipette tips and so on in a 500-ml bottle containing 0.1 M HCl (prepared with metal-free water), and place on a mixer overnight. Pour off the HCl and rinse thoroughly with metal-free water. Refill the bottle with metal-free water and place on a mixer for a further 2 h. Remove the vials and disposables from the water, and dry in an oven.

**HPLC method for peptide purification** Set up a general HPLC method for peptide separation on a C18 column, as in the following example: 0–20 min, 5–60% Solvent B; 20–25 min, hold at 60% Solvent B; 25–30 min, 60–90% Solvent B; 30–35 min, 90–5% Solvent B. It might be necessary to optimize the separation of the conjugated peptide from any unconjugated peptide or chelator. Inject the starting materials to determine their elution times. Inject small amounts of the crude product and decrease the solvent gradient if necessary to improve the peak resolution. Try not to decrease the gradient to $<1\%$ ACN per min, as this might cause peak broadening.

## PROCEDURE

### Conjugation reaction

**1|** Calculate the amount of DOTA-($t$Bu)$_3$ to give a threefold to fourfold molar excess over the amount of peptide to be used (1–5 mg).

**2|** Add equimolar amounts of HATU and DIPEA along with NMP (approximately 30 μl NMP per mg peptide to be used) to the DOTA-($t$Bu)$_3$ chelator, and incubate at room temperature for 20 min.

**3|** Transfer the mixture to a 1-ml Reacti-Vial.

**4|** Dissolve the peptide in NMP (30 μl mg$^{-1}$ peptide) and add the same amount of DIPEA as was added to the chelator.

**5|** Transfer this solution to the 1-ml Reacti-Vial, add a Reacti-Vial magnetic stirrer and stir for 4 h at room temperature.

### Sep-Pak purification

**6|** Activate a C-18-Sep-Pak cartridge with methanol and then pre-equilibrate the column with water.

**7|** Load the reaction mixture onto the column and wash with 5 ml water.

**8|** Elute the peptide conjugate into a 1.5-ml microcentrifuge tube with 1 ml of 0.1% TFA (vol/vol) in ACN.

**9|** Evaporate the solvent using a centrifugal evaporator (this should take approximately 15 min at room temperature).
■ PAUSE POINT The protected peptide-conjugate crude material can be stored in a refrigerator overnight at 4 °C.

### Deprotection

**10|** Dissolve the crude product in 282 μl TFA, 12 μl thioanisole and 6 μl water, and react for 4 h.

**11|** Evaporate off the deprotection mixture using a centrifugal evaporator (this should take approximately 15–20 min at room temperature).

**12|** Wash the residue with ice-cold ether (3 × 1 ml) and re-dissolve in 20% ACN (vol/vol) in water.

### HPLC purification

**13|** Purify the peptide conjugate by RP-HPLC using a Jupiter C18 column (or similar) equilibrated on 5% Solvent B.
▲ CRITICAL STEP The first time this step is carried out for any peptide, it might be necessary to optimize the separation method, before injection of the entire crude mixture. Refer to the advice given in the EQUIPMENT SETUP. Be aware that any residual unconjugated DOTA-($t$Bu)$_3$ might have also come through the SPE column, so there might be some deprotected DOTA in this HPLC purification mixture. If there is more than one unprotected primary amine group on the peptide, this will lead to a mixture of products, which will again complicate the purification procedure. In this case, the products should be separated by HPLC, collected and structural analysis carried out in order to identify the required product.

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**15|** Collect the HPLC eluate containing the peptide conjugate and evaporate to dryness in a weighed microcentrifuge tube using a centrifugal evaporator. Re-weigh the microcentrifuge tube to determine the yield of conjugate.

**16|** Redissolve the product in water (with added base or acid to achieve solubility if necessary) to a concentration of 1 mg $ml^{-1}$, divide into suitable sized aliquots (10–50 µg depending on application) in metal-free tubes and store at below –20 °C until required.
■ **PAUSE POINT** The peptide conjugate can be stored for months or years under these conditions.

**Radiolabeling**
! **CAUTION** Use appropriate radiation safety measures at this stage.
**17|** Thaw out an aliquot of the peptide conjugate.

**18|** Pipette the required volume of radionuclide solution into a metal-free polypropylene screw-top tube (such as a 2-ml Corning cryotube). The quantity will depend on the amount of radioactivity needed for the desired application.

**19|** Add a volume of 1 M ammonium acetate buffer (pH 5.5–6) equal to 10% of the volume of the radioactive isotope.

**20|** Add a volume of peptide conjugate corresponding to the required specific activity (MBq $nmol^{-1}$) of the radiolabeled product. Check the pH of the labeling reaction using pH indicator strips (it should be in the range of 4 to 6).
▲ **CRITICAL STEP** If the pH is $<4$, it is probable that 1 M ammonium acetate buffer has been omitted from the reaction mixture or insufficient buffer has been added. This will reduce the rate of metal complexation and more buffer should be added to compensate for this. If the pH is $>6$, the pH of the ammonium acetate buffer is probably incorrect. This could lead to the formation of insoluble metal hydroxides.

**21|** Mix well and heat the labeling reaction in a dry-block heater for up to 30 min at up to 98 °C. The exact conditions depend on the radioisotope (e.g., $^{111}$In requires more heating than $^{90}$Y) and the peptide (the temperature or duration of heating might have to be minimized if there are any thermolabile residues, e.g., if it is necessary to prevent the oxidation of methionine).

**22|** Remove the vial from the heating block and allow to cool for a few minutes. Quench the reaction by adding 10% of the total reaction volume of 0.1 M ammonium acetate/50 mM EDTA solution.

**Measurement of radiolabeling efficiency by thin-layer chromatography (TLC)**
**23|** Pour 0.1 M ammonium acetate containing 50 mM EDTA solution into a glass beaker (10–15 cm tall) or similar container until it is 0.5 cm deep. Cover the beaker with a Petri dish lid, aluminum foil or similar.

**24|** Repeat this procedure using a 1:1 ratio of 3.5% (vol/vol) ammonia/methanol solution in a separate container.

**25|** Place a 1-µl spot of the radiolabeled peptide sample to be analyzed onto the centre of the pencil mark on each of two chromatographic strips and allow the spots to dry.

**26|** Using forceps, gently place the strips upright in each beaker with the pencil mark at the lower end just above the solvent level. Cover the beaker and allow the solvent to run up the support material.

**27|** When the solvent is about 5 mm from the top of the strip, remove it from the beaker using forceps and lay it on a clean piece of tissue to dry.

**28|** Cut the strips in two equal parts across the short axis. Place the upper and lower halves into separate gamma counter tubes and measure the radioactive counts from each tube in a gamma counter.

**29|** In the case of the ammonium acetate/EDTA strip, unbound radionuclide migrates with a retardation factor ($R_f$) of 1, while labeled peptide remains at the origin. Calculate the proportion of unbound radionuclide as follows:

$$\%\ \text{unbound radionuclide (A)} = \frac{\text{Counts on upper half}}{\text{Counts on lower plus upper half}} \times 100\%$$

**30|** In the case of the 1:1 ratio of 3.5% (vol/vol) ammonia/methanol strip, calculate the amount of insoluble metal hydroxides (colloidal material) present in the radiolabeling mixture as follows:

$$\%\ \text{insoluble hydroxides (B)} = \frac{\text{Counts on lower half}}{\text{Counts on lower plus upper half}} \times 100\%$$

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**Measurement of radiolabeling efficiency by HPLC**

**31|** Dilute radiolabeling reaction with water or saline. Inject on RP-HPLC using the same column and conditions as those used for the purification of the peptide conjugate.

● **TIMING**

Steps 1–5: peptide conjugation, 5 h.

Steps 9–15: deprotection and purification, 6.5 h.

Steps 17–31: Radiolabeling and analysis, 1 h.

**? TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

| PROBLEM | POSSIBLE CAUSES |
|---|---|
| Poor peptide conjugate yields. | If an EtOAc extraction step is used, the product can be lost. Use NMP as a reaction solvent instead of DMF, and use SPE purification instead of EtOAc extraction. |
| | Loss of peptide during HPLC purification. This can be due to peptide binding to free silanols on the C18 column. Use a column that tolerates low pH, such as Phenomenex Jupiter C18. |
| Low labeling efficiency. | Reaction mixture too dilute (i.e., peptide concentration too low). |
| | Reaction conditions not optimal for particular peptide-chelator-metal combination. Vary reaction time, temperature, volume and pH (do not exceed 6.5 due to formation of insoluble metal hydroxides). |
| | Contamination with metal ions. Make sure that all reagents and disposables are metal free. Use a polypropylene reaction vial for the radiolabeling reaction. |
| | Contamination of buffers with chelating material, such as Chelex. If high specific activities cannot be achieved without the use of Chelex, introduce a post-labeling Sep-Pak purification to remove the radiometal–Chelex complexes. |
| High specific activity not achievable. | Contamination with metal ions. Make sure that all reagents and disposables are metal free. Use a polypropylene reaction vial for the radiolabeling reaction. |

## ANTICIPATED RESULTS

Yields of conjugated peptide after deprotection and purification should be in the region of 60%. Radiolabeling efficiencies >95% should be routinely achieved.

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**COMPETING INTERESTS STATEMENT** The authors declare that they have no competing financial interests.

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