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Improved synthesis and biological evaluation of chelator-modified α-MSH analogs prepared by copper-free click chemistry

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ABSTRACT

Radionuclide chelators (DOTA, NOTA) functionalized with a monofluorocyclooctyne group were prepared. These materials reacted rapidly and in high yield with a fully deprotected azide-modified peptide via Cu-free click chemistry under mild reaction conditions (aqueous solution, room temperature). The resulting bioconjugates bind with high affinity and specificity to their cell-surface receptor targets in vitro and appear stable to degradation in mouse serum over 3 h of incubation at 37 °C.

Radiolabeled peptides are increasingly recognized as effective tools for targeted molecular imaging and therapy of cancer.[1–4] A significant challenge in preparing these compounds is selective attachment of chelators for coupling of radionuclides, while maintaining the high affinity binding of the peptide for its in vivo molecular target. A number of protection/deprotection schemes are employed to enable selective installation of the chelator moiety.[5–7] Although these methods have proven effective, there is potential for loss of chemical yield with each synthetic step. Thus, simplified methods that enable selective attachment of the chelator, while preserving radiometal coupling, serum stability, and high affinity binding of the peptide for the molecular target can be advantageous.

Click chemistry techniques that employ copper-catalyzed [3+2] cycloaddition between azides and terminal alkynes have received considerable attention recently for use in bioconjugation strategies.[8–10] These reactions are characterized by supreme selectivity, high yields, and rapid kinetics, while being amenable to mild aqueous reaction conditions. Although these characteristics have been investigated for the addition of polyamino-macrocyclic chelators to molecular targeting vectors,[9,11] use of the copper-catalyzed reaction for this application is complicated by the need to remove copper that is associated strongly with the chelator moiety prior to radiolabeling.[9,11,12]

An alternative to the copper catalyzed cycloaddition reaction is the use of cyclooctyne agents that undergo cycloaddition reactions with azides spontaneously as a consequence of ring strain. In particular, fluoro-substituted cyclooctynes and dibenzocyclooctynes have been shown to participate in [3+2] cycloadditions with azides under mild conditions while exhibiting reaction profiles (chemoselectivity, yields, kinetics) comparable with their copper catalyzed counterpart.[13–16] Although synthesis of cyclooctyne derivatives suitable for this application has been challenging,[17] we recently introduced a versatile monoflouro-substituted cyclooctyne (MFCO, **1**) that can be conveniently prepared in only four steps from readily available cyclooctanone in ~40% overall yield (Scheme 1).[18] We also demonstrated that **1** could be coupled to a *t*-butyl protected DOTA (*t*-DOTA) derivative for selective addition to a resin-bound

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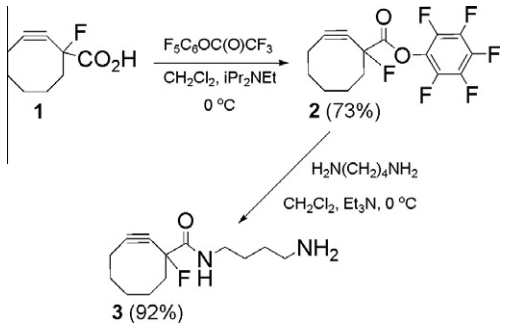


Scheme 1. Synthesis of MFCO-amine (**3**).

azide-modified peptide via copper-free click chemistry, followed by deprotection and cleavage by standard methods.[19] With success in our initial proof of concept, we embarked to develop an improved bioconjugation strategy that would allow direct attachment of the chelator to bioligands under aqueous conditions without the need for protecting groups. Toward this end, we report here the preparation of fully-deprotected DOTA- and NOTA-MFCO chelators that can be conveniently and selectively attached to fully deprotected azide-modified peptides in aqueous solution at room temperature. To evaluate the potential of the approach for molecular imaging and radionuclide therapy, the DOTA- and NOTA-MFCO were conjugated to an azide-modified variant of a well-characterized peptide under investigation of molecular imaging and therapy of metastatic melanoma (see below). The chelator modified bioconjugates were efficiently radiolabeled with gallium-68 (^{68}Ga) and copper-64 (^{64}Cu). Competitive binding assays were conducted to evaluate the potential effect of the fused-triazole linkage on the binding affinity and the stability of the chelator-modified peptide conjugates was examined in mouse serum. We further evaluated the effect of metallation on binding affinity by labeling the chelator-modified peptides with stable Ga^{3+} and comparing the resulting affinity to the unmetallated variant.

The synthesis of DOTA- and NOTA-MFCO[†] began with conversion of the acid (**1**) to the corresponding pentafluorophenyl ester **2** (Scheme 1). This material was previously prepared and used without isolation in the synthesis of a biotin-MFCO conjugate.[17] In the present case, however, it was found that **2** could be easily isolated/purified and was stable for months at −20 °C. With the purified MFCO-PFP ester in hand, amine-MFCO (**3**) was readily obtained upon treatment of **2** with an excess of 1,4-diaminobutane in CH_2Cl_2. A simple aqueous workup of the reaction mixture afforded the desired MFCO-amine (**3**) as a pale yellow gummy solid in 92% yield. Material obtained in this manner proved suitable for use in subsequent coupling reactions without further purification.

The preparation of MFCO-functionalized DOTA and NOTA derivatives (Scheme 2) proceeded smoothly by reaction of **3** with commercially available DOTA and NOTA *N*-hydroxysuccinimide esters (NHS esters) **4** and **5**. These couplings were performed in DMF/DMSO at room temperature, and each reaction proceeded to completion within 2 h as determined by HPLC monitoring. The desired products **6** and **7** were isolated and purified by HPLC in good chemical yield (85% and 47%, respectively) and their identities were confirmed by mass spectrometry.

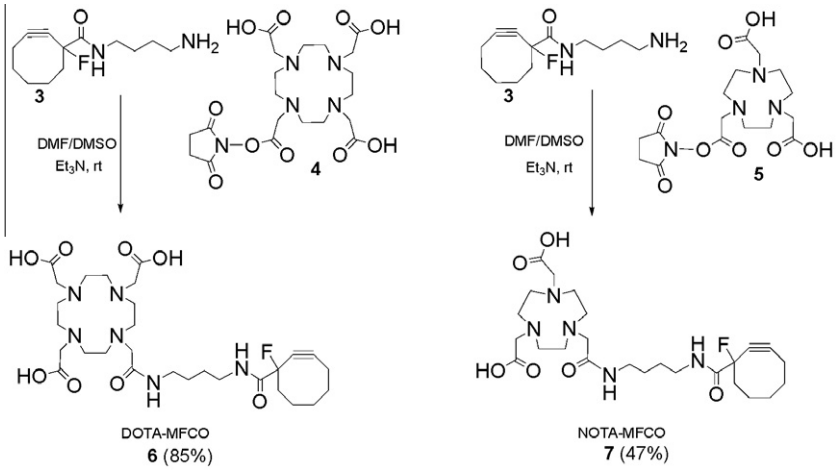
With **6** and **7** in hand, selective attachment of these new chelators to an azide-modified peptide via Cu-free click chemistry was next explored. The peptide chosen is a variant of α-melanocortin-stimulating hormone (α-MSH) in which two residues have been replaced with unnatural amino acids (Nle^4,D-Phe^7) to form NDP-α-MSH, which exhibits enhanced affinity for melanocortin receptors relative to the natural ligand. Since binding assays involving NDP-α-MSH are well established,[20–27] this ligand offers a means to test the effect of DOTA(NOTA)-MFCO conjugation via fused triazole linkages on binding affinity to its cell-surface receptor target (i.e., melanocortin receptor subtype 1; MC1R).

An azide-modified derivative of NDP-α-MSH (**8**) was prepared by standard solid phase peptide synthesis. Incorporation of the azide function was accomplished by coupling 6-azido hexanoic acid to the *N*-terminus of the fully side-chain protected peptide on resin, followed by deprotection and cleavage using routine procedures and purification/characterization by RP-HPLC and LC–MS.

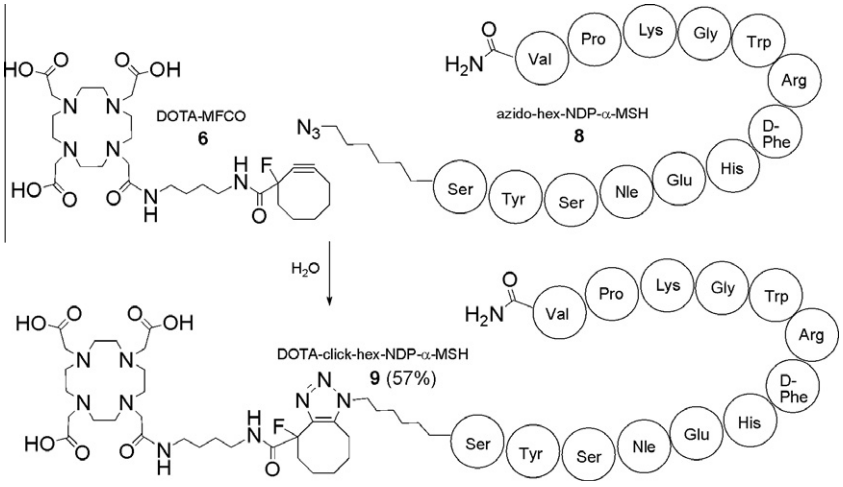
DOTA-click-hex-NDP-α-MSH (**9**) was synthesized by reacting 100 nmol of azido-hex-NDP-α-MSH (**8**) with a 10-fold excess of DOTA-MFCO (**6**) in 0.5 mL of H_2O at rt (Scheme 3). The corresponding NOTA analog was prepared similarly by treating **8** with a 20-fold excess of **7**. Reaction progress was monitored by observing the disappearance of starting material using RP-HPLC (214 nm). The Cu-free click reaction between DOTA-MFCO (**6**) and azido-hex-NDP-α-MSH (**8**) formed the desired product (**9**) in 2 h while complete formation of NOTA-click-hex-NDP-α-MSH (**10**, not shown in Scheme 3) required 5.5 h. The differences in reaction kinetics under these conditions are unclear, but may be related to differences in solubility of the NOTA- and DOTA-MFCO bifunctional chelators. Further optimization of the reaction parameters are the subject of ongoing research. No evidence of side reactions was observed by monitoring the 214 nm absorbance of the HPLC trace during the reaction period. The peptide conjugates were purified by HPLC, lyophilized, converted to the acetate form, and stored at −20 or −80 °C (see Supplementary data). HPLC purification provided excellent isolated chemical yields and purity for these reactions for both DOTA-click-hex-NDP-α-MSH (**9**, 57.1%; ESI-MS: observed 2370.4 amu; theoretical: 2370.7 amu); and NOTA-click-hex-NDP-α-MSH (**10**, 70.3%; ESI-MS: observed 2269.4 amu; theoretical: 2269.58 amu). While the formation of 1,4 and 1,5-triazole regioisomers was expected, the species were not distinguishable by radio- or UV-HPLC methods applied here (see Fig. 1; and Supplementary Fig. 4). Although radioHPLC traces for ^{64}Cu suggested the presence of the expected regioisomers, efforts to separate these species further by modification of HPLC parameters proved unsuccessful (data not shown). On the other hand, mass spectral analysis of the resulting bioconjugates indicated that the fluorine substituent is labile over a period of months despite storage at −20 and −80 °C (see Supplementary Fig. 4). However, subsequent radiolabeling and in vitro competitive binding assays and stability analysis in serum indicate that loss of fluorine (as HF) neither degrades the binding affinity of the peptides for their cognate receptor target, nor promotes degradation of the chelator-peptide bioconjugate.

The ability to incorporate radionuclides into the purified bioconjugates was next determined through radiolabeling with positron emitters ^{64}Cu and ^{68}Ga. Radiolabeling reactions were carried out by methods described previously.[18,28,29] Briefly, ^{64}Cu (Washington University in Saint Louis, USA) was incubated for 45 min at 70 °C with 5–10 nmol of peptide in 50 μL acetate buffer (pH 6.0). For ^{68}Ga, radiolabeling was carried out through the use of a ^{68}Ga/^{68}Ge generator system IGG100 (Eckert Ziegler, GmbH, Berlin, Germany) with a total ^{68}Ge activity of approximately 900 MBq at the time of the experiments presented here. ^{68}Ga was eluted in 10 mL 0.1 M HCl, purified by cation exchange and incubated with 5–10 nmoles of chelator modified peptide at 100 °C for 12 min. Preparation of ^{68}Ga labeled variants required the insertion of a final purification step using a disposable C-18 cartridge post-radiolabeling to achieve suitable radiochemical purity (Fig. 1A and B). In each case radioHPLC analysis demonstrated excellent radiochemical purity (>98%) and specific activity (^{68}Ga, 48 MBq nmole^{-1} and

[†] DOTA – 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; NOTA – 1,4,7-triazacyclononane-1,4,7-triacetic acid.



Scheme 2. Synthesis of DOTA-MFCO (**6**) and NOTA-MFCO (**7**).



Scheme 3. Synthesis of DOTA-click-hex-NDP-α-MSH (**9**).

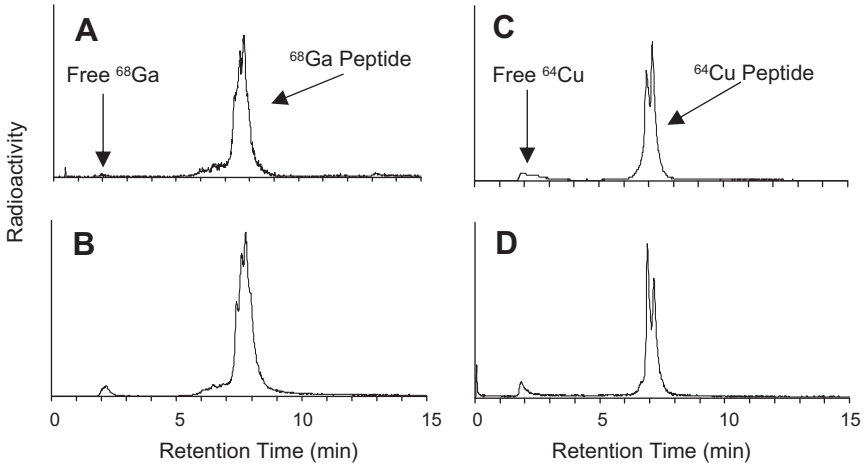


Figure 1. Radiochemical purity of ^{64}Cu and ^{68}Ga labeled DOTA- and NOTA-click-hex-NDP-a-MSH (**9**, **10**): (A) [^{68}Ga]DOTA-peptide; (B) [^{68}Ga]NOTA-peptide; (C) [^{64}Cu]DOTA-peptide; (D) [^{64}Cu]NOTA-peptide. Radiochemical purity in excess of 98% is achieved in all cases.

^{64}Cu, 35 MBq nmole^{-1}). It is anticipated that the specific activity of the ^{64}Cu variant could be improved as no final purification step was required to obtain >98% radiochemical purity at specific activity of 35 MBq nmole^{-1} (Fig. 1C and D).

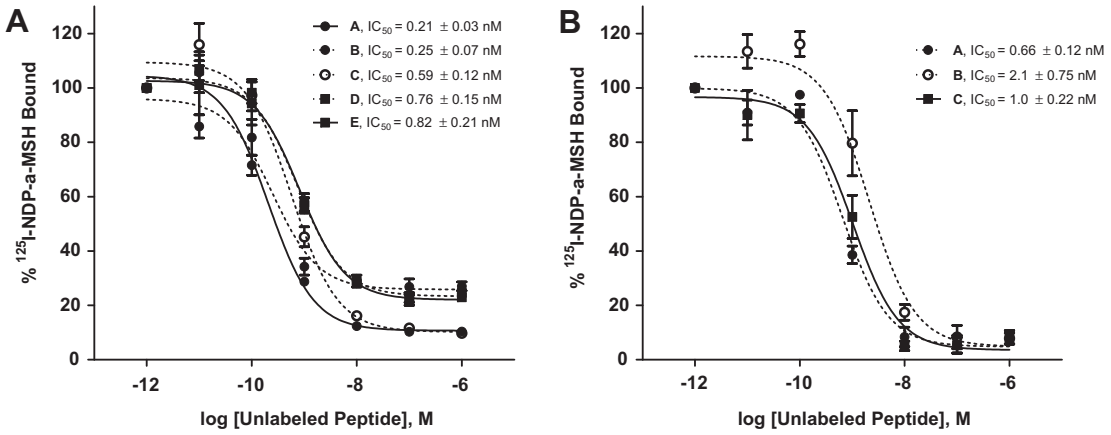


Figure 2. Competitive inhibition of [^{125}I]-NDP-α-MSH binding to B16-F10 mouse melanocytes by α-MSH peptides. (A) Peptides A through E ($n = 4$) in the figure legend correspond to the following: (A) NDP-α-MSH; (B) azido-hex-NDP-α-MSH (**8**); (C) DOTA-amide-NDP-α-MSH; (D) DOTA-click-hex-NDP-α-MSH (**9**); and (E) NOTA-click-hex-NDP-α-MSH (**10**). (B) Peptides A through C in the figure legend correspond to the following: A—[Ga]DOTA-NDP-α-MSH, B—[Ga]DOTA-click-NDP-α-MSH, and C—[Ga]NOTA-click-NDP-α-MSH.

Finally, the biological activities of DOTA and NOTA-click-hex-NDP-α-MSH peptide conjugates **9** and **10** were evaluated through in vitro cell-binding assays. For these experiments, the effect of the fused triazole-MFCO linkage on receptor binding was determined by comparison to binding affinities exhibited by α-MSH peptide derivatives lacking this motif. The effect of metallation on receptor binding affinity was examined using stable Ga^{3+} labeled DOTA-click-hex-NDP-α-MSH. Specifically, the binding of **9** (metallated and unmetallated) and **10** to B16-F10 murine melanoma cells expressing MC1R was compared to binding affinities obtained for NDP-α-MSH, azido-hex-NDP-α-MSH (**8**), and a DOTA-NDP-α-MSH derivative prepared by standard *N*-amide bond conjugation. By using stable Ga^{3+} for these experiments, we were able to make this comparison using the same iodine-125 (^{125}I) labeled native α-MSH competitor and melanoma cells, thus enabling a fair comparison of observed binding affinities of the unmetallated species conducted by us and according to methods described previously.[30] Briefly, cell suspensions were incubated with a constant quantity of ^{125}I labeled NDP-α-MSH ([^{125}I]-[Nle^4,D-Phe^7]-α-MSH) and increasing concentrations of peptide analog ranging from 10^{-6} to 10^{-11} M. Following incubation, cells were pelleted by centrifugation, the media was aspirated, pellets were transferred to 12 × 75 mm glass tubes, and the radioactivity of the cell pellet was determined by gamma counter. Each experiment was performed in quadruplicate and binding curves were plotted; IC_{50} values and their associated standard errors were calculated with GraphPad Prism 5 curve-fitting software (GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, CA). The observed IC_{50} values for the DOTA- and NOTA-click-hex-NDP-α-MSH (**9** and **10**) calculated in this way were less than 1 nM (0.82 and 0.76 nM, respectively, Fig. 2). No significant difference in binding affinity was observed for Ga^{3+} metallated NOTA- click-hex-NDP-α-MSH (1.0 ± 0.22 nM) and the unmetallated species (0.82 ± 0.21 nM). Comparison of affinity values for Ga^{3+} metallated DOTA-amide-NDP (0.66 ± 0.12 nM) and unmetallated conjugate (0.59 ± 0.12 nM) also yielded no significant difference associated with metallation of the chelator modified peptide. The metallated-species affinity values are slightly elevated in comparison to unmetallated conjugates and overall are slightly higher than standard *N*-terminal amide bond formation, although the difference is not statistically significant. Furthermore, **9** and **10** display IC_{50} concentrations comparable to those of azido-hex-NDP-α-MSH (**8**) and NDP-α-MSH.[21,24,27,31–33] On the other hand, the binding affinity of the Ga^{3+} metallated DOTA-click-hex-NDP-α-MSH conjugate (2.1 ± 0.75 nM) appeared somewhat higher than the unmetallated counterpart (0.76 ± 0.15), although the uncertainty obtained for the metallated conjugate is rather large in comparison to other experimental results suggesting the potential for an artifact that requires further investigation. In general, these findings suggest that the presence of fused triazole-MFCO linkages does not significantly alter the binding affinity or selectivity of the molecular targeting vector in vitro. It is anticipated that this quality will be preserved for conjugation of the MFCO-family of chelators to other molecular targeting vectors as well.

In summary, two novel chelator moieties (DOTA-MFCO **6** and NOTA-MFCO **7**) were successfully synthesized in high yield and used for copper-free click conjugation to an azide-modified peptide. Specifically, these MFCO-modified chelators were selectively attached to an azide-modified fully-deprotected analog of NDP-α-MSH under aqueous conditions at room temperature within 2 h (DOTA-MFCO) and 5.5 h (NOTA-MFCO). The new NOTA and DOTA peptide variants were each radiolabeled with ^{64}Cu and ^{68}Ga in excellent radiochemical purity and specific activity. In vitro binding assays suggest that the fused-triazole conjugation strategy provides a stable coupling and does not significantly alter the binding affinity of the peptide for its cognate MC1R receptor. Metallation with Ga^{3+} did not significantly alter the binding affinity of the NOTA-click-hex-NDP-α-MSH or DOTA-click-hex-NDP-α-MSH for their molecular target. The "click" bioconjugates were stable in mouse serum when incubated at 37 °C for 3 h (a relevant time frame for peptide-based molecular targeting), with evidence of a stability advantage over an amide-linked DOTA-NDP-α-MSH variant that is the subject of ongoing research (see Supplementary Figs. 5 and 6). Thus, the DOTA-MFCO (**6**) and NOTA-MFCO (**7**) entities represent a new class of bifunctional chelator that enables selective attachment to fully-deprotected azide-modified peptides under aqueous conditions at room temperature with excellent chemical yields.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.08.017.

References and notes

1. Bushnell, D. L., Jr.; O'Dorisio, T. M.; O'Dorisio, M. S.; Menda, Y.; Hicks, R. J.; Van Cutsem, E.; Baulieu, J. L.; Borson-Chazot, F.; Anthony, L.; Benson, A. B.; Oberg, K.; Grossman, A. B.; Connolly, M.; Bouterfa, H.; Li, Y.; Kacena, K. A.; LaFrance, N.; Pauwels, S. A. *J. Clin. Oncol.* **2010**, *28*, 1652.
2. Menda, Y.; O'Dorisio, M. S.; Kao, S.; Khanna, G.; Michael, S.; Connolly, M.; Babich, J.; O'Dorisio, T.; Bushnell, D.; Madsen, M. *J. Nucl. Med.* **2010**, *51*, 1524.
3. Miao, Y.; Quinn, T. P. *Crit. Rev. Oncol. Hematol.* **2008**, *67*, 213.
4. Virgolini, I.; Ambrosini, V.; Bomanji, J. B.; Baum, R. P.; Fanti, S.; Gabriel, M.; Papathanasiou, N. D.; Pepe, G.; Oyen, W.; De Cristoforo, C.; Chiti, A. *Eur. J. Nucl. Med. Mol. Imaging* **2010**, *37*, 2004.
5. De Leon-Rodriguez, L. M.; Kovacs, Z. *Bioconjugate Chem.* **2008**, *19*, 391.
6. Brechbiel, M. W. *Q. J. Nucl. Med. Mol. Imaging* **2008**, *52*, 166.
7. Dijkgraaf, I.; Liu, S.; Kruijtzer, J. A.; Soede, A. C.; Oyen, W. J.; Liskamp, R. M.; Corstens, F. H.; Boerman, O. C. *Nucl. Med. Biol.* **2007**, *34*, 29.
8. El-Sagheer, A. H.; Brown, T. *Chem. Soc. Rev.* **2010**, *39*, 1388.
9. Lebedev, A. Y.; Holland, J. P.; Lewis, J. S. *Chem. Commun.* **2010**, 1706.
10. Moses, J. E.; Moorhouse, A. D. *Chem. Soc. Rev.* **2007**, *36*, 1249.
11. Knor, S.; Modlinger, A.; Poethko, T.; Schottelius, M.; Wester, H. J.; Kessler, H. *Chemistry* **2007**, *13*, 6082.
12. Nwe, K.; Brechbiel, M. W. *Cancer Biother. Radiopharm.* **2009**, *24*, 289.
13. Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. *PNAS* **2007**, *104*, 16793.
14. Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *JACS* **2008**, *130*, 11486.
15. Sletten, E. M.; Bertozzi, C. R. *Org. Lett.* **2008**, *10*, 3097.
16. Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253.
17. Jewett, J. C.; Bertozzi, C. R. *Chem. Soc. Rev.* **2010**, *39*, 1272.
18. Schultz, M. K.; Parameswarappa, S. G.; Pigge, F. C. *Org. Lett.* **2010**, *12*, 2398.
19. Martin, M. E.; Parameswarappa, S. G.; O'Dorisio, M. S.; Pigge, F. C.; Schultz, M. K. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4805.
20. Miao, Y.; Gallazzi, F.; Guo, H.; Quinn, T. P. *Bioconjugate Chem.* **2008**, *19*, 539.
21. Miao, Y.; Whitener, D.; Feng, W.; Owen, N. K.; Chen, J.; Quinn, T. P. *Bioconjugate Chem.* **2003**, *14*, 1177.
22. Raposinho, P. D.; Correia, J. D.; Oliveira, M. C.; Santos, I. *Biopolymers* **2010**, *94*, 820.
23. Ren, G.; Liu, S.; Liu, H.; Miao, Z.; Cheng, Z. *Bioconjugate Chem.* **2010**, *21*, 2355.
24. Wei, L.; Butcher, C.; Miao, Y.; Gallazzi, F.; Quinn, T. P.; Welch, M. J.; Lewis, J. S. *J. Nucl. Med.* **2007**, *48*, 64.
25. Wei, L.; Miao, Y.; Gallazzi, F.; Quinn, T. P.; Welch, M. J.; Vavere, A. L.; Lewis, J. S. *Nucl. Med. Biol.* **2007**, *34*, 945.
26. Yang, J.; Guo, H.; Gallazzi, F.; Berwick, M.; Padilla, R. S.; Miao, Y. *Bioconjugate Chem.* **2009**, *20*, 1634.
27. Yang, J.; Guo, H.; Padilla, R. S.; Berwick, M.; Miao, Y. *Bioorg. Med. Chem.* **2010**, *18*, 6695.
28. Anderson, C. J.; Wadas, T. J.; Wong, E. H.; Weisman, G. R. *Q. J. Nucl. Med. Mol. Imaging* **2008**, *52*, 185.
29. Rockey, W. M.; Huang, L.; Kloepping, K. C.; Baumhover, N. J.; Giangrande, P. H.; Schultz, M. K. *Bioorg. Med. Chem.* **2011**, *19*, 4080.
30. Eberle, A. N.; Verin, V. J.; Solca, F.; Siegrist, W.; Kuenlin, C.; Bagutti, C.; Stutz, S.; Girard, J. *J. Recept. Res.* **1991**, *11*, 311.
31. Miao, Y.; Hoffman, T. J.; Quinn, T. P. *Nucl. Med. Biol.* **2005**, *32*, 485.
32. Miao, Y.; Quinn, T. P. *Front. Biosci.* **2007**, *12*, 4514.
33. Quinn, T.; Zhang, X.; Miao, Y. *G. Ital. Dermatol. Venereol.* **2010**, *145*, 245.