REVIEWS

Synthesis of Target-Specific Radiolabeled Peptides for Diagnostic Imaging

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INTRODUCTION

Radiopharmaceuticals are drugs containing atoms of some radioactive elements. They are designed for diagnostic or therapeutic purposes, to deliver small doses of ionizing radiation to the disease sites in the body. Radiopharmaceuticals, unlike classical chemotherapeutics, act against malignant cells with high specificity. Radiopharmaceuticals are mostly small organic molecules, such as peptides or peptidomimetics, but they can also be macromolecules, for example antibodies.

Biodistribution of radiopharmaceuticals can be determined either by their chemical and physical properties or by their biological interactions. Radiopharmaceuticals, which act through receptor binding, are called target-specific. Ideally, these radiopharmaceuticals are designed to locate with high specificity at cancerous tumors, even if their location in the body is unknown, while producing minimal radiation damage to normal tissues (*1*−*6*). In the past decade significant progress has been made in the development of peptide-based target-specific radiopharmaceuticals, which have become an important class of imaging agents for the detection of various diseases, such as tumors, thrombosis, and inflammation.

Many excellent reviews have been published discussing different aspects of radionuclide chemistry and therapy (*7*−*9*) and the use of radiopharmaceuticals for diagnosis and treatment of different pathological conditions (*10*−*19*). Most popular technetium radiopharmaceuticals (*20*−*28*), as well as those incorporating other radionuclides (*29*, *30*), have been reviewed. Antibodies (*24*, *25*), peptides (*23*, *30*−*35*), and steroids (*36*) as targeting molecules have been described.

In this review, which is limited to the use of small peptides as targeting molecules, we attempt to summarize, from the chemical point of view, the development of labeling methods, in particular the application of different bifunctional chelating agents. We also give a short description of radionuclides used with these agents. Radiopharmaceuticals based on small peptides, which are in clinical use or under investigation in preclinical and clinical trials, are also mentioned.

LABELING METHODS

Direct Labeling. Direct labeling methods (Table 1) are mostly based on the binding of a radionuclide to thiol groups in the targeting molecule, that seems relatively easy to perform (*37*, *38*). However, such a labeling process is difficult to control, for its detailed chemistry is unknown and may lead to unplanned changes in the structure, stability, and pharmacokinetic properties of the labeled molecule. Furthermore, very little is known about the number of donor atoms in the labeled molecule and the geometry of radionuclide coordination. The stability in vivo of a synthesized complex also remains uncertain.

The direct labeling approach is rather unsuitable for small peptides, which either do not possess disulfide linkage or are unable to maintain their activity after reduction. For example Thakur (*39*) has reported the alteration of the receptor binding properties of the radiolabeled somatostatin analogues, when the disulfide bridge was reduced to free thiol groups and subsequently radiolabeled with ^{99m}Tc. However, direct labeling has been successfully applied for labeling of the platelet receptor-binding peptide with ^{99m}Tc (*40*, *41*) and for high molecular weight proteins such as antibodies and their fragments (*42*, *43*).

Chelate Methods. In chelate methods (Table 1) a radionuclide is bound to the targeting molecule indirectly, through a bifunctional chelating agent (BFCA) (Figure 1).

In general, a radiopharmaceutical containing a BFCA consists of the following parts: a targeting molecule, BFCA, radionuclide, and a linker (*22*). The targeting molecule is a carrier of a radionuclide to the receptor site in vivo. A radionuclide serves as a radiation source. A BFCA, covalently attached to the targeting molecule, functions as the coordinator of the radionuclide. A linker, not always necessary, is a spacer residue, which separates a targeting molecule from a chelating agent.

Functional groups, naturally present in the peptide or introduced synthetically, are responsible for covalent attachment of BFCA. Naturally occurring functional groups include terminal, as well as side-chain amino and carboxy groups, thiol groups from cysteine, and *p*-hydroxyphenyl from tyrosine. BFCA must contain a conjugation group, which is used for attachment of the peptide.

Several types of conjugation groups, active esters, isothiocyanates, maleimides, hydrazides, and α-haloamides are used to form BFCA−peptide linkages (Figure 2).

Active esters can be used to form an amide bond between a carboxy group of a BFCA and an amino group in a peptide ligand. Since they are at a low level of activation, side-reactions during coupling are generally less of a problem than with most amide bond forming procedures. Commonly used are *p*-nitrophenyl, pen-

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Table 1. General Overview of Applied Labeling Methods

labeling method	main principle	targeting molecule	advantages	disadvantages	ref
direct labeling	radionuclide binds directly to active groups present in the targeting molecule	high molecular weight molecules	easy to perform	unknown chemistry	(*32*, *35*, *37*, *38*)
				unknown geometry of a radionuclide-targeting molecule complex	
				possible damage to targeting molecule during labeling process	
chelate methods					
prelabeling	labeling of BFCA followed by conjugation with the targeting molecule	small peptides	relatively easy to control, well-defined chemistry	time-consuming	(*26*, *32*)
			targeting molecule functional groups remain unlabeled	complicated purification of obtained radiopharmaceutical	
postlabeling	conjugation of BFCA to targeting molecule, followed by labeling of conjugate	small peptides	most popular method	possible damage to targeting molecule during labeling process	(*35*, *44*)
			well-defined chemistry possible use of classical solid-phase or solution methods of the peptide synthesis		

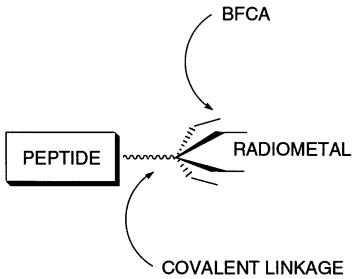


Figure 1. Schematic structure of a radiopharmaceutical. BFCA can complex a metal and also contains a functional group which forms a covalent linkage to a biological molecule, such as peptide.

tafluorophenyl, *N*-hydroxysuccinimide, and sulfo-*N*-hydroxysuccinimide active esters. The *N*-hydroxysuccinimide esters are very reactive with high selectivity toward aliphatic amines (*22*, *45*). The choice of an active ester to be used for BFCA−peptide bond formation is partly dictated by sheer reactivity, but the ease of coproduct removal is also an important consideration. Thus, for water-insoluble BFCA−peptide conjugates, a succinimide ester coupling is especially convenient because both *N*-hydroxysuccinimide and sulfo-*N*-hydroxysuccinimide are very water-soluble (*46*, *47*) and easy to remove. But for water-soluble BFCA−peptide conjugates *p*-nitrophenyl or pentafluorophenyl esters, which are ether-soluble, may be a better choice (*48*).

Isothiocyanates, like active esters, react with amino groups of a peptide forming thiourea bonds. Since they react best in the higher pH, they cannot be used with peptides susceptible to alkaline conditions.

The third class are maleimides, which react with thiols and form thioether bonds. The optimum pH for the reaction is near 7. At higher pH maleimides may hydrolyze to form nonreactive maleanic acids (*49*, *50*).

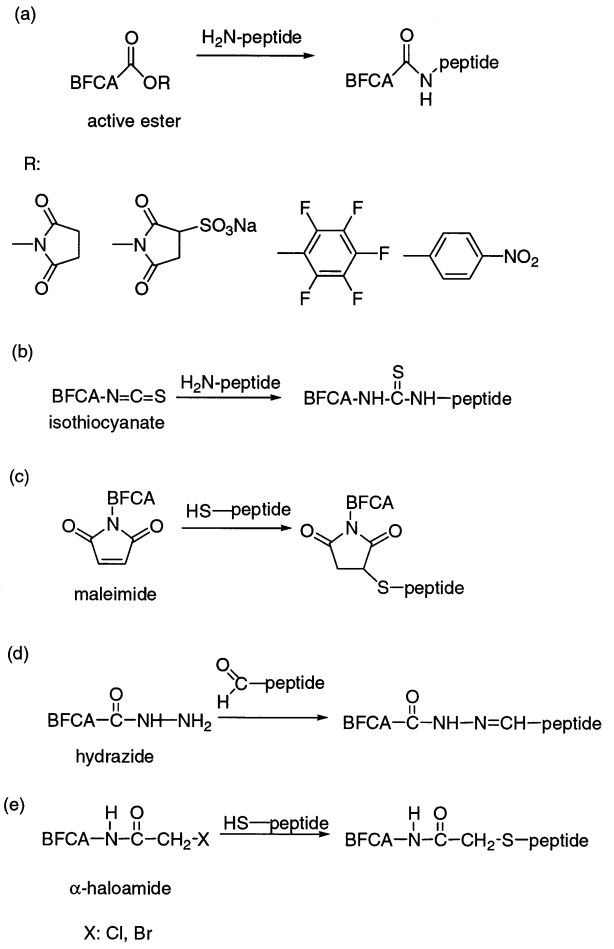


Figure 2. Reaction schemes for BFCA−peptide conjugation.

peptide conjugate. They are suitable for peptides contain-

by periodate at pH = 7 to generate an aldehyde. The use of a low molar ratio of periodate to peptide minimizes the potential for side-reactions during the oxidation. The formed hydrazones are stable at pH 6−8 for at least 12 h at 22 °C (*51*).

α-Haloamides are suitable for conjugation with peptides containing a free SH group. Chloroacetyl- or bromoacetylamides are used (*52*, *53*).

The bifunctional approach is often associated with the term 'pharmacokinetic modifier' (PKM). Liu and Edwards (*22*) define PKM as a linker between a targeting molecule and a BFCA. PKM is a spacer residue, separating the radionuclide from the binding site of the molecule to minimize the risk of its undesired modification. The use of a linker helps to choose a BFCA and a radionuclide more independently. The most popular linkers are long poly(ethylene glycol) (PEG) or hydrocarbon chains to increase the lipophilicity and polyamino acid sequences, such as polyglycine, to increase the hydrophilicity, as well as esters and disulfides capable of rapid metabolism.

The distinction between chelate methods can be made depending on the sequence of the steps used for the synthesis of a radiolabeled peptide.

Prelabeling Method. The pre-labeling method (preformed chelate approach) is based on the labeling of a BFCA with its subsequent activation and conjugation, through covalent bonds, to a peptide. In this approach the chemistry of a process is well defined and easily controlled. As the labeling and conjugation steps are separated, it can be ensured that the radionuclide is attached directly to a chelate moiety and the peptide amino groups remain unlabeled (*22*). However, the prelabeling of a BFCA may impair the conjugation process and complicate purification of a radiopharmaceutical. The method is time-consuming, so not appropriate for use with short-lived isotopes, generally associated with imaging. As such the prelabeling method is rarely used for synthesis of radiolabeled peptides.

Postlabeling Method. The postlabeling method (indirect labeling approach), the most popular approach in the synthesis of radiopharmaceuticals, requires the synthesis of a BFCA−peptide conjugate and is followed by its labeling. In this method BFCA may be attached to N- or C-terminus, as well as to a side chain of a peptide or it can be even incorporated into a peptide backbone. The postlabeling method is characterized by a well-defined chemistry and relative simplicity. For convenient, high yield synthesis of radiopharmaceuticals, applied BFCA should be compatible with the solid-phase or solution methods of peptide synthesis. However, harsh conditions required for effective labeling of conjugates may sometimes cause changes in the amino acid sequence or peptide backbone conformation and even begin the decomposition of the whole radiopharmaceutical (*35*).

BIFUNCTIONAL CHELATING AGENTS

BFCAs are used to connect a radionuclide and a targeting molecule to form a radiopharmaceutical. An ideal BFCA should coordinate the radionuclide with a high yield, to form a relatively stable complex. The agent must comply with the nature and oxidation state of a radionuclide and should prevent any accidental changes in its redox potential.

It is important to carefully choose a proper BFCA, as the conjugation with targeting molecule requires specific conditions: pH, temperature, reaction time. The stere-

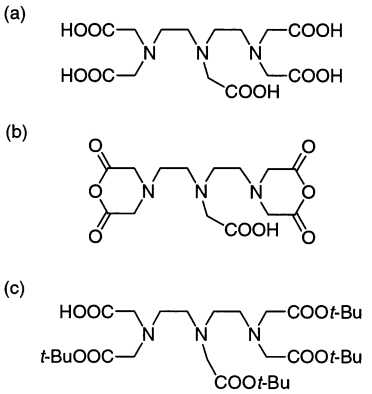


Figure 3. Structure of DTPA and its analogues: (a) DTPA; (b) cDTPA; (c) mDTPA.

DTPA. DTPA (N^{α}-diethylenetriaminopentaacetic acid) belongs to the group of polyaminocarboxy chelates (Figure 3a). It is a strong chelating group, mostly linked with ^{111}In, a trivalent radionuclide. It can be attached to larger proteins, e.g., albumins and antibodies (*49*, *50*, *54*), as well as to small peptides, like somatostatin analogues (*55*, *56*). The conjugation of DTPA with macromolecules has been successfully performed by the use of isobutyl chloroformate as a coupling reagent (*57*). For small peptides, however, DTPA derivatives such as DTPA bicyclic anhydride (cDTPA) and monoreactive DTPA derivative, 3,6-bis(carboxymethyl)-9-(((2-maleimidoethyl)-carbamoyl)methyl)-3,6,9-triazaundecanedioic acid (mDTPA), have been applied (Figure 3b and 3c).

Hnatowich et al. (*54*) have developed a simple method of covalent coupling of cDTPA to peptides at their amino groups. The efficiency of this method is relatively high and it has several advantages. The coupling reaction runs in an aqueous solution and is a simple, one-step process. The side product of the reaction, a double substituted DTPA derivative, and unreacted material can be both easily separated from the main product by gel chromatography. The conjugated peptide maintains its affinity toward specific receptors. The sample, purified before the addition of a radionuclide, can be stored and labeled only when required. For peptides containing lysine the conjugation occurs especially at its ϵ-amino group, as more basic than N-terminal amino group. The method is therefore inappropriate for somatostatin analogues, as the lysine residue is situated within the active site of the molecule and the conjugation may result in the loss of receptor binding activity. For that reason a modified method, proposed by Bakker et al. (*55*) for DTPA-octreotide, is used to conjugate the somatostatin analogue with cDTPA. In this approach lysine residue within the active site of the peptide is protected with *tert*-butyloxycarbonyl group (Boc), before the reaction with cDTPA and deprotected after the conjugation (Figure 4). This method enables a selective reaction of the N-terminal amino group with BFCA, whereas the lysine residue within the bioactive site remains unsubstituted.

A monoreactive DTPA derivative, mDTPA, with four carboxy groups protected as *tert*-butyl esters, was introduced by Arano et al. (*57*) (Figure 5). Since mDTPA possesses only one free carboxy group, the formation of undesired intermolecular linkages with peptides is prevented. High solubility of mDTPA in various solvents makes this BFCA appropriate for both liquid- and solid-phase peptide synthesis.

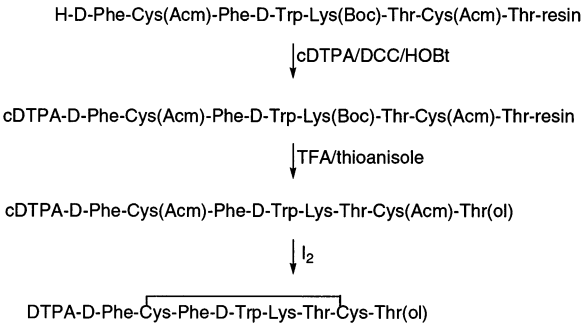


Figure 4. Synthesis of DTPA−octreotide, starting with cDTPA.

tion, as they compete with radionuclides in the process of labeling. For that reason a significant, 40- to 70-fold molar excess of peptide conjugate and ultrapure radionuclide derivative of the highest possible specific activity are required (*55*).

DTPA conjugates have been shown to form ^{111}In-chelate structures (Figure 6), which are eight coordinate, using all three amino and four carboxy groups, while the eighth position around the radionuclide is occupied by the amide carbonyl oxygen (*58*). ^{111}In−DTPA conjugates possess excellent in vivo stability (*59*−*61*).

Many research groups put much effort in the synthesis of kinetically stable DTPA−peptide conjugates that form complexes with ^{90}Y (*62*). Substitutions, particularly in the carbon atoms of the DTPA backbone, sterically hinder the opening of the chelate ring that must occur during radionuclide complex dissociation and increase the in vivo stability of the radiopharmacutical. The first class of modified DTPA conjugates was constructed by attaching *p*-isothiocyanatobenzyl moiety to one DTPA backbone ethylene group and appending methyl to another ethylene group in the same backbone (Figure 7a,b). The second class of modified DTPA conjugates was developed by replacing one of the ethylene groups by a cyclohexyl moiety (Figure 7c). Such modifications increase the rigidity in the DTPA backbone and the in vivo stability of obtained radiopharmaceuticals (*62*−*64*). Synthesis of new derivatives constructed on DTPA core has been recently reported (*65*, *66*).

^{99m}Tc is less suitable for the labeling of DTPA−peptide conjugates, as this radionuclide, even at high concentrations, has low affinity and poor selectivity to the binding sites of this BFCA (*67*).

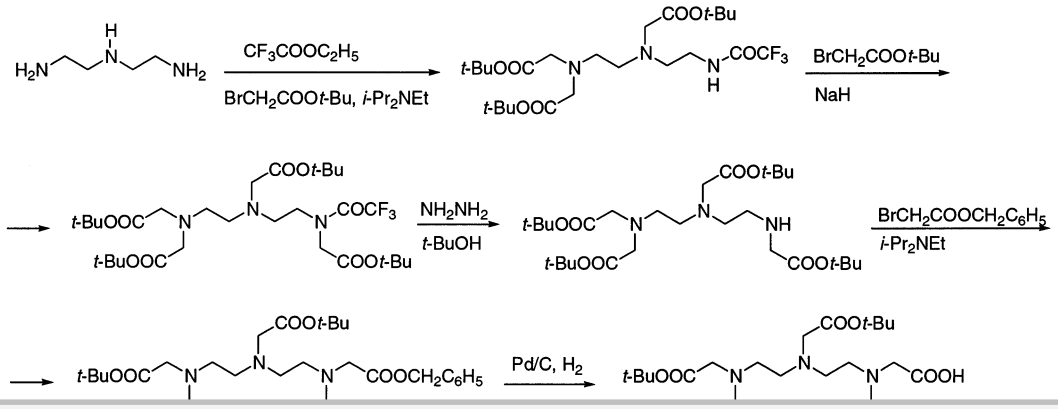
DOTA. DOTA (1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) (Figure 8a) and its derivatives proved to be a good alternative for DTPA. They play an important role in clinical applications, as they form very stable complexes with a variety of trivalent radionuclides, such as ^{66,67,68}Ga,^{86,90}Y, ^{111}In, ^{149}Pm, ^{177}Lu (*68*−*73*) and divalent radionuclides, ^{27}Mg,^{47}Ca,^{64}Cu (*74*).

Two different approaches for DOTA conjugation with peptides have been developed. In the first approach one of the four carboxy groups in DOTA is activated to facilitate the reaction with primary amines in the peptide and form a stable amide bond linkage. In the second approach DOTA derivatives with additional side chains are used. A peptide ligand is attached to the side chain of DOTA derivative. Several DOTA derivatives have been synthesized so far, like PA−DOTA (α-[2-(4-aminophenyl)-ethyl]-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and *p*-NCS-Bz−DOTA ((2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) (Figure 8b and 8c) (*69*). Recently Eisenwiener et al. (*44*) have introduced two new DOTA derivatives, DOTASA(*t-Bu*)$_4$, (1-(1-carboxy-2-carbo-*tert*-butoxyethyl)-4,7,10-(carbo-*tert*-butoxymethyl)-1,4,7,10-tetraazacyclododecane) and DOTAGA(*t*-Bu)$_4$, (1-(1-carboxy-3-carbo-*tert*-butoxypropyl)-4,7,10-(carbo-*tert*-butoxymethyl)-1,4,7,10-tetraazacyclododecane) (Figure 8d), which convenient synthesis is outlined in Figure 9. The conjugation of all DOTA derivatives to a peptide is performed through an amino group of a peptide.

Fully eight coordinate structure has been reported for all DOTA complexes (*60*) using four amino and four carboxy groups. In the case when one carboxy group is used for conjugation, the amide carbonyl oxygen occupies the eighth position around the radionuclide.

DOTA and derivatives were successfully conjugated to a number of somatostatin analogues, and obtained radiopharmaceuticals had good pharmacological parameters (*72*−*78*). DOTA conjugates are especially suitable for radionuclide therapy, as they can be radiolabeled with ^{67}Ga (*75*), ^{90}Y (*71*, *76*), and ^{111}In (*73*). De Jong et al. (*68*) have demonstrated that ^{90}Y−DOTA conjugates have very good pharmacokinetic properties in vivo. However, in these conjugates the chelate is situated closer to the peptide, the labeled conjugate is more rigid and less flexible, which makes binding with the receptor more difficult. Reubi et al. (*79*) reported the best pharmacological properties for ^{67}Ga−DOTA complexes. The radionuclide coordination geometry, including the number of uncomplexed carboxy and amino groups, increases the flexibility of a ligand and allows its better adjustment to the receptor binding site.

TETA. TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,-11-tetraacetic acid) (Figure 10) is one of the most studied chelating agents for copper in peptide targeted radio-



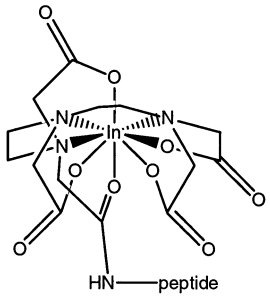


Figure 6. Possible structure of In^{3+}−DTPA−peptide.

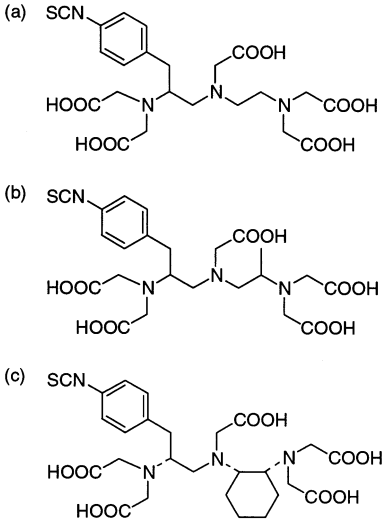


Figure 7. Structures of DTPA derivatives: (a) 2-(*p*-isothiocyanatobenzyl) diethylenetriaminopentaacetic acid; (b) 2-(*p*-isothiocyanatobenzyl)-6-methyldiethylene-triaminopentaacetic acid (1B4H-DTPA); (c) 2-(*p*-isothiocyanatobenzyl) cyclohexyldiethylene-triaminopentaacetic acid (CHX-DTPA).

therapy. TETA has been successfully used as a BFCA with somatostatin analogues (*30*).

NOTA. NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) (Figure 11a), its phosphonate analogue NOTP (1,4,7-triazacyclononane-*N*,*N*′,*N*″-tris(methylenephosphonic) acid) (Figure 11b) and the monoethyl ester of NOTP, NOTPME (1,4,7-triazacyclononane-*N*,*N*′,*N*″-tris(methylenephosphonate-monoethyl ester)) (Figure 11c) were studied for possible use in radiopharmaceuticals. Complexes with ^{67}Ga (*80*) and ^{111}In (*81*) were reported.

A monoreactive NOTA derivative, NODAGA(tBu)$_3$ (1-(1-carboxy-3-carbo-*tert*-butoxypropyl)-4,7-(carbo-*tert*-butoxymethyl)-1,4,7-triazacyclononane) (Figure 11d) was synthesized by Eisenwiener et al. (*82*). The synthesis is outlined in Figure 12. This BFCA is useful for the coupling to the N-terminus of peptides on solid-phase and in solution. The NODAGA−peptide conjugates were labeled with ^{67}Ga and ^{111}In in high yields and good specific activities. NODAGA-based derivatives carry a spacer function between the BFCA and the peptide which improves the receptor binding affinity.

HYNIC. HYNIC (2-hydrazinonicotinic acid) (Figure 13), first described by Abrams et al. (*83*) has been used as a BFCA for radiolabeling of different groups of molecules, such as γ-globulins (*83*, *84*), chemotactic peptides (*85*, *86*), and somatostatin analogues (*87*−*90*). Structural organization of HYNIC determines its application, as it can only occupy one or two coordination sites of the radionuclide. That is why a coligand such as

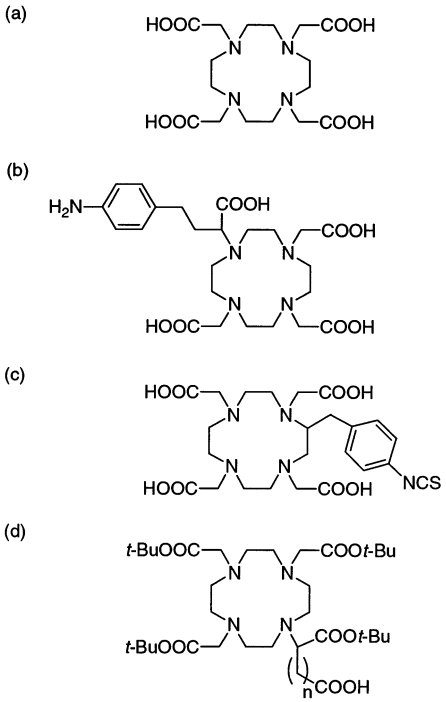


Figure 8. Structures of DOTA and its derivatives: (a) DOTA; (b) PA−DOTA; (c) *p*-NCS-Bz−DOTA; (d) DOTASA(t-Bu)$_4$, n = 1 and DOTAGA(t-Bu)$_4$, n = 2.

of a radionuclide (*91*, *92*) (Figure 14). The conjugation of coligands helps in modifying the properties of obtained radiopharmaceutical, such as hydrophilicity or pharmacokinetics. However, the requirement for the use of coligands makes the chemistry of the synthesis more complicated, and multiple possible products and side-products can be obtained.

HYNIC−coligand conjugates were reported to have low stability (*22*). The search for stable HYNIC−coligand complexes is now carried on and phosphines seem to be the most promising coligands so far (*92*, *93*). HYNIC derivatives, together with phosphines and tricine, form ternary complexes [^{99m}Tc(HYNIC-TM)(tricine)(phosphine)] (TM-targeting molecule). Such complexes are stable in solution, and their hydrophilicity can be modified by changing functional groups attached to phosphine backbone or by substitution of tricine by other glycine derivatives.

HYNIC is often used as a BFCA for somatostatin analogues. The desired amide bond formation should occur between the carboxy group of HYNIC and the N-terminal amino group of a peptide. However, in somatostatin analogues the presence of lysine makes it difficult to obtain a monosubstituted product. Krois et al. (*87*) have compared available methods of HYNIC−octreotide conjugation, and none of them seemed efficient enough. The method of protecting the lysine amino group with $(Boc)_2O$ reported by Bakker et al. (*55*) proved to be unsatisfactory, as the final product was contaminated with Boc-disubstituted derivative, difficult to separate. The activation of HYNIC to *N*-hydroxysuccinimide ester to facilitate the conjugation step, a method suggested by Abrams et al. (*83*), also produced poor results. An improved method of HYNIC−octreotide conjugation, reported by Krois et al. (*87*), is based on the incorporation of the chelator at an early stage of ab ovo peptide synthesis performed by solution method. That approach

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