Conjugation of 1,4,7,10-Tetraazacyclododecane-1,4,7,10-Tetracetic Acid (DOTA) and its Derivatives to Peptides: Synthesis, Applications and Future Prospects

Z. Kovacs[†] and L. M. De León-Rodríguez[‡]*

[†]*UT Southwestern Medical Center, Advanced Imaging Research Center, 2201 Inwood Road NE 4.2, Dallas, TX 75390-8568 USA*

[‡]*Instituto de Investigaciones Científicas, Universidad de Guanajuato, Cerro de la Venada s/n, Guanajuato, Gto., C.P. 36040, México*

Abstract: The universal chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetracetic acid (DOTA) and its bifunctional derivatives are widely used to label peptides with various metal ions for biomedical applications. Bifunctional DOTA derivatives and their conjugation to peptides has become an established strategy for constructing target-specific metal containing agents including targeted MRI contrast agents and diagnostic and therapeutic radiopharmaceuticals. This review covers the synthesis of DOTA-peptide conjugates, their potential use in biomedicine and the problems related to their applications.

Keywords: DOTA, peptides, conjugation, labeling, diagnostic, therapy.

INTRODUCTION

Peptides as pretargeted carrier units labeled with reporter molecules such as fluorescent probes and ligands capable of complexing metals play an important role in the development of molecular sensors and probes. Peptides labeled with various metal chelates have particular importance as potential diagnostic and therapeutic agents. The number of peptide-metal chelate based molecular sensors, diagnostic and therapeutic agents is rapidly increasing and some reviews have recently appeared focusing on diagnostic and therapeutic applications [1]. Among the ligands used for biomedical applications 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetracetic acid (DOTA) and its derivatives offer the highest thermodynamic and kinetic stability with a wide selection of metal ions. In general, metal labeled DOTA-peptide conjugates consist of four main components: 1) peptide, 2) linker, 3) DOTA or derivative and 4) metal.

Target-specific peptide sequences can be generated either through phage display [2] or chemical libraries [3]. The former is more advantageous since it allows monitoring different degrees of target-specificity depending on where the peptide is translated in the bacteriophage. Additionally, current progress in the incorporation of non-natural aminoacids [4] into the genetic code of microorganisms has significantly widened the possibilities of producing modified peptides. In this respect, the first example of a phage display library containing the non-natural aminoacid azido phenylalanine has recently been reported [5]. This could potentially be used to tag the library using a "click chemistry" approach [6]. Peptides can be obtained by three general methodologies: chemical synthesis, recombinant peptide synthesis and transgenics [7]. Currently the synthesis of relatively short peptides (up to 16 aminoacids) is the most efficient using Fmoc based SPPS [8] and most of the labeled peptides have been assembled by this way. Recombinant peptide synthesis is an emerging new technique and could be a very useful approach to long peptide sequences. Introduction of non-natural aminoacids in recombinant synthesis may be possible in the near future [9].

DOTA or a derivative can be either directly attached to the peptide or a linker may be included between the peptide chain and the chelator. The selection of the linker is dependent on the desired pharmacokinetic properties of the conjugate as it has been discussed elsewhere [10]. Methodologies for labeling peptides with DOTA complexes are continuously improving parallel with the growing number of potential applications, and therefore in this review we focus on the recent progress in the synthesis of DOTA-peptides as well as on the perspective of applications and future conjugation methodologies.

Bifunctional Ligands

A bifunctional ligand (BFL) is defined as a chelator capable of forming a covalent bond with a biological vector or macromolecule through a reactive functional group while maintaining the complexing properties of the chelator moiety unaltered. The reactive functional group is usually an activated ester or an aromatic isothiocyanate that will react with an aliphatic amino group under slightly basic conditions. Thiol reactive bifunctionals (maleimide derivatives) have also been developed [11]. The ideal DOTA BFL has the four macrocyclic amino and pendant carboxylic acid groups freely available for metal complexation. The stability of the metal-DOTA complexes is due to the chelate and macrocyclic effect [12] and both the thermodynamic stability and the kinetic inertness of the complexes usually decreases when less than four of the carboxylate pendant arms is available for chelation. However, the stability of the complexes is not compromised significantly when one of the carboxylate sidearms is converted to an amide as a result of conjugation because the amide oxygen still coordinates to

*Address correspondence to this author at the Instituto de Investigaciones Científicas, Universidad de Guanajuato, Cerro de la Venada s/n, Guanajuato, Gto., C.P. 36040, México; Tel/Fax: +52-473-7326252; E-mail: lmdeleon@quijote.ugto.mx

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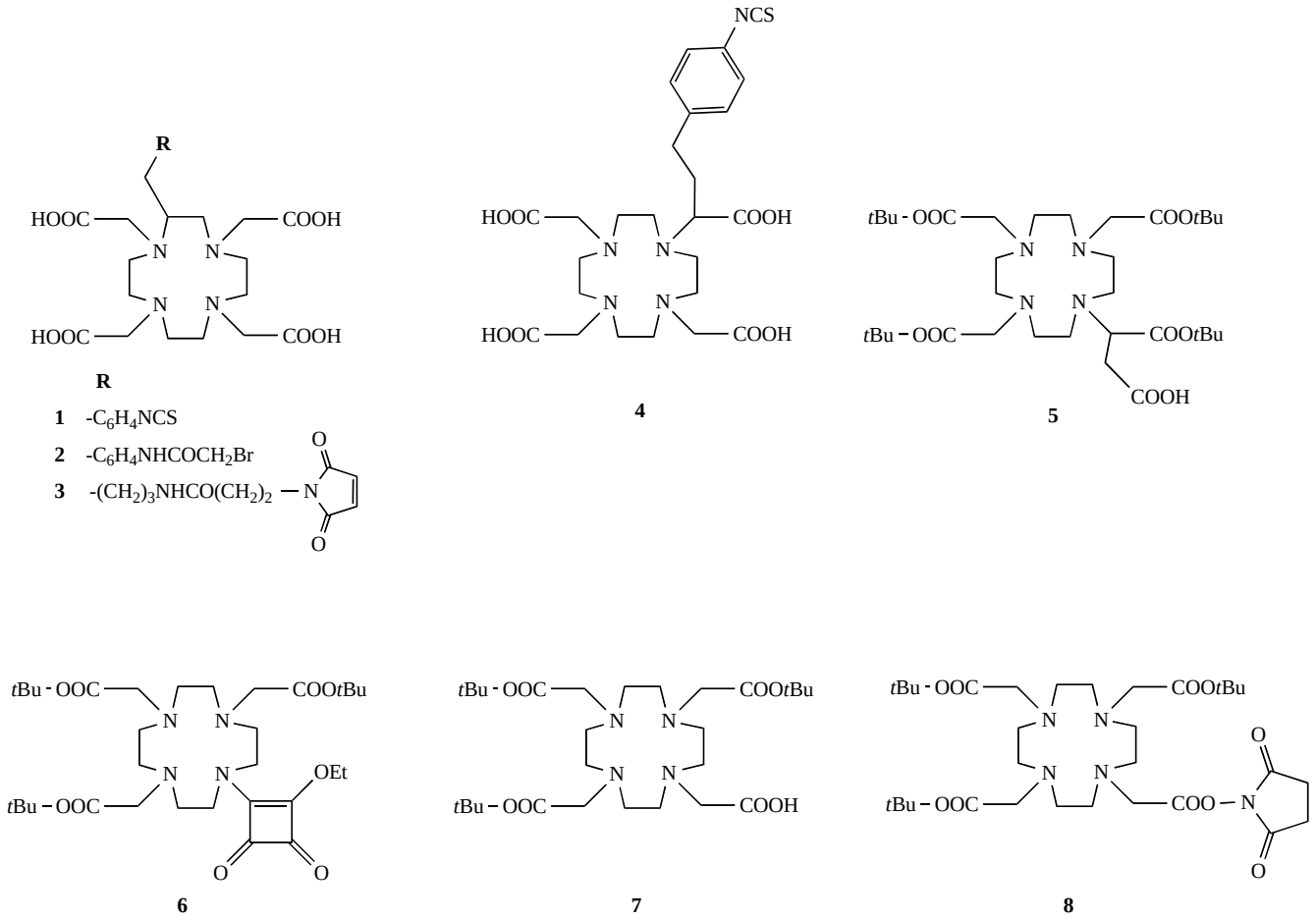


Fig. (1). Bifunctional DOTA ligands.

the metal, at least in the case of lanthanide and Y(III) complexes. Several DOTA derivatives have been prepared (Fig. (**1**)), which can be classified depending on the position of the bifunctional linker. C-functionalized DOTA bifunctional ligands have a reactive functional group attached to the macrocyclic backbone (Fig. (**1**)), (**1**-**3**) [13,14] while the point of attachment in N-functionalized derivatives is off the pendant arm (**4**-**8**) [15,16]. Initial strategies to label peptides with DOTA (**9**) derived from the earlier methodologies developed to conjugate this ligand to antibodies. The simplest procedure is based on the coupling of DOTA to free amino groups of the antibody by the *in situ* activation of one of the carboxylic acids *via* a succinimide ester (**10**) or a mixed anhydride derivative (Fig. (**2**)), (**11**) [17-18]. However, this approach have some disadvantages when applied to label peptides; for instance DOTA and DOTA activated derivatives are poorly soluble in organic solvents and the active compounds are rapidly hydrolyzed in water or water-solvent mixtures such as DMF-water and DMSO-water. Additionally, other problems such as cross-linking might arise which can be partially overcome by performing the coupling under high dilution conditions.

To minimize the hydrolysis problems mentioned above, several DOTA mono esters were obtained from various phenols and DOTA (Fig. (**2**)), (**12**-**14**) [19]. Some of these derivatives showed higher hydrolytic stabilities when compared to DOTA-NHS (**10**) as well as good labeling efficiencies with albumin.

Synthesis of DOTA Labeled Peptides

DOTA can be added to peptides while the chain is still attached to the solid support (pre-labeling) or after cleavage, when the peptide is in solution (post-labeling). Specific labeling of unprotected peptides with monoactivated DOTA derivatives is restricted to sequences containing only one free reactive group, although the differences in the basicities between Lys epsilon and the terminal amino group could potentially be exploited for selective functionalization.

Later, suitably protected DOTA derivatives (Fig. (**1**)), (**6**-**8**) that are compatible with routine Fmoc chemistry based SPPS have been developed. The advantage of these compounds over the *in situ* activation of DOTA Fig. (**2**), (**10**-**14**) is that they are soluble in most organic solvents and fully compatible with the reagents and conditions used in SPPS, and due to the protection of the acetate sidearms cross-linking [20] is avoided. Therefore, compounds like DOTA-tris-*tert*-butyl ester (**7**) and the corresponding succinimide ester (**8**) [21] have become the most popular DOTA based BFL for SPPS. Compound (**6**) has been used to label the Nε amino groups of polylysines [22], and its application might be extended to other peptides.

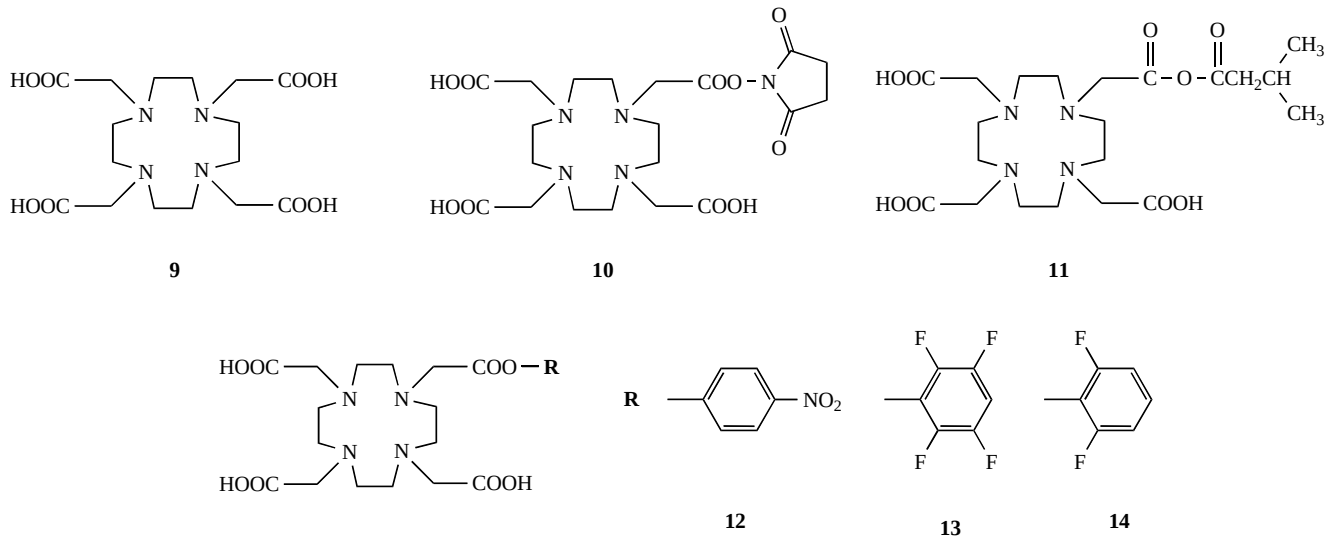
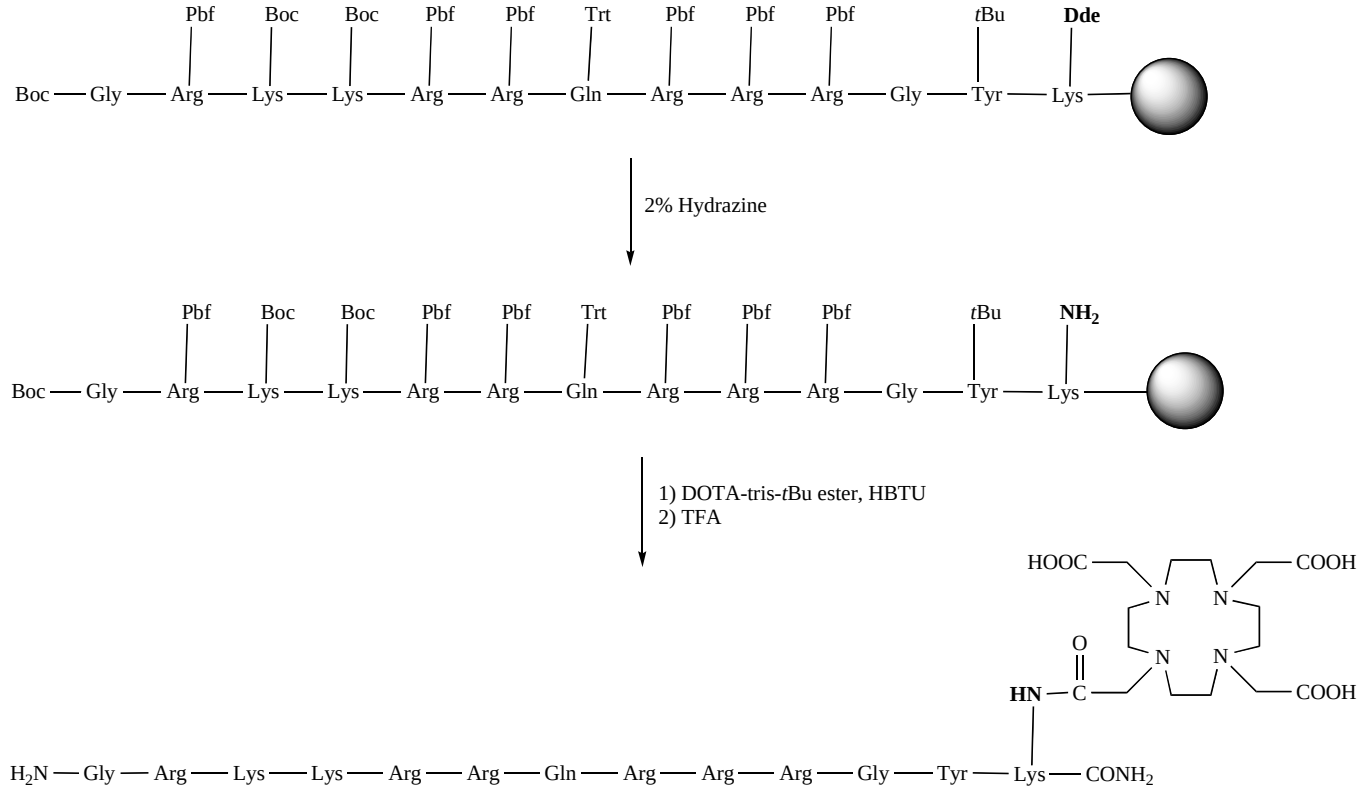


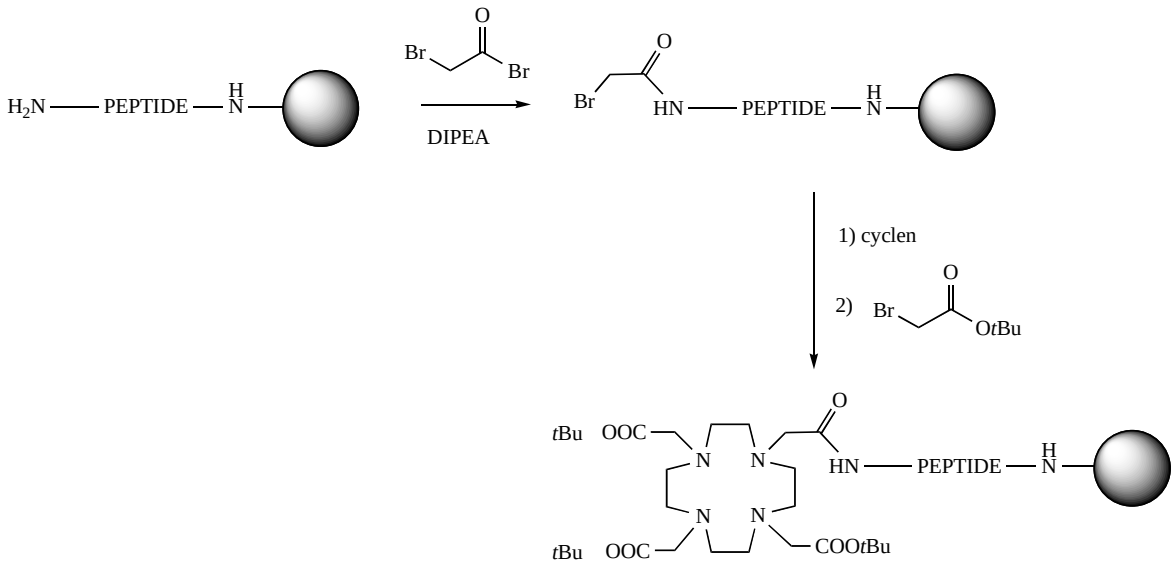
Fig. (2). DOTA derivatives used for conjugation to macromolecules.

DOTA-tris-*tert*-butyl ester, in which three of the acetate sidearms are protected in the acid labile *tert*-butyl ester form and the fourth is available for conjugation, can be added to the N-terminus of the resin bound peptide chain in an automated peptide synthesizer [23]. This is the most convenient way to attach a DOTA moiety to the N-terminus and in addition, lysine epsilon amino groups can be labeled if the peptide is synthesized using an orthogonal protection strategy involving the selective removal of a non-acid or non-base labile protecting group (for example ivDde, cleavable with hydrazine) of a specific Lys residue. This is demonstrated by the synthesis of a cell permeable HIV-tat peptide DOTA conjugate (Scheme **1**).

Obviously, conjugation of DOTA by this method is limited to the N terminal position or Lys sidechains. It is also possible to attach DOTA to different positions using other orthogonal protection strategies and amino acid derivatives designed specifically for the desired target peptides as it was demonstrated by the use of a p-formylamido phenylalanine for the specific conjugation of DOTA to Phe residues [24] and the C and N functionalization of Tyr^3-octreotate with DOTA [25]. However, these methods are limited in scope.



Scheme 1. Orthogonal protection approach for DOTA conjugation on Nε-Lysine residues.



Scheme 2. DOTA synthesis in solid phase support.

Introduction of DOTA into any desired position in a peptide sequence without the need of orthogonal protection was achieved using Fmoc protected DOTA- amino acids (Nα-Fmoc lysine [26,27] and phenylalanine [27]). These derivatives allowed the generation of DOTA-peptide libraries via SPPS [27].

An alternative and conceptually different approach of synthesizing DOTA-peptides involves the formation of the DOTA unit by functionalizing the protected peptide with bromoacetyl bromide followed by a treatment with excess cyclen and alkylation of the cyclen-peptide derivatives with *tert*-butyl bromoacetate [28] (Scheme **2**). Although cross-linking due to the reaction of the cyclic tetramine with more than one bromoacetyl peptide chain was not observed the slow alkylation of the peptide bound cyclen lead to significant amount of impurities.

Certain peptide sequences can form reasonably stable complexes with metal ions including lanthanides [29]. Although the thermodynamic stability of the DOTA chelates is several orders of magnitude higher than that of the peptide-metal complexes, DOTA chelates display slow formation kinetics and complications might arise during complexation. Conditions such as pH, temperature, ligand to metal ratio and the choice of buffer have to be carefully optimized to achieve maximum labeling efficiency [30].

Applications

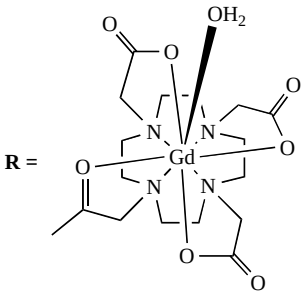
The excellent and almost universal chelating ability of the DOTA unit renders DOTA-peptides very useful in biomedical applications. Depending on the metal potential applications include targeted T_1 MRI contrast agents (Gd^{3+}), PARACEST agents (Eu^{3+}), diagnostic PET agents ($^{64}Cu^{2+}$), SPECT agents ($^{111}In^{3+}$) [31] and therapeutic radiopharmaceuticals ($^{67}Cu^{2+}$, $^{90}Y^{3+}$, ^{177}Lu) [32].

For therapeutic radiopharmaceuticals the tumor size largely determines the choice of the radionuclide. Low energy beta emitters such as ^{177}Lu have short tissue penetrations and are better suited to treat small tumors while high energy beta emitters such as ^{90}Y should be used for larger tumors. Other characteristics such as the ratio of penetrating to nonpenetrating radiation, the physical half-life ($t_{1/2}$), the chemical properties and availability of the radionuclide, the stability of any daughter isotopes, tumor uptake, pharmacokinetics and metabolism of the vector peptide have to be considered as well [33]. In particular the half-life of the radioisotope has to be long enough to permit full chelation because the formation of DOTA complexes is relatively slow [34,35].

Specific Contrast Agents in Magnetic Resonance Imaging (MRI)

Gd(III) complexes of DOTA-peptides have been explored as cell permeable contrast agents as well as targeted MRI agents. One of the first examples of a Gd^{3+}-DOTA-peptide derivative is a DO3A-monoamide-HIV-tat peptide conjugate (Fig. (**3**)), (**15**) [36]. The tat protein encoded by the HIV virus consists of 86 residues and is responsible for breaching the membranes of host cells and facilitating entrance of the virus into the cell. It was found that only the sequence from residue 48 to 57 was required for the translocation of the peptide which then localizes in cytoplasmatic and nuclear compartments [37]. MR images of cells loaded with Gd-(**15**) showed significantly higher signal intensities than control cells treated with Gd-DOTA. This was the first demonstration of cellular internalization of a MRI contrast agent. Later, an all D-aminoacid TAT-DOTA peptide was also synthesized (**16**) to improve the biological half life of the peptide. The Gd complex was internalized by human leukemia cells and MRI studies performed in mice gave enhanced liver, kidney and mesenteric images [38]. Cationic peptides (protein transduction domains) can efficiently enter mammalian cells by endocytosis [39] and the internalization of several Gd-DOTA-polyarginine conjugates (**17**) have recently been demonstrated [40].

Gd^{3+}-(Nε-DOTA)$_x$ - polylysine derivatives (1**8**) were the first DOTA-peptides prepared. The aim was to circumvent the inherently low sensitivity of MRI by increasing the number of Gd(III) ions that could be conjugated to proteins and antibodies [17,41]. However, the expected sensitivity



AHA = 6-aminohexanoic acid

$\mathbf{R}_1$ = protein, antibody

H_2N-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Tyr-Lys(R)-$CONH_2$ **15**

CH_3CONH-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-AHA-Gly-Lys(R)-$CONH_2$ **16**

n = 8,12,16 **R**-NH-(Arg)$_n$-COOH **17**

H_2N-(Lys($\mathbf{R}_1$)-Lys(NH_2)-Lys(R)-Lys(NH_2))$_x$-COOH **18**

R-NH-Thr-Phe-Asp-Asp-Leu-Phe-Trp-Lys-Glu-Gly-His-Arg-$CONH_2$ **19**

H_2N-Thr-Phe-Asp-Asp-Leu-Phe-Trp-Lys(R)-Glu-Gly-His-Arg-$CONH_2$ **20**

H_2N-Thr-Phe-Asp-Asp-Leu-Phe(R)-Trp-Lys-Glu-Gly-His-Arg-$CONH_2$ **21**

Fig. (3). DOTA-peptides as contrast agents in MRI.

increase upon binding to their biological target (increase in relaxivity) was not reached, and the observed increase was merely attributed to a partial decrease in the molecular tumbling (more accurately an increase in the rotational correlation time, τ_R, for a further insight into the theory of relaxivity of MRI contrast agents see cited reviews) [42,43] of the macromolecule.

One of the major goals of molecular imaging by MRI is the detection of sparsely expressed cell surface receptors and enzymes [44]. Due to their high target specificity, Gd labeled DOTA peptides are excellent candidates for this task. The feasibility of molecular MRI was demonstrated by detecting the binding of a Gd-DOTA labeled 12-mer peptide (**19**), (**R = H**) previously identified by phage display to the galactose regulatory protein Gal80 [45]. Additionally, a small DOTA-peptide library was created by SPPS using DOTA-phenylalanine and DOTA-lysine protected derivatives. Conjugates (**20**) and (**21**) did not show specific binding to Gal80 [27], while (**19**), (**R**=Gd-DOTA) showed high affinity allowing easy detection of the binding event by MRI at concentration levels as low as 10 μM. Phage display techniques have the ability to generate specific peptide sequences for virtually every protein making this approach quite general. Including membrane translocation sequences such as HIV-tat, should allow the detection of specific intracellular binding events by MRI.

However, the practical application of target specific contrast agents in MRI is quite limited due to the inherently poor sensitivity (mM concentration of the agent) of the method, although the high spatial resolution of the technique (microns) could be very useful in obtaining specific and detailed morphological information [46].

According to the Solomon Bloembergen theory, the relaxivity of a Gd-DOTA based contrast agent is determined by coordinated water residence life time (τ_M), the electronic relaxation time of the Gd ion and the rotational correlation time (τ_R) of the Gd-complex. The relaxivity attains its maximum value at slower Gd-DOTA tumbling rate (larger rotational correlation times, τ_R) at optimal τ_M [20]. In principle, molecular recognition event between a Gd-DOTA-peptide and the target protein will result in a slower molecular tumbling due to the formation of an engaged supramolecular complex. Unfortunately, the tumbling rate of the Gd-DOTA moiety in the reported systems so far have not reached the optimal value likely due to rotationally not constrained linkers used to bind DOTA to the peptide.

Radiopharmaceuticals

A large number of receptors have been identified as targets for radiodiagnostic and therapy. These include receptors for somatostatine (SSN), gastrin releasing peptide (GRP), α-melanocyte stimulating hormone (α-MSH), guanylate cyclase-C (GC-C), neurotensin, integrin and many others [47]. Radiopharmaceuticals that incorporate vectors that target these receptors are likely to be significant in future therapy and diagnostic applications. These receptors typically recognize short peptides or specific sequences within a polypeptide, so conjugation of such a peptide to a bifunctional chelator should yield a suitable targeting complex. As many of these targeting peptides are degraded rather quickly *in vivo*, it has been found that substituting naturally occurring L-amino acids with D-amino acids often dramatically increases the *in vivo* stability of the peptide. Binding and/or receptor uptake is in some cases actually improved by making small structural alterations to the peptide, i.e., by altering the terminal group or an amino acid side chain. It is important to mention that the transition of peptide radiopharmaceuticals from the research to the medical field has been growing steadily. Some examples of

radiopharmaceuticals currently approved by the US FDA for human clinical applications are; (^{111}In)-pentetreotide (^{111}In-DTPA-octreotide, Octreoscan®), which binds to somatostatin receptors (SSTRs); technetium-99m (^{99m}Tc)-depreotide (NeoTect^{TM}), a ^{99m}Tc-labeled somatostatin receptor (SSTR)-binding analog, and ^{99m}Tc-apcitide (AcuTect^{TM}). ^{99m}Tc-apcitide, which has high affinity for glycoprotein IIb-IIIa receptors is useful for clot detection in deep vein thrombosis. Up to date, the only targeted metal based radiopharmaceutical for therapy is Zevalin, an ^{90}Y labeled antibody, for the treatment of non-Hodgkin's lymphoma. As mentioned before, DOTA shows a marked advantage over the linear derivatives (DTPA) in terms of kinetic and thermodynamic stability of the corresponding metal complexes, with the only disadvantage of slow metal complexation. In the litera ture there is a large number of reports dealing with various DOTA-peptide radiopharm-aceuticals and their applications [32,48-50], some of which will be reviewed here.

Somatostatin DOTA Peptides

Somatostatin is a cyclic neuropeptide of 14-amino acid residues (Fig. (**4**)), (**22**). It acts as a neurotransmitter in the brain and its hormonal activities include the inhibition of the physiologic and tumorous release of growth hormone, insulin, glucagon, gastrin, serotonin, and calcitonin [51]. In addition to being present in normal tissues, somatostatin receptors are expressed in the majority of tumors of neuroendocrine origin, in neuroblastomas, some medullary thyroid cancers, pheochromocytomas, and in small cell lung cancers. When tissues that contain somatostatin receptors become cancerous, these receptors can be over-expressed in the tumor cells and serve as a potential target for radiolabeled somatostatin analogs [52].

To date, five different subtypes of human somatostatin receptors (hsstr) have been identified and cloned [53]. The naturally occurring somatostatin-28 and somatostatin-14 peptides possess high affinity for all these receptors but are vulnerable to rapid *in vivo* enzymatic degradation which precludes their use *in vivo* [54]. To circumvent this problem, eight-amino acid somatostatin analogs (Fig. (**4**)), (**23-27**) (**R** = **H**), with enhanced resistance to *in vivo* enzymatic degradation and preservation of the biological activity of the original somatostatin peptide, have been developed by modifying the original somatostatin peptide.

Several radiolabeled DOTA-somatostatin derivatives have been reported in the literature; and their potential applications have been discussed in other reviews [49,55]. Therefore, in this review, we will just discuss those peptides which structure has been modified relative to the commonly studied somatostatin octapeptides. Thus, DOTA-somatostatin (**23-27**) complexes with ^{111}In, ^{90}Y, ^{177}Lu, ^{66}Ga, ^{67}Ga and ^{68}Ga have been reported [53,56-66]. Additionally, a new somatostatin analog labeled with DOTA *via* D-Tyr^{1} residue of the peptide was prepared and radiolabeled with ^{64}Cu. The resulting ^{64}Cu complex, ^{64}Cu-DOTA-D-Tyr^{1}-octreotate showed moderate tumor uptake in AR42-J tumor-bearing rats and poor pharmacokinetic behavior [67]. In another report, DOTA D-Phe^{1}(αNal^{3})–octreotide (DOTA-NOC) (**28**), a new somatostatin derivative was made and its ^{111}In and ^{90}Y complexes had low affinity towards sstr1 and 4 receptors when compared to DOTA-lanreotide, and three to four times higher binding affinity to sstr2 than ^{111}In,^{90}Y-DOTA- Tyr^{3}-octreotide. ^{111}In-DOTA-NOC showed a specific and fast internalization into AR4-2J rat pancreatic tumor cells which, after 4 h, was about two times higher than that of ^{111}In-DOTA-TOC and three times higher than that of ^{111}In-DOTA-octreotide. Biodistribution studies in CA 20948

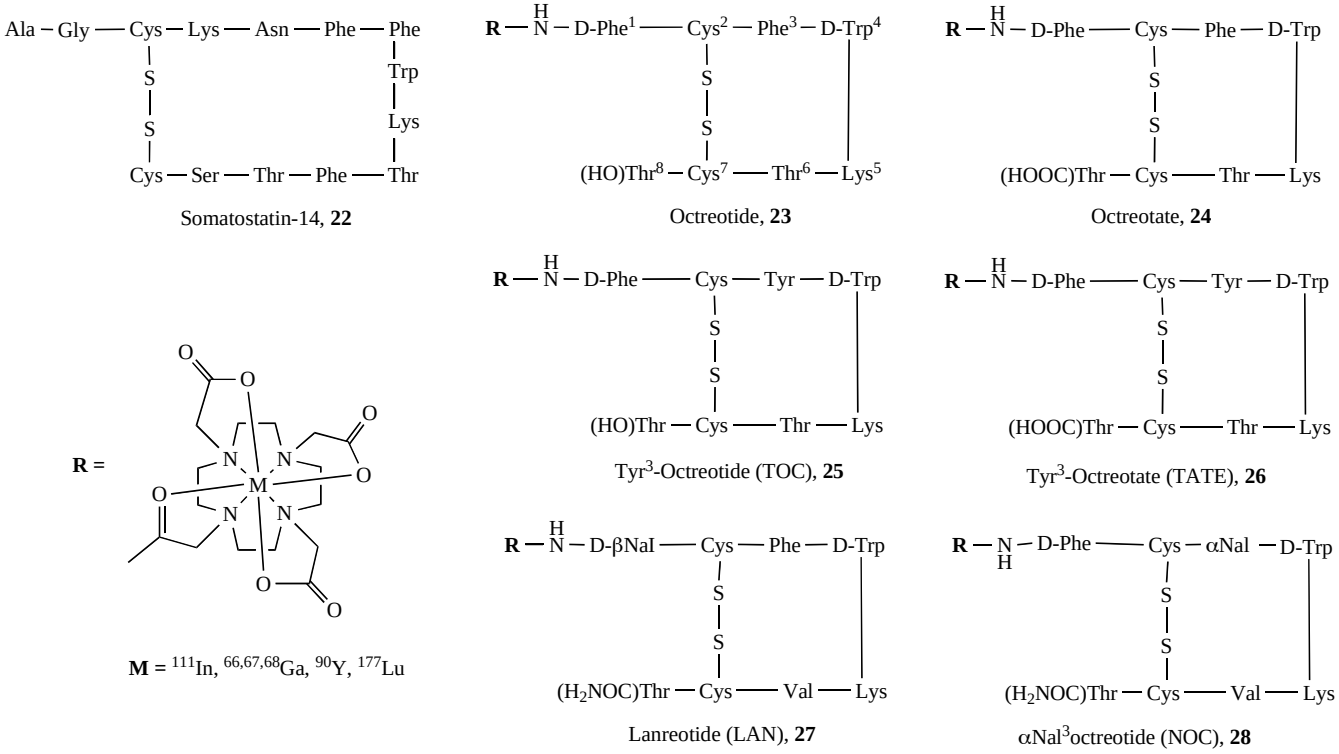


Fig. (4). DOTA-Somatostatin peptide derivatives.

tumor-bearing rats showed rapid clearance from all sstr-negative tissues except the kidneys [68]. These preclinical data suggest that DOTA-NOC could become an important radiopharmaceutical in the future.

Bombesin/Gastrin Releasing Peptides

Bombesin (BBN), first isolated from the skin of the frog Bombina bombina, is a 14-amino acid neuropeptide (Fig. (**5**)), (**29**) with high affinity for the gastrin-releasing peptide (GRP) receptors [69]. BBN and its mammalian counterpart GRP [70], which is a 27- amino acid peptide, have similar biological properties and almost the same C-terminal amino acid sequence.

Four subtypes of BBN/GRP receptors have been identified [71]. Specific receptors for BBN, also known as GRP receptors, are expressed on a variety of human tumors, including prostate, breast, gastric, colon, pancreatic cancers, and glioblastoma [72]. The overexpression of BBN/GRP receptors in various tumors, suggests that radiolabeled BBN analogs could be used for their detection and treatment. GRP, like somatostatin, has a half life in the plasma of approximately 2–3 min. Therefore, several BBN analogs with higher biological half life and *in vivo* potency have been developed and evaluated. It has been shown that the C-terminal amino acid sequence, Trp^8-Ala^9-Val^{10}-Gly^{11}-His^{12}-Leu^{13}- Met^{14}-NH_2, is necessary for retaining receptor binding affinity and preserving the biological activity of BN-like peptides [73]. Hence the N-terminal region of the peptide can be used for radiolabeling. Therefore, several radiolabeled DOTA-BBN derivatives have been reported including ^{111}In-DOTA-Pro^1-($Gln^2Arg^3Tyr^4$)-BBN (**30**) and ^{111}In-DOTA-NεLys^3-(Tyr^4)-BBN (**31**) [74]. ^{111}In-DOTA-Linker-(Gln^7-Met^{14})-BBN analogs (**32**) with linkers such as β-alanine (β-Ala), 5-aminovaleric acid (5-Ava), 8-aminooctoanoic acid (8-Aoc), 11-aminoundecanoic acid (11-Aun) or without linker have been synthesized [75]. ^{149}Pm, ^{177}Lu and ^{153}Sm radioderivatives of DOTA-(Gln^7-Met^{14})-BBN, DOTA-βAla-(Gln^7-Met^{14})-BBN have also been reported [76]. Additionally, ^{177}Lu-DOTA-8-Aoc-(Gln^7-Met^{14})-BBN [77], ^{64}Cu-DOTA-PEG-(Gln^7-Met^{14})-BBN [78], ^{64}Cu- DOTA-NεLys^3-(Tyr^4)-BBN [79], as well as ^{68}GaDOTA-BBN analogs [80] and other derivatives are also known.

Other DOTA-Peptides

Several other DOTA-peptide derivatives have been reported in the literature and a comprehensive review is beyond the scope of this publication. Some interesting examples include ^{111}In-DOTA-peptide analogs with high affinity to α-MSH receptors showing promising results as candidates for melanoma detection and therapy (Fig. (**6**)), (**33**-**35**) [81]. ^{111}In and ^{90}Y-DOTA-neurotensin (NT) derivatives are good candidates for imaging and therapy of exocrine pancreatic cancers (**36**) [82]. Integrins play a key role in the angiogenesis of growing solid tumors and radiolabeled DOTA-RGD (**37**) derivatives that are specific for $\alpha_V\beta_3$ integrin are suitable for diagnosis and therapy of solid tumors [83]. Finally, labeled DOTA conjugates of *Escherichia coli* heat-stable peptide (STh) analogues (**38**) exhibited high guanylin/guanylate cyclase-C (GC-C)

pGlu^1–Gln^2–Arg^3–Leu^4–Gly^5–Asn^6–Gln^7–Trp^8–Ala^9–Val^{10}–Gly^{11}–His^{12}–Leu^{13}–Met^{14}–$CONH_2$

Bombesina (BBN), **29**

R–N(H)-Pro–Gln–Arg-Tyr-Gly–Asn-Gln–Trp–Ala-Val–Gly-His–Leu–Met–$CONH_2$

DOTA-Pro^1-(Gln^{2}Arg^{3}Tyr^4)-BBN, **30**

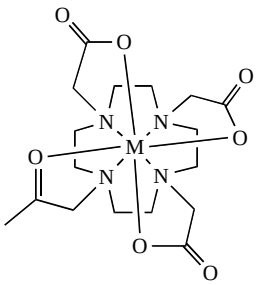
R–NH-pGlu–Gln–Lys-Tyr-Gly–Asn-Gln–Trp–Ala-Val–Gly-His–Leu–Met–$CONH_2$

DOTA-NεLys^3-(Tyr^4)-BBN, **31**

R–N(H)–Linker–Gln–Trp–Ala-Val–Gly-His–Leu–Met–$CONH_2$

DOTA-Linker-(Gln^7-Met^{14})-BBN, **32**

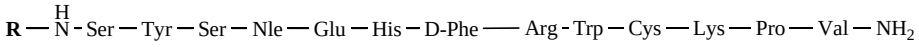
R =



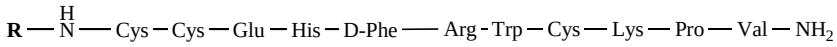
M = ^{111}In, ^{68}Ga, ^{64}Cu, ^{177}Lu, ^{149} Pm, ^{153}Sm

Linker = none, βAla, 5-Ava, 8-Aoc, 11-Aun, PEG

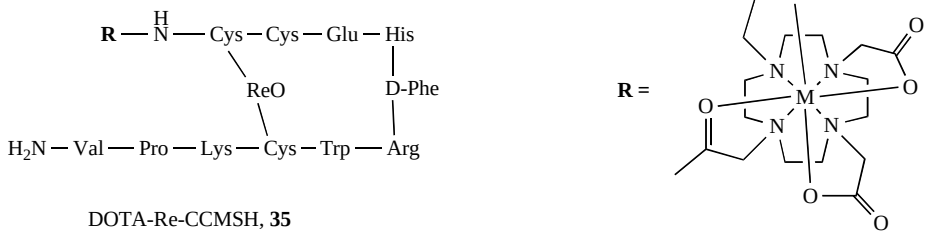
Fig. (5). Radiolabeled DOTA-Bombesin-conjugates.



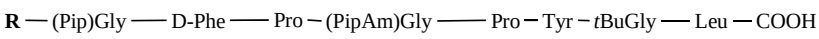
DOTA-NDP, **33**



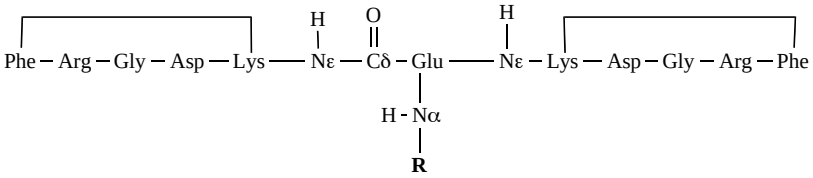
DOTA-CCMSH, **34**



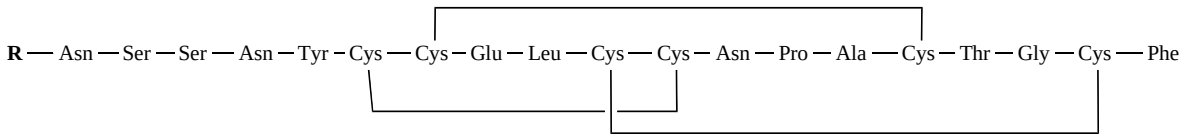
DOTA-Re-CCMSH, **35**



DOTA-NT, **36**



DOTA-E [c(RGDFK)]$_2$, **37**



DOTA-Phe^{19}-ST$_h$, **38**

Fig. (6). Potential DOTA-peptide radiopharmaceuticals.

receptor binding affinities present in T-84 human colon cancer cells [84,85].

Multifunctional Conjugates and Future Applications

The term "multifunctional conjugate" (MC) is used here to describe molecules that are capable of performing more than two functions such as targeting, crossing cell membranes and killing cells. To the best of our knowledge the first example of a DOTA containing MC was a peptide-DOTA-polyamide nucleic acid (PNA) [86] conjugate with the DOTA-lysine derivative [26,87,88] inserted between the peptide and the PNA sequence. The antisense peptide nucleic acid complementary to the first six codons of the B-cell lymphoma/leukemia-2 gene (*bcl-2*, a proto-oncogene associated with the transformation of benign lesions to malignancy), was synthesized by manual solid phase synthesis followed by the coupling of the DOTA-Lys derivative. Next the membrane-permeating peptide (PTD-4-Gly) sequence was added by automated SPPS (Fig. (**7**)). The ^{111}In and ^{90}Y complexes of the resulting MFS bound specifically to as little as 50 fmol of bcl-2 mRNA showing potential for diagnostic imaging and targeted radiotherapy applications in bcl-2-positive cancers [26].

More recently, Maecke *et al.* [89] reported the design and synthesis of new trifunctional conjugates of a truncated analog of somatostatin capable of receptor binding and internalization (TOC), targeting of the cell nucleus (NLS) and cell killing by emitting Auger electrons (^{111}In-DOTA) (Fig. (**8**)), (**39**-**41**). These conjugates were designed to enhance cellular and nuclear uptake in sstr-positive tissues, to have prolonged retention time in tumors and to localize in the cell nucleus to improve the efficiency of cell killing by the low energy Auger electrons [90]. From preliminary results it was observed that C-terminus modification of DOTATOC (see compound (**41**) in Fig. (**8**)) causes a significant loss in binding affinity for either rat or human sstr2, but the N-terminal functionalization fully preserves the pharmaco-logical activity.

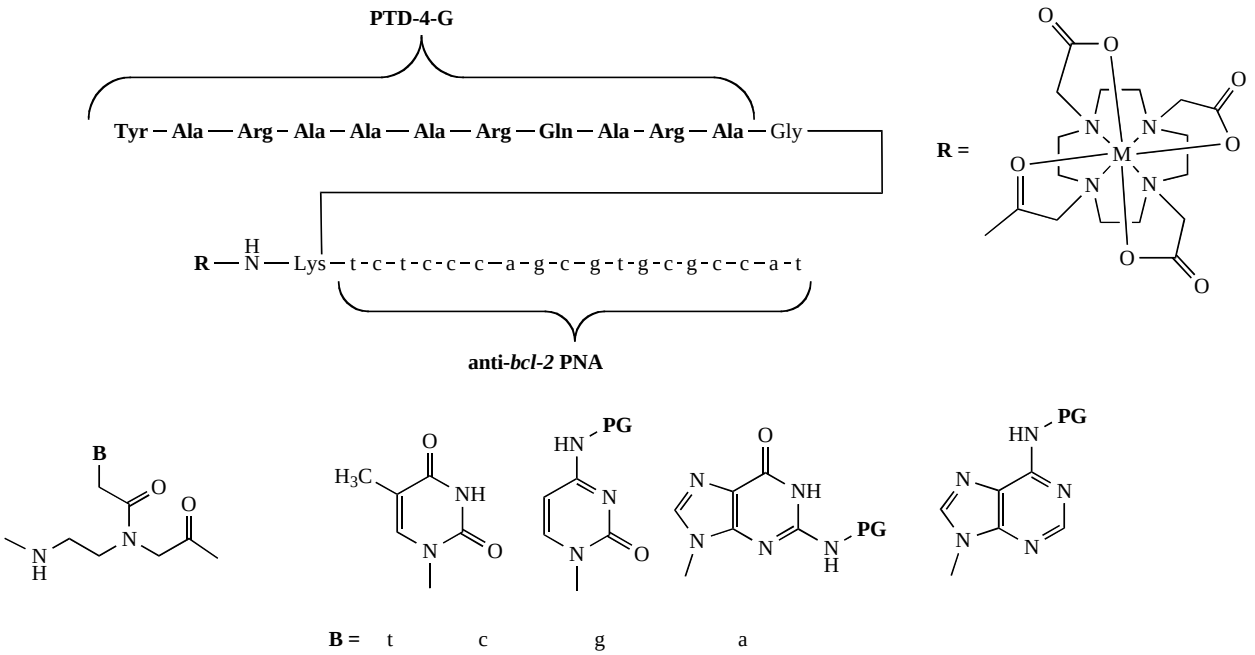
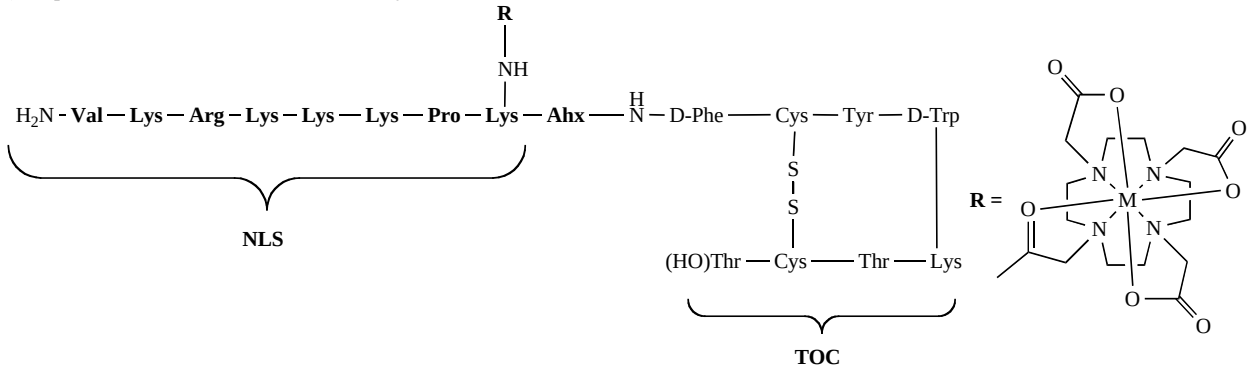
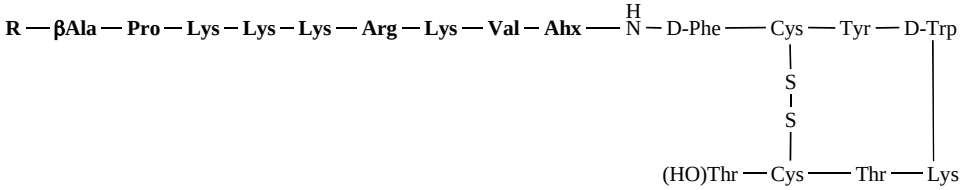


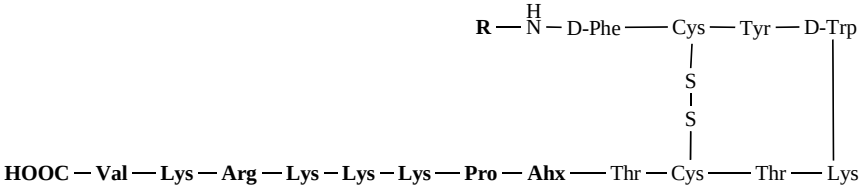
Fig. (7). Peptide-DOTA-PNA multifuncional system.



H_2N-NLS-(NεLys^{8}DOTA)-TOC(OH), **39**



DOTA-NLS-Ahx^9-TOC(OH), **40**



DOTA-TOC-Ahx^9-NLS(COOH), **41**

Fig. (8). DOTA-TOC trifunctional systems.

Finally, the possibility of using DOTA-peptide multi-functional quantum-dots as powerful multifunctional imaging systems have also been suggested [91].

It is obvious that most DOTA-peptide systems are quite complicated and future MC will be most likely even more complex generating problems that have to be solved before these compounds can move forward into practical applications. For example, the synthesis of DOTA bifunctional ligands is tedious and expensive and the yield of DOTA-peptides by SPPS is relatively low (ranging from 1.5% for (**38**) to 20% for (**39**) for final purified compound) requiring lengthy purifications.

Judging by the recent activity in this field as hopefully reflected in this review, we predict that DOTA-peptides will have a bright future as diagnostic and therapeutic agents. It appears that recombinant and transgenic peptide synthesis will allow the generation of complicated (MC) peptide systems in acceptable yields and relatively large amounts [7]. The possibility of expanding the genetic code by introducing non-natural aminoacids may lead to modified peptides *via* recombinant and transgenic synthesis [4,92]. This could lead to the wider availability of peptides with improved targeting properties and longer biological half lifes. Incorporation of non-natural aminoacids (e.g. azide derivatives) and the development of DOTA derivatives compatible with techniques such as "click chemistry" [93,94] will allow to label peptides with DOTA in any desired position and possibly to label them with preformed DOTA-metal complexes.

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