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The Use of Solid-Phase Synthesis Techniques for the Preparation of Peptide–Metal Complex Conjugates

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This microreview summarizes recent reports on the preparation of metal complex peptide conjugates by solid-phase synthesis methods. Procedures for such conjugates are in many cases different from standard solid-phase peptide synthesis protocols. Specific advantages of general strategies, the synthesis of peptide–ligand conjugates and complexation with excess metal ions on solid support or the incorporation of previously prepared metal complex amino acid derivatives are discussed.

Introduction

Solid-phase synthesis is a convenient and established method for the preparation of peptide-based compounds. However, solid-phase synthesis of inorganic complexes is a rather new discipline, established by Heinze, Metzler-Nolte, Reedijk and others.[1] Earlier attempts to use solid-phase synthesis to obtain, for example, (2,2′-bispyridine) dichloro complexes of platinum(II) by Gallop failed at the cleavage step,[2] due to the more labile metal–ligand bonds of organometallic building blocks in relation to typical covalent bonds of organic molecules.

Coordination and organometallic chemistry on solid-phase have typically been studied in the context of catalyst performance.[3] Recently, solid-phase synthesis using insoluble resins as solid support has been used to synthesize metal complexes based on peptide backbone ligands. These coordination compounds find applications in biochemistry as well as in medicinal chemistry. Resin-bound chelates were prepared in such a manner that upon the addition of suitable metal salts the target metal complexes were selectively released from the resin and used in, for example, fluorescence or radio imaging or oligonucleotide DNA/RNA binding studies. Other approaches have incorporated previously prepared metal complex building blocks in solid-phase peptide synthesis to afford, for example, peptide–platinum complex conjugates with anticancer activity. This versatile approach to the incorporation of pendant protected amino acid functionalities offers several advantages over solution-phase or post-solid-phase peptide synthesis conjugation.[4] It provides the flexibility to incorporate a metal ion chelator with exclusive site specificity in any amino acid sequence,

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Burkhard König received his doctorate in 1991 from the University of Hamburg under the direction of Prof. Meijere. He continued his scientific education as a postdoctoral fellow with Prof. M. A. Bennett, Canberra, Australia, and Prof. B. M. Trost, Stanford, U.S.A. In 1996 he obtained his "Habilitation" at the University of Braunschweig, Germany. Since 1999 he has been full professor of organic chemistry at the University of Regensburg. His current research interests focus on the development of synthetic receptors for peptide and protein recognition

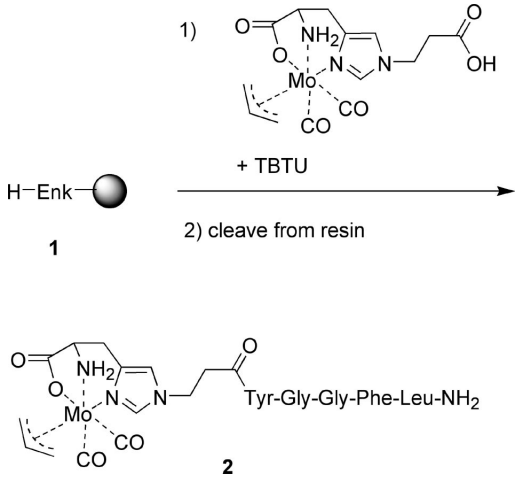
not just terminally or at one or more lysine or cysteine side chains.[5] Additionally, peptides are often prepared most effectively by automated solid-phase synthesis.

In this review we summarize recent reports on the preparation of metal complex–peptide conjugates by solid-phase synthesis methods. The focus of the review lies on the synthetic methodology used to prepare the building blocks and peptides rather than on applications of the metal complex–peptide conjugates. We have structured our survey by the metal ions used for complex formation, and distinguish in the discussion between examples of solid-phase ligand synthesis with subsequent metallation[6] and the use of metal-containing amino acids for synthesis.

1. Chromium, Molybdenum and Tungsten (Group 6) Metal Complex–Peptide Conjugates

1.1 N_δ,*N*,*O*-L-Histidinate (His) Molybdenum Conjugate

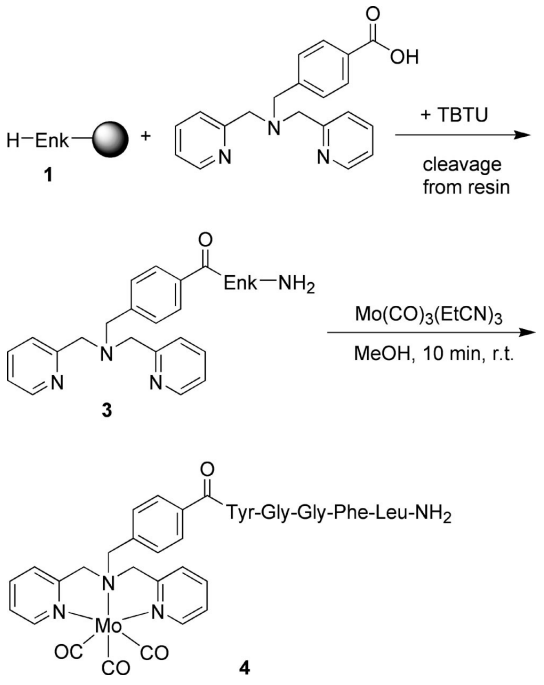
Metzler-Nolte and co-workers reported oligopeptide bioconjugates with organometallic Mo carbonyl complexes.[7] The conjugates were prepared in excellent yields and purities by two different solid-phase synthesis strategies. In one approach the neuropeptide enkephalin (enk) Tyr-Gly-Gly-Phe-Leu, which is a natural ligand to the opiate receptor, was synthesized by standard Fmoc solid-phase methods on NovaSyn TGA resin with an HMBA linker. The metal complex Mo(N_ε-$C_2H_4CO_2H$-His)(allyl)$(CO)_2$ was coupled to the resin-bound, fully deprotected enkephalin **1** and afterwards cleaved from the resin by treatment with saturated NH_3 solution in MeOH (Scheme 1).



Scheme 1. Solid-phase synthesis of Mo(His)Enk conjugate **2** using a metal complex acid.

1.2 Bis(2-picolyl)amine (bpa) Molybdenum Conjugate

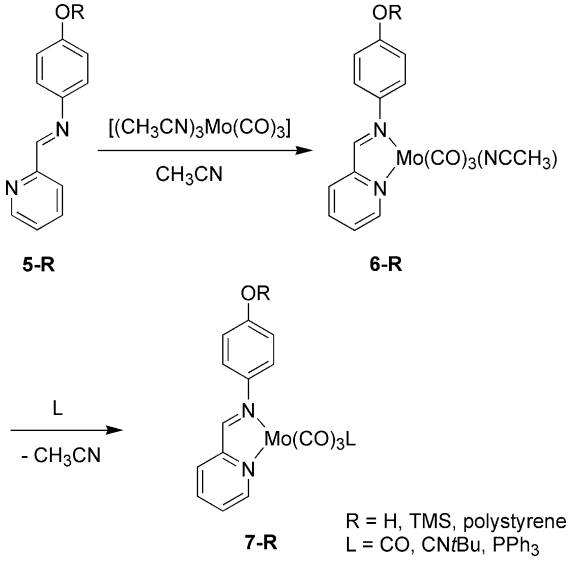
In cases in which the attachment of a metal complex to the peptide on the solid support is not desirable – with radioactive metal isotopes, for example – an innocent anchoring group can be attached to the peptide during solid-phase synthesis. The ligand–peptide conjugate is then cleaved from the resin and purified, and the metal label is only added in solution immediately prior to use of the bioconjugate. Metzler-Nolte et al. provided an example of this procedure using the $Mo(CO)_3$ fragment and bpa as a ligand (Scheme 2).[7]



Scheme 2. Synthesis of Mo(bpa)-Enk conjugate **4** by subsequent metallation.

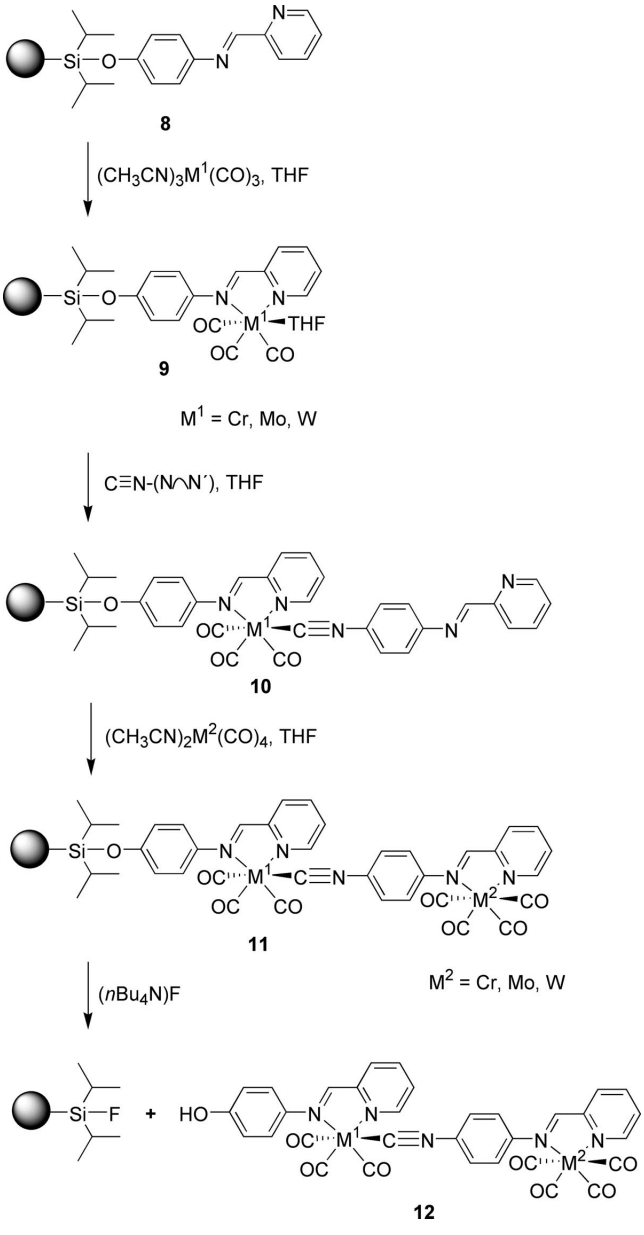
1.3 Bidentate Schiff Base Metal Conjugates

A solid-phase synthesis approach for molybdenum carbonyl complexes was developed by Heinze (Scheme 3).[8]



Scheme 3. Synthesis of molybdenum tricarbonyl complexes on solid support.

We have included this example, although neither peptide coupling nor metallated amino acids are used, because it illustrates that complex organometallic transformations are possible on solid support. A specific resin and linker system allows coordination and organometallic chemistry under solid-phase reaction conditions and the cleavage of the metal complex from the solid support. Bidentate Schiff base **5-R** was used as the ligand. The phenolic hydroxy group allows the attachment to the solid support. A silyl ether-based[9] linker was chosen, due to its stability under basic and acidic conditions and the potential to cleave it with fluoride ions, which are expected to be unreactive towards most metal complexes. In solution, high temperatures and rather harsh oxidative reaction conditions are necessary to synthesize the desired tricarbonyl compounds. Such harsh conditions have to be avoided in solid-phase chemistry with polystyrene resins as the molybdenum precursors can react with the aromatic residues of the support. Heinze and coworkers used $[(CH_3CN)_3Mo(CO)_3]$ as a $Mo(CO)_3$ source, and under mild reaction conditions the intensely blue coloured complexes **6-R** and **7-R** were formed rapidly and cleanly in excellent yields. However, acetonitrile, a rather poor solvent for resin-swelling, had to be used in a mixture with toluene. Otherwise the complexation led to the formation of the immobilized tetracarbonyl complex instead of the desired tricarbonyl complex. The cleavage was performed with tetra-*n*-butylammonium fluoride in dichloromethane and resulted in deeply coloured solutions of the deprotonated complexes.



Scheme 4. Synthesis of mixed-metal dinuclear complexes on solid support.

Heinze et al. used their molybdenum carbonyl complexes, as the molybdenum–carbonyl and molybdenum–isocyanide bonds are substitutionally inert metal–ligand bonds, to synthesize di- and trimetallic homonuclear complexes (Scheme 4).[10] Finally, mixed-metal dinuclear complexes prepared from chromium, molybdenum and tungsten and a directional bridging ligand were assembled stepwise on solid-phase and cleaved from the support.[11]

Solution synthesis, although straightforward, requires purification of the products and intermediates, which is rather difficult, and makes this approach less suitable for longer-chain complexes. The solid-phase synthesis needs more reaction steps (ligand immobilization and product release) and differently optimized reaction conditions. However, it is much easier to accomplish, and solubility problems and purification of intermediates can be disregarded.

2. Manganese, Technetium and Rhenium (Group 7) Metal Complex–Peptide Conjugates

The manganese family includes the most widely used metals for peptide complexation. Its applications range from rhenium- and technetium-labelled radiopharmaceuticals[12] to organometallic PNA oligomers with rhenium and their interaction with complementary DNA and to peptide–manganese complexes with catalytic activity.

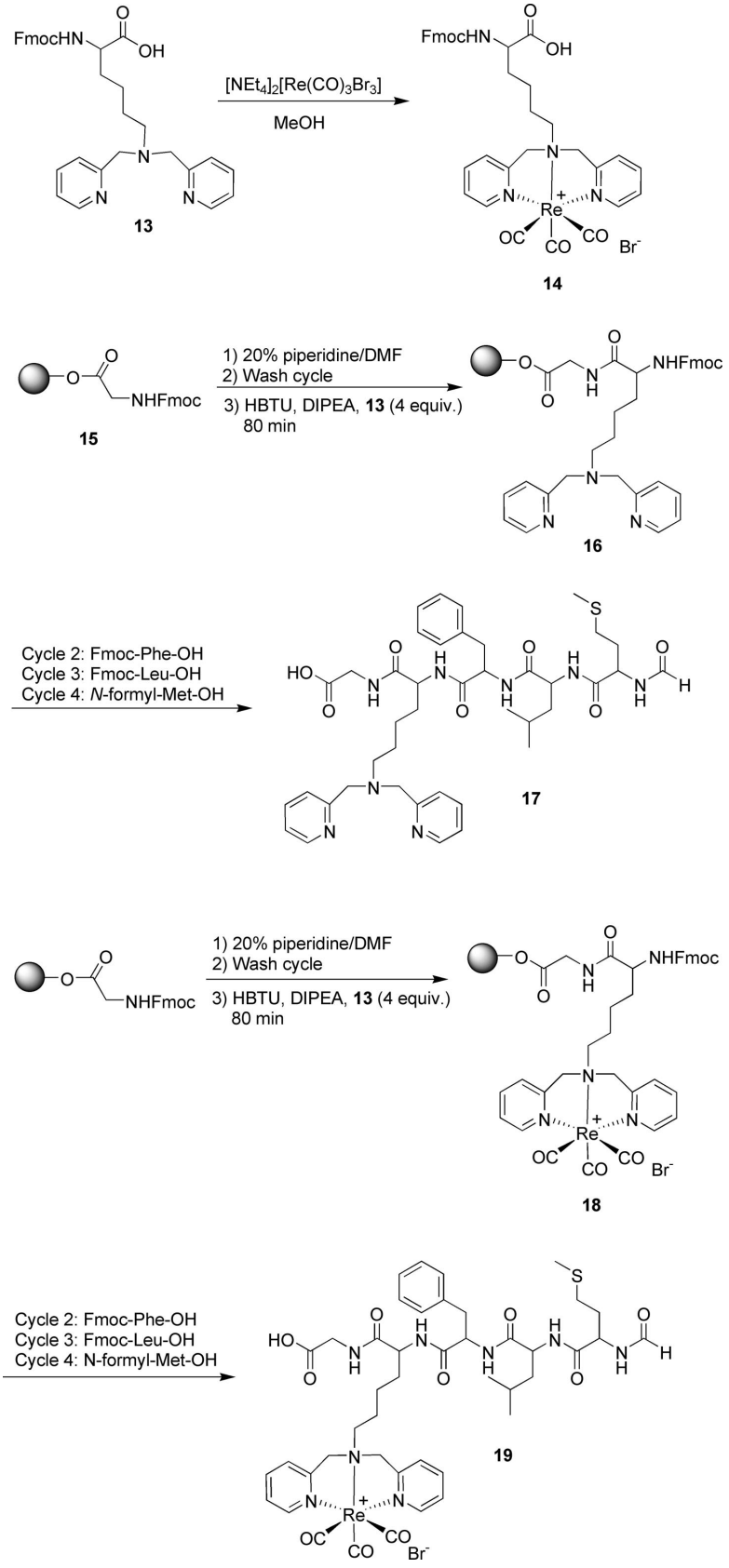
The transition metals technetium and rhenium are among the most commonly used radioisotopes in medicine, due to the favourable emission energies and decay properties of radioactive isotopes Tc-99m, Re-186 and Re-188.[13] As a result, methods of attaching these radionuclides to peptide sequences have been developed. A solid-phase synthesis strategy was employed to optimize the receptor binding affinity and biodistribution of technetium-labelled peptides[14] as it allows the preparation of analogues of a particular peptide–ligand bioconjugate in parallel.[15]

2.1 Bpa Metal Conjugate

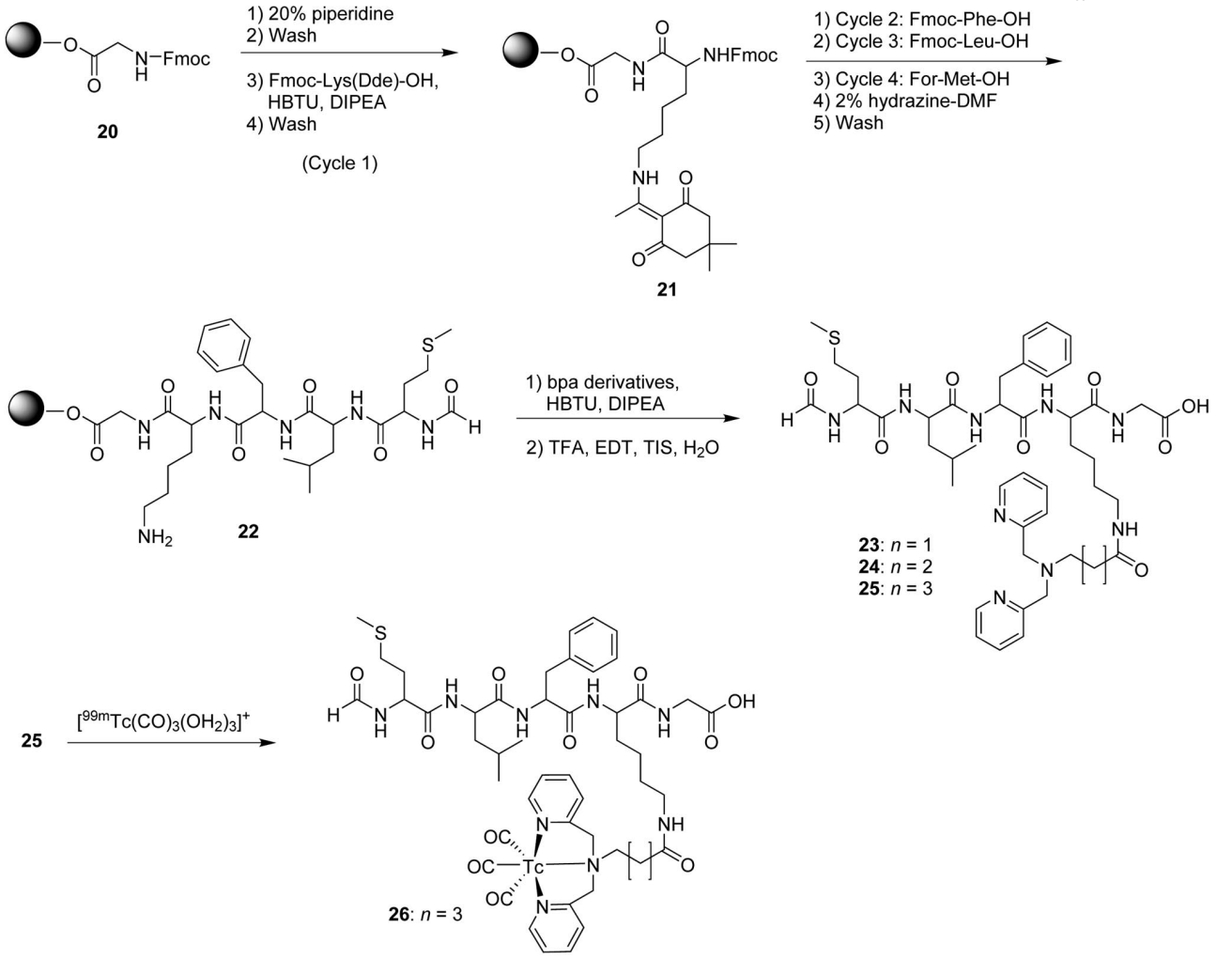
Valliant and co-workers prepared the single amino acid chelate (SAAC) **17** and Re-SAAC-peptide derivatives **19** using solid-phase synthesis.[16] Fmoc-protected dipyridyl chelate **13** and its Re complex **14** were incorporated into

the growing peptide linked to a SASRIN resin using HBTU as the coupling agent (Scheme 5).

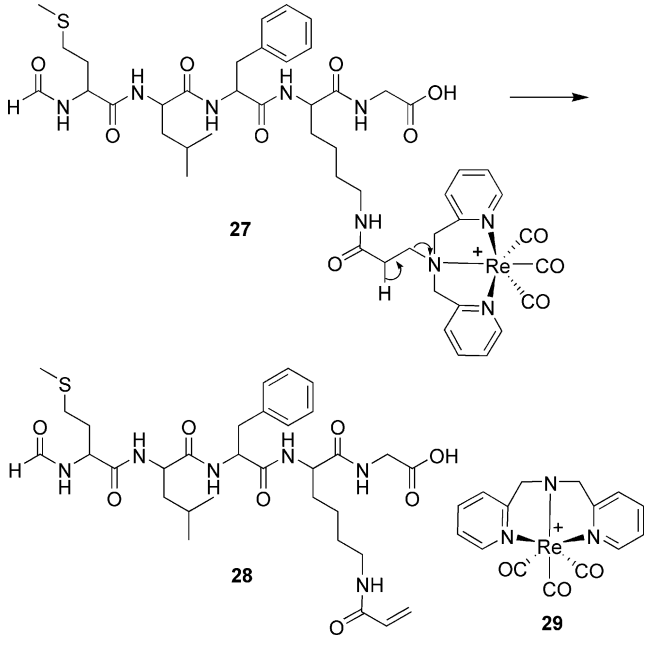
In 2005 Valliant et al.[17] published a solid-phase methodology that aimed to incorporate lysine into the backbone



Scheme 5. Solid-phase synthesis of ligand **17** and rhenium complex **19** using a metallated amino acid.



Scheme 6. Synthesis of peptide–technetium conjugate **26** by metallation after solid-phase ligand preparation.



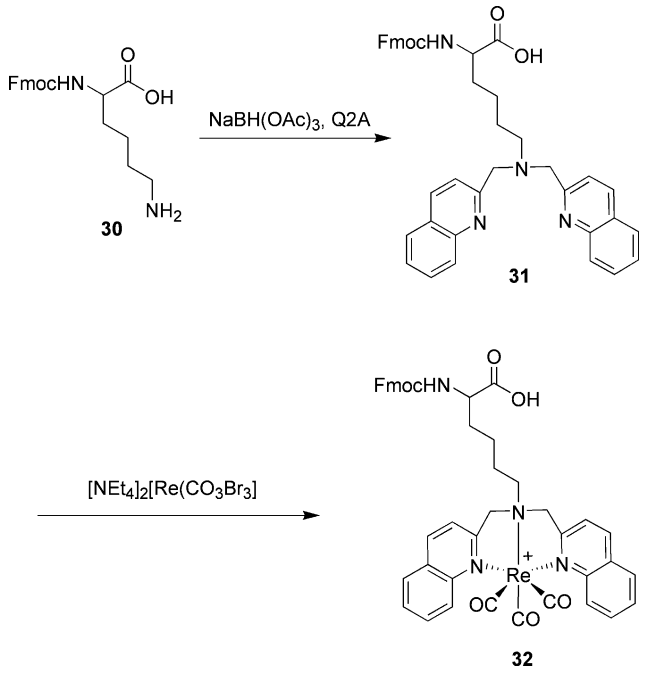
Scheme 7. Proposed mechanism for the degradation of the Re^{I}-peptide conjugate **27**.

of a peptide in such a manner that the ε-nitrogen could be selectively liberated and a metal-bpa-chelate added while the peptide was still linked to the resin. Dde was used as lysine side chain protecting group, because it is stable to the conditions used in typical Fmoc solid-phase synthesis, and it can be selectively liberated without affecting Boc protecting groups.[18] This approach is applicable to bifunctional chelating systems containing a pendent acid group. After the removal of the Dde protecting group, a series of dipyridylamine ligands **23**–**25** with linker arms varying in length were coupled to the resin-bound peptides using HBTU and DIPEA (Scheme 6). However, stable Tc^{I} and Re^{I} complexes were not obtained for all of the ligands. In the case of peptide conjugate **27**, degradation is probably caused by elimination to give an α,β-unsaturated amide **28**, which concomitantly results in liberation of a neutral metal complex **29** (Scheme 7).

2.2 Quinoline-2-aldehyde (Q2A) Metal Conjugate

To obtain a fluorescent SAAC-type Re complex with retention of its ability to bind ^{99m}Tc, Valliant et al. treated Fmoc-L-lysine with Q2A in the presence of $Na(OAc)_3BH$

to yield the bifunctional ligand **31** (Scheme 8).[19] The objective was to develop a method for preparing bioconjugates that can deliver the ligand to specific receptors. The SAACQ ligand and the SAACQ–Re complex represent such amino acid analogues that can be incorporated into peptide sequences by solid-phase peptide synthesis. The rhenium complex **32**, prepared by complexation with $Re(CO_3)Br_3$, was integrated into the peptide fMLF (*N*-formyl-L-methionine-L-leucine-L-phenylalanine), a targeting sequence that has been used to guide radiopharmaceuticals to the formyl peptide receptor (Figure 1). The work is an example of the use of metal-containing amino acids in solid-phase peptide synthesis.



Scheme 8. Synthesis of fluorescent SAAC-type Re complex **32**.

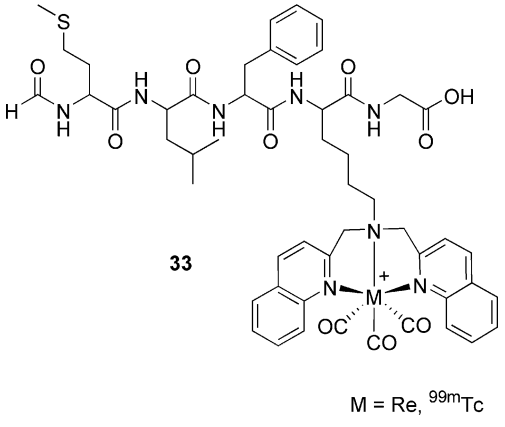
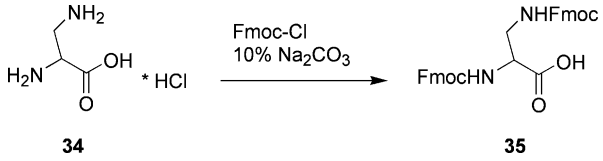


Figure 1. Bioconjugate complex fMLF[(SAACQ-$M(CO)_3)^+$]G **33**.

2.3 N_xS_y Metal Conjugates

Many studies have shown that ligand systems containing nitrogen and thiol sulfur atoms are effective for the coordination of Tc and Re.[20] In 1997, Quinn and co-workers appended a rhenium-bound peptide to the N termini of receptor-binding α-melanocyte stimulating hormone fragments as the last step of a conventional solid-phase peptide synthesis.[21] This diaminedithiol (N_2S_2) chelator was also assembled at the N termini of short peptides in a two-step procedure by Gariépy et al.[22] The deprotected terminal amino group was first treated with di-Fmoc-diaminopropionic acid **35** (Scheme 9), and the two protected amino groups were then simultaneously deprotected and subsequently treated with *S*-benzoylthiolglycolic acid to generate a protected N_2S_2 chelator **38**. The resulting constructs were cleaved from the resin support and labelled with ^{99m}Tc-pertechnetate (Scheme 10).



Scheme 9. Preparation of di-Fmoc-protected amino acid derivative **35**.

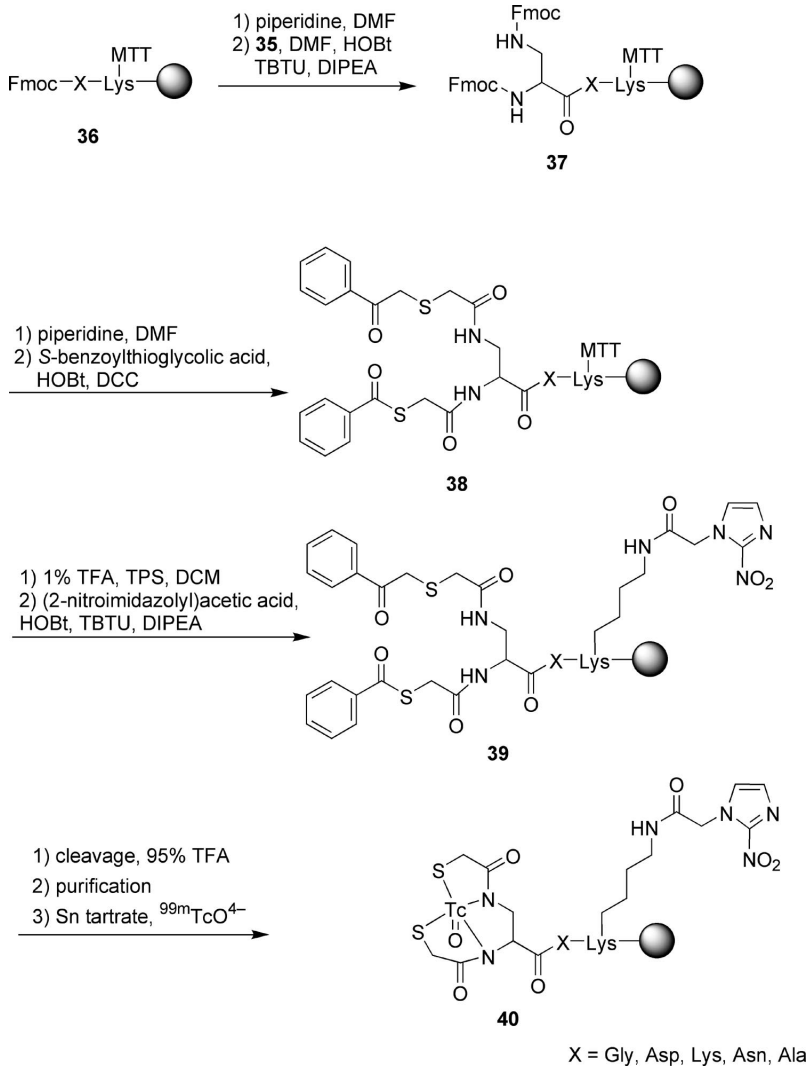
Okarvi used a pre-labelling method[23] in which the radionuclide binds to the chelate in a separate step prior to the attachment of a peptide.[24]

Monoamide monoamine (MAMA)[25] forms neutral, stable and well defined complexes with both Tc^V and Re^V, and it can be easily derivatized, regioselectively and with a wide range of different functional groups.[26] Resin-bound peptide–MAMA conjugates were prepared in such a manner that upon the addition of suitable Re^V and Tc^V precursor **42** the target metal complexes **43** were selectively released from the resin (Scheme 11).[27]

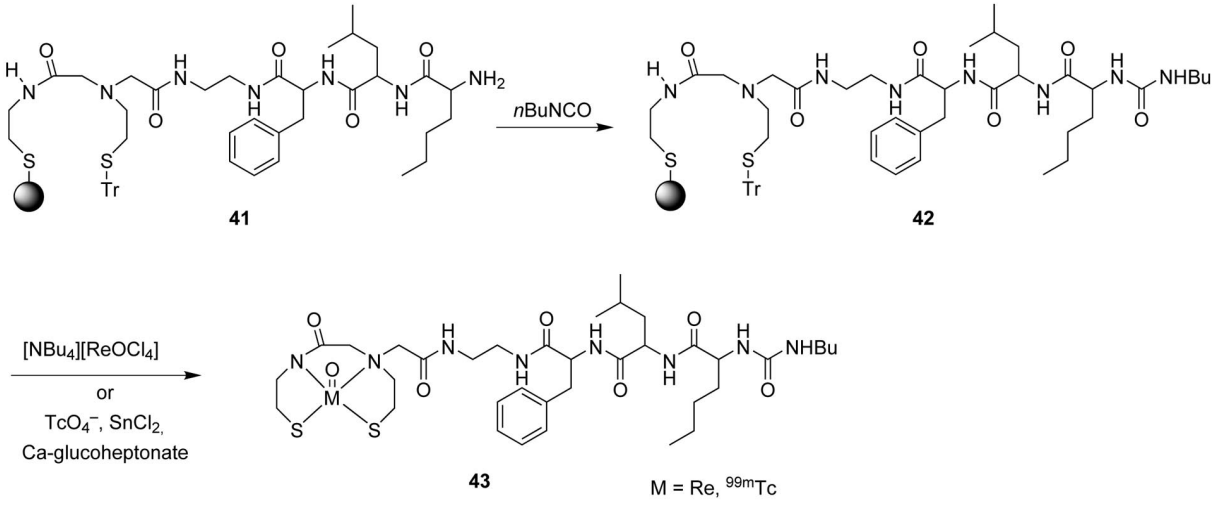
Although it is conceivable to build peptides using the carboxylic acid-functionalized MAMA derivative, automated peptide synthesis is typically performed starting from a primary amine using Fmoc-protected amino acids. Valliant et al. therefore prepared a primary amine-functionalized MAMA chelate by coupling a diamine to the carboxylic acid of **44**[25] (Scheme 12). Compound **45** was used to synthesize a model peptide with standard Fmoc/HBTU protection and coupling methods. The peptide-functionalized resin **47** was subsequently treated with [TBA][$ReOCl_4$], and heating released the complex **48** from the resin. The target peptide is the *n*-butylurea derivative of Phe-Leu-Nle, which is an antagonist for the formyl peptide receptor (FPR).[28]This ligand is of interest because radiolabelled compounds that are capable of binding selectively to the FPR on white blood cells can be used to image sites of infection and inflammation.[29]

Valliant and co-workers also prepared a bombesin-derived peptide-^{99m}Tc chelate conjugate **55** using solid-phase synthesis methodology.[30] Bombesin is a 14-amino acid peptide hormone (Scheme 13).

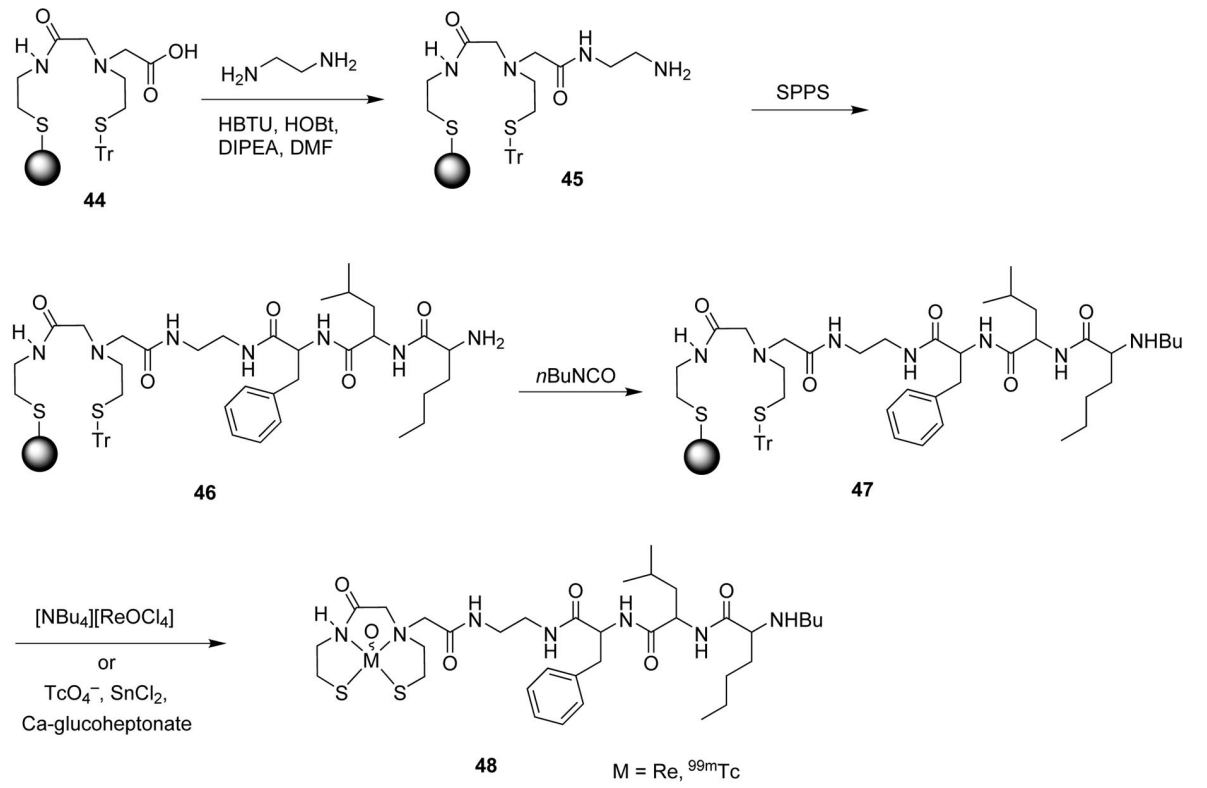
The reported approach involved linking of a prefabricated bifunctional N_2SN' technetium chelate complex **51** to a resin-bound peptide sequence **53** derived from bombesin,



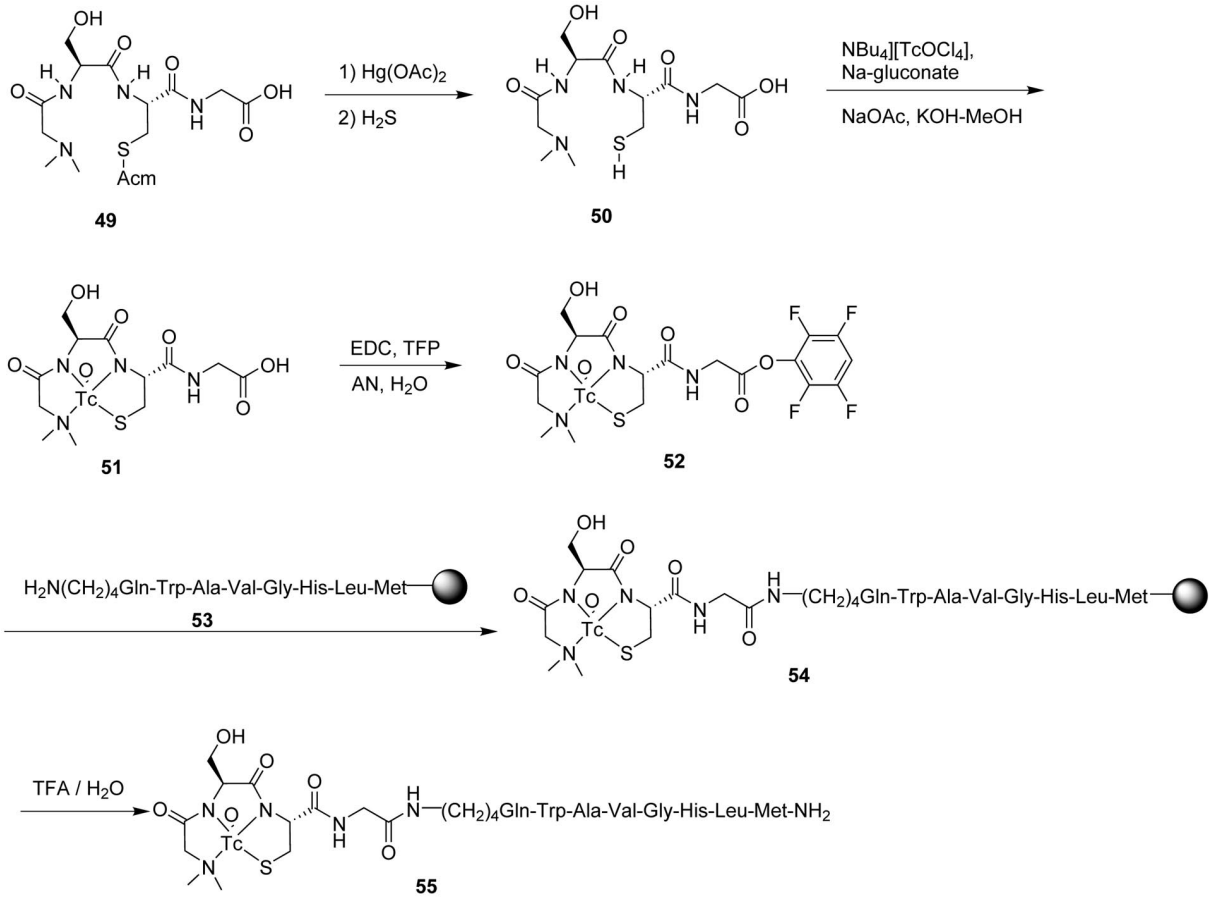
Scheme 10. Synthetic scheme for the preparation of $N_2S(benzoyl)_2$-containing peptides by ligand synthesis on solid support and subsequent metallation.



Scheme 11. Synthesis of Re^V and Tc^V peptide conjugates on solid support; release from resin occurs on metallation.



Scheme 12. Synthesis of Re^{V}- and Tc^{V}-Mama peptide conjugates **48**; metallation releases the complex from the solid support.



Scheme 13. Solid-phase synthesis of a N_2SN' technetium chelate peptide conjugate **55** derived from bombesin using the metal-containing amino acid **52** for peptide coupling.

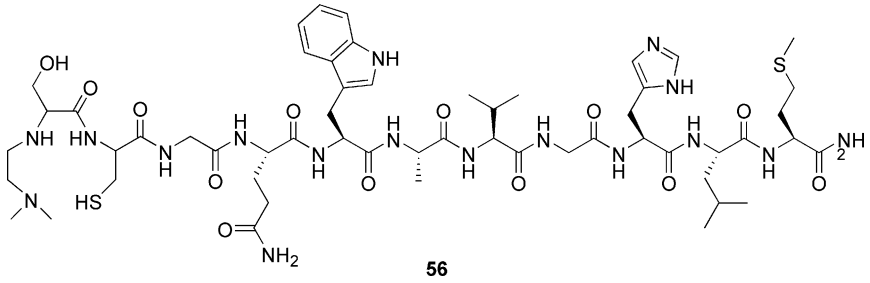


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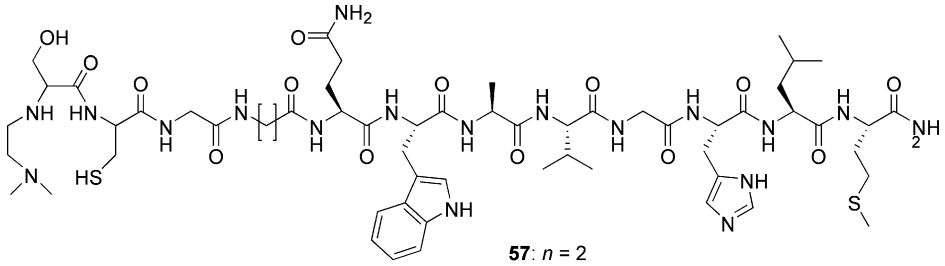
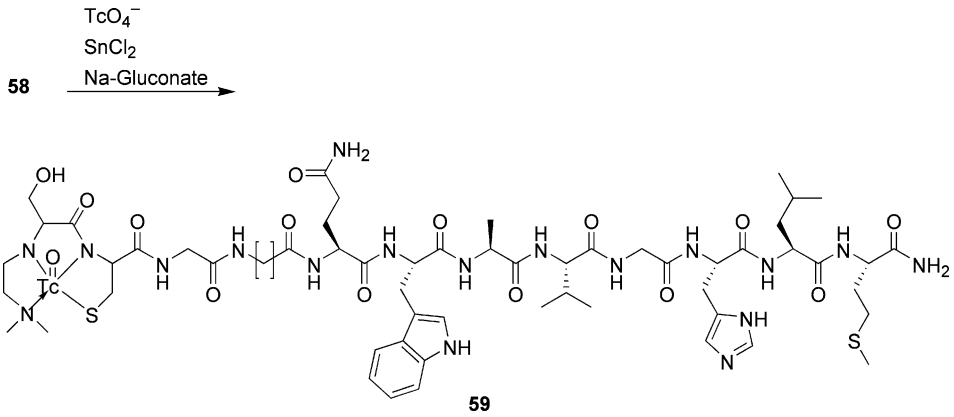
Figure 2. Radiometal chelate linked directly to the N-terminal amine group of BBN[7–14]NH_2.



57: *n* = 2
58: *n* = 4
59: *n* = 7
60: *n* = 10

Figure 3. Radiometal chelate linked to the N-terminal amine group of BBN[7–14]NH_2 via hydrocarbon spacer groups.



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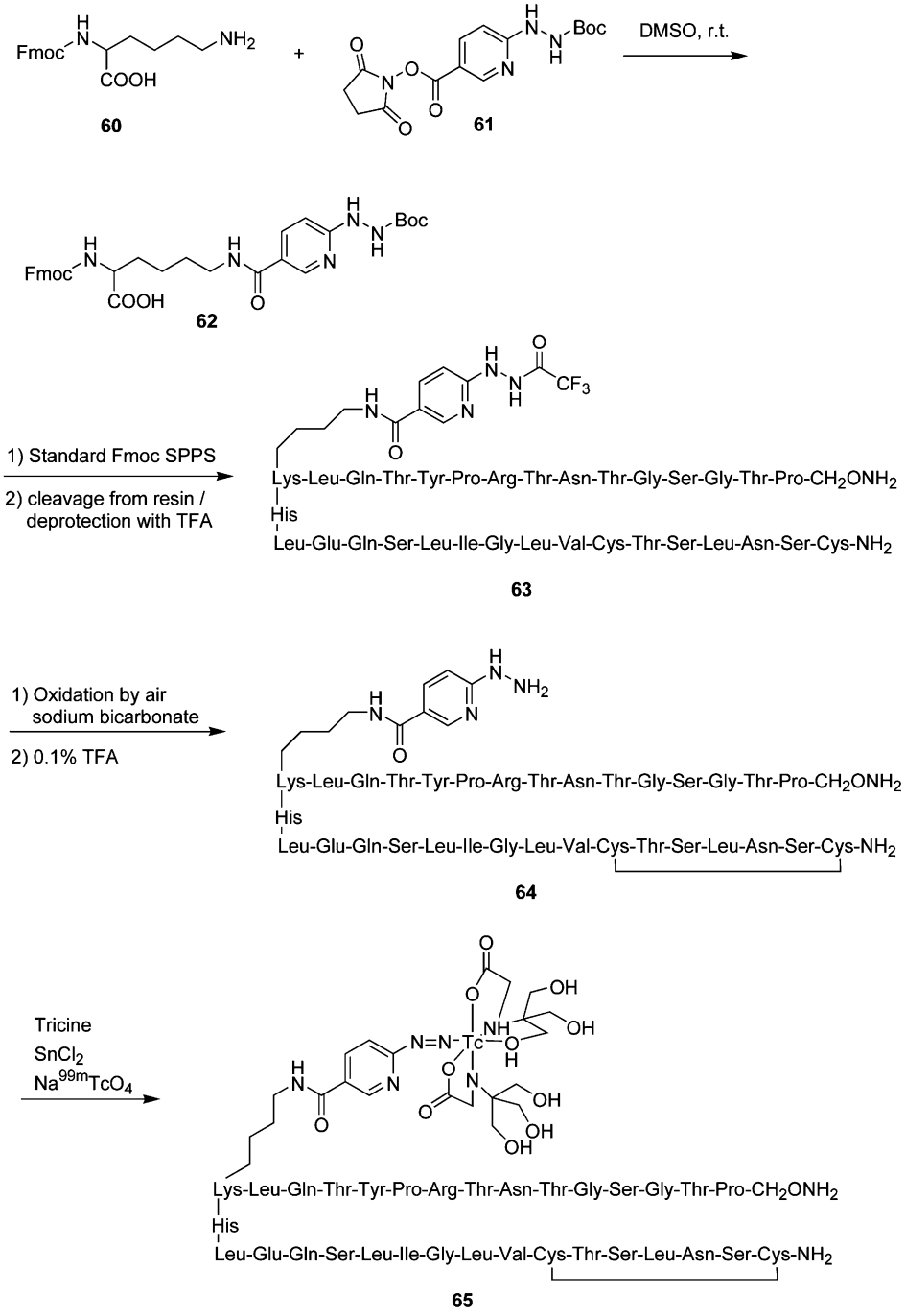
Scheme 14. Post-transmetallation of triamido-thiol bifunctional chelate with ^{99m}Tc.

which has been shown to bind to the gastrin-releasing peptide (GRP) receptor. Bombesin (BBN) is an analogue of human GRP that binds to GRP receptors (GRPrs) with high affinity and specificity.[31] The GRPr is overexpressed on a variety of human cancer cells, including prostate, breast, lung and pancreatic cancers. The synthesis of a series of bombesin derivatives was reported by Hofman et al.[32] They describe the design of BBN agonist analogues in which the radiometal chelate is linked to the N-terminal amine group of BBN[7-14]NH_2 either directly (**56**; Figure 2) or through hydrocarbon spacer groups (**57**–**60**; Figure 3). In a "post-transmetallation" manner, ^{99m}Tc was introduced onto the triamido-thiol (N_3S) bifunctional chelating agent and the effects of varying the length of hydrocarbon spacer groups were determined (Scheme 14).

2.4 Hydrazinonicotinyl Acid (HYNIC) Technetium Conjugate

Blower and co-workers recently described a novel solid-phase synthesis approach in which a HYNIC derivative (**61**) of Fmoc-lysine was used as a metal-binding amino acid analogue.[33] The *N*-protected HYNIC derivative was successfully incorporated into a bioactive peptide by standard Fmoc solid-phase peptide chemistry. Fmoc–*N*-ε-(Hynic–Boc)–Lys is a highly versatile technetium-binding amino acid and it was used to synthesize a technetium-99m-labeled salmon calcitonin with the HYNIC-linked amino acid in place of lysine-18. α-Fmoc-protected lysine **60** was treated with the NHS (*N*-hydroxysuccinimide) ester of Boc-protected HYNIC **61** to give the α-Fmoc-protected amino acid

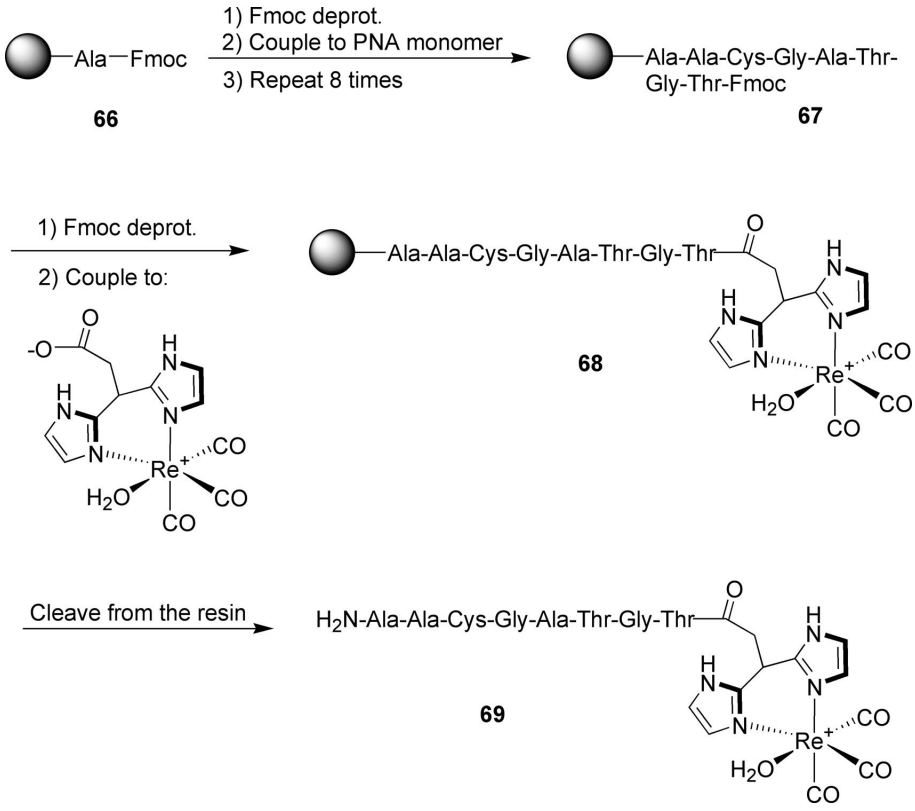


Scheme 15. Synthesis of Fmoc lysine–HYNIC derivative **62** and its use in peptide synthesis and subsequent Tc-99m labelling.

62 (Scheme 15). A trifluoroacetate group protected the HYNIC during alkaline oxidation to the cyclic disulfide and was readily removed by mild acid treatment. After deprotection and cleavage of the 32-amino acid sequence from the resin, the peptide **63** was oxidized with air in $NaHCO_3$ (0.1 M) under high-dilution conditions to form the corresponding disulfide-cyclized peptide **64**. After removal of the TFA protecting group the peptide conjugate was labelled with Tc-99m.

2.5 3,3-Bis(2-imidazolyl) Propionic Acid (bip-OH) Rhenium Conjugate

Metzler-Nolte et al. have reported the preparation of an organometallic metal–PNA conjugate.[34] Solid-phase synthesis was used to couple $Re(bip)(CO)_3$ fragments to PNA decamers on Tentagel resin with PAL linker and their interaction with complementary DNA was studied (Scheme 16). Such metal–PNA conjugates are of interest for the detec-



Scheme 16. Synthesis of rhenium–PNA conjugate by application of a rhenium-containing carboxylic acid in solid-phase peptide synthesis.

tion of complementary DNA or RNA, due to the excellent hybridization properties of PNA.

3. Iron, Ruthenium and Osmium (Group 8) Metal Complex–Peptide Conjugates

3.1 4′-Aminomethyl-2,2′-bipyridyl-4-carboxylic Acid (Abc) Ruthenium Conjugate

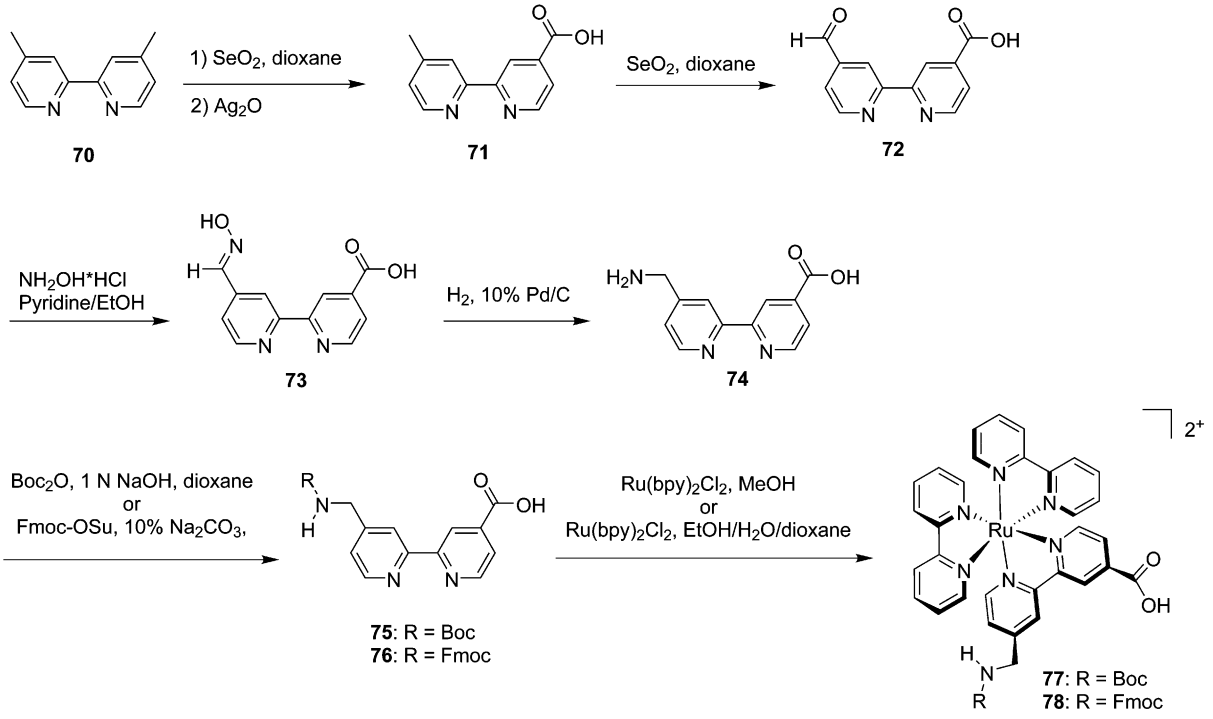
Tris(diimine) metal complexes of 4′-aminomethyl-2,2′-bipyridyl-4-carboxylic acid (Abc) are of interest, since they possess a number of favourable properties, including high stability, inertness to ligand exchange reactions, tuneable electronic structures, long lifetimes in fluid solution, and high quantum yields. Site-specifically labelled ruthenium oligonucleotides have been prepared by DNA solid-phase synthesis using a ruthenium-nucleoside phosphoramidite,[35] but this example does not lie within the scope of this review. Another approach used bipyridyl amino acids and in particular Boc- and Fmoc-protected Abc, which were incorporated into a hexapeptide.[36]

Solid-phase synthesis of these metallopeptides was performed on MBHA resin using BOP and ByBOP as coupling reagents to provide high-affinity binding sites for ruthenium(II). Metal complexation occurred in solution, and was followed by cleavage of the peptide from the solid support. The Abc residue bears the bipyridyl group not in a side chain but in the main peptide chain and is used as a tetradentate ligand for octahedral coordination and asymmetric encapsulation of a ruthenium(II) ion, creating a novel peptide-caged redox-active metal complex.

To prepare the Abc **74**, a dual oxidation strategy was employed (Scheme 17). Firstly, 4,4′-dimethyl-2,2′-bipyridine (**70**) was selectively oxidized to the 4′-monocarboxylic acid derivative **71**. Secondly, the 4′-methyl group of **71** was oxidized with excess selenium dioxide to form the aldehyde acid 4′-formyl-2′,2-bipyridine-4-carboxylic acid (**72**). Oxime formation with hydroxylamine in ethanol/pyridine smoothly converted **72** into compound **73**. Lastly, oxime acid **73** was transformed into the desired amino acid Abc **74** by catalytic hydrogenation.

Amino acid **74** was converted into both Boc and Fmoc derivatives for use in solid-phase peptide synthesis. Treatment of the Abc·HCl salt with bis(*tert*-butyl)dicarbonate provided Boc-Abc-OH (**75**), while treatment of Abc·HCl with Fmoc-succinimide similarly furnished Fmoc-Abc-OH (**76**). The metal complexation properties of building blocks **75** and **76** for bipyridyl solid-phase peptide synthesis were confirmed by the synthesis of their corresponding ruthenium(II) octahedral mixed-ligand complexes. Treatment of **75** and **76** with dichlorobis(2,2′-bipyridine)ruthenium(II) (Rub_2Cl_2) gave the bis-heteroleptic complexes **77** and **78**.

To demonstrate the utility of Abc **74** in solid-phase peptide synthesis, a heptapeptide containing two Abc residues was synthesized to serve as a tetradentate caging peptide ligand for ruthenium(II) ions (Scheme 18). Two aminohexanoic acid (Ahx) residues were arranged as a bridging tether just long enough to form *cis*-bridged meridonal metal com-



Scheme 17. Synthesis and metal complexation of Abc **74** and Boc-/Fmoc-protected derivatives.

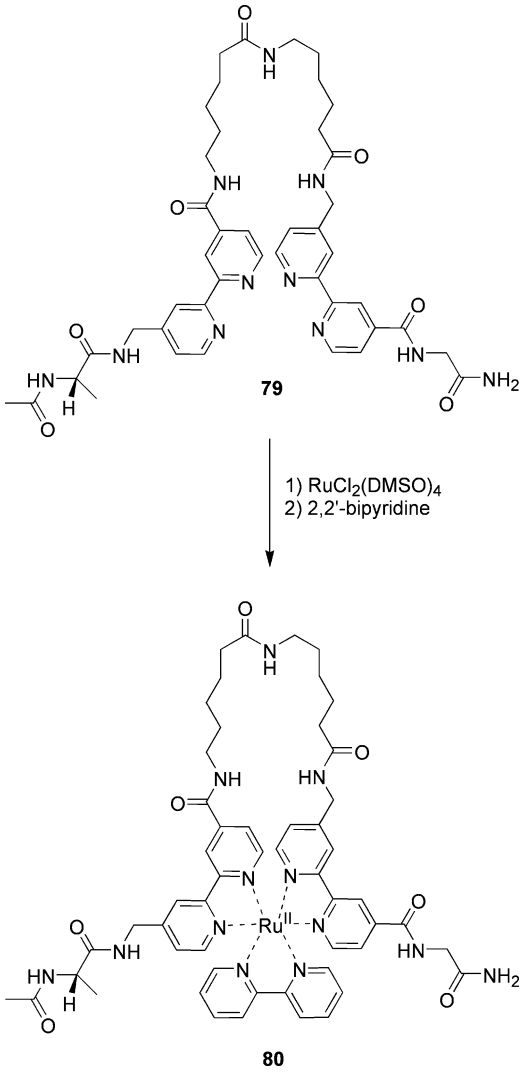
plexes. The C-terminal Gly residue was included to facilitate attachment of the bipyridine **77** or **78** to the sterically hindered MBHA resin, since direct coupling of Abc-OH to MBHA resin proved sluggish. The acetylated hexapeptide amide Aha **79** was prepared by a Boc/TFA strategy from Boc-Abc-OH (**75**) and other Boc amino acids using conventional reagents and procedures for manual solid-phase peptide synthesis. Reaction times and yields for the coupling of **77** to the Gly-MBHA resin were remarkably improved by addition of stoichiometric amounts of the acylation catalyst DMAP. Following the assembly, apopetide **79** was cleaved from the resin with anhydrous HF, and subsequent conversion of $Ru^{II}(Aha)Cl_2$ into the heteroleptic tris(bipyridyl) complex $Ru^{II}(Aha)(bpy)$ (**80**) was performed in solution.

3.2 Metallocene (Ferrocene)[37] Conjugate

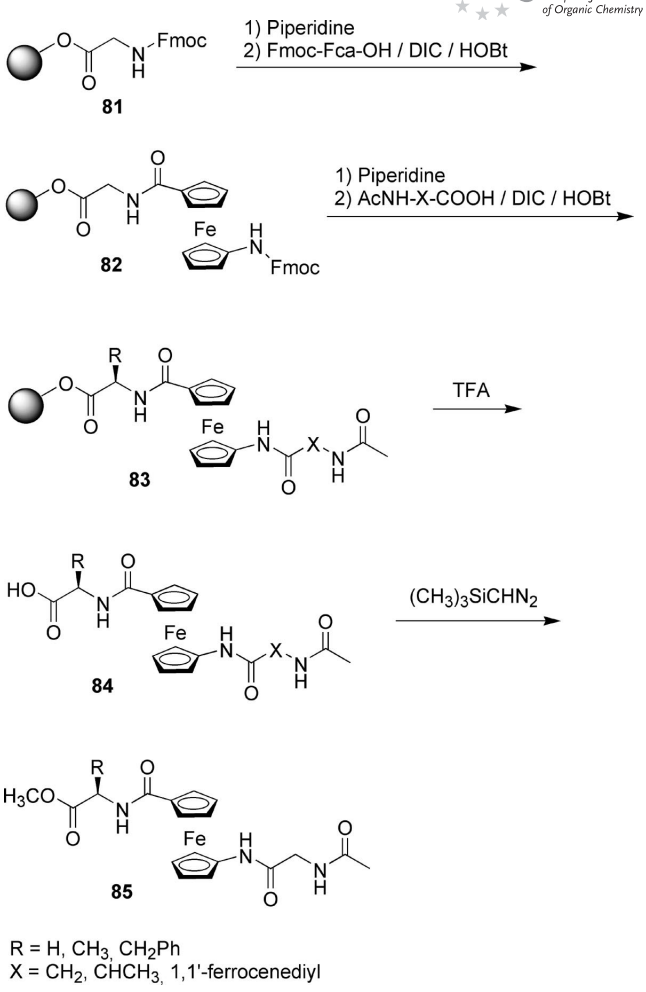
Ferrocene-containing tripeptides containing one or two ferrocene building blocks have been prepared by solid-phase peptide synthesis.[38] Heinze et al. incorporated the solid-phase peptide synthesis-compatible ferrocene building block Fmoc-protected 1′-aminoferrocene-1-carboxylic acid (Fca)[39] into the backbones of tripeptides. The coupling was performed using DIC/HOBt for activation and TentaGel-Wang, which turned out to be superior to polystyrene/divinyl resin, as solid support. Cleavage of the resulting tripeptides from the support with trifluoroacetic acid gave the mono- (Scheme 19) or diferrocene peptides. Reversible on-bead oxidation allows switching between the neutral ferrocene (low-affinity state) and charged ferrocenium ion (high-affinity state), which results in superior anion-binding affinities.

Metallocene-modified tri- to pentapeptides were identified as having antibacterial activities,[40] although the highest activity is still one order of magnitude lower than the minimum inhibitory concentration (MIC) values found for most naturally occurring antimicrobial peptides (AMPs). First, Metzler-Nolte and co-workers synthesized metallocene-peptide bioconjugates in which the amino acid sequence ranged from three to five residues by solid-phase peptide synthesis (Scheme 20). The ferrocene and the cobaltocenium groups were introduced at the N terminus by coupling of ferrocenecarboxylic acid hexafluorophosphate with the free amino group of the peptide **87** while the peptide was attached to the solid support. Care has to be taken during the cleavage from the Rink amide resin. Decomposition – that is, loss of a ferrocenoyl moiety – occurs when the TFA/H_2O/TIS cleavage mixture is used. However, this problem can be circumvented by the use of phenol rather than water.

Later, Metzler-Nolte and co-workers hoped to arrive at small, readily available artificial AMPs with activities comparable to those of the best naturally occurring AMPs by addition of metallocenes to more active peptide sequences.[41] Arg- and Trp-containing hexapeptide sequences that had been shown to have good antibacterial properties[42] were selected and modified by replacement of the N-terminal amino acid with a ferrocenyl (or a cobaltocenium) group. The metallocene peptide conjugates were prepared on Rink amide resin, whereas the ferrocenecarboxylic acid

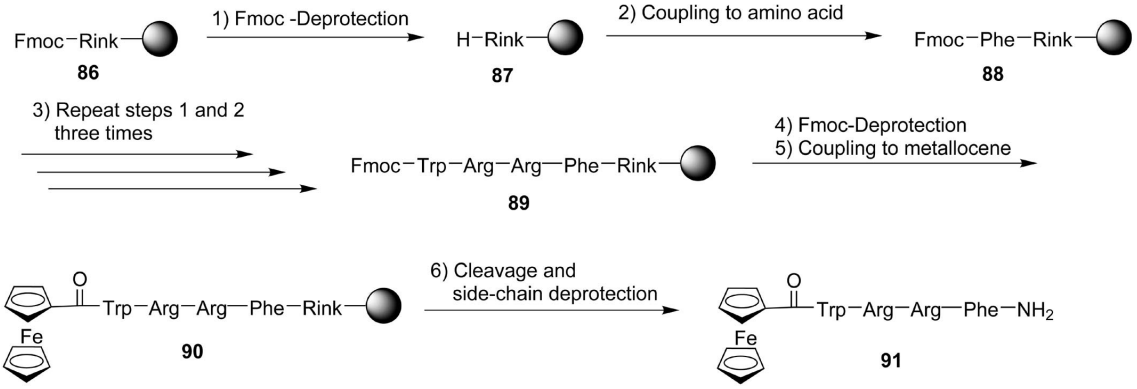


Scheme 18. Preparation of heteroleptic tris(bipyridyl) complex Ru^{II}(Aha)(bpy).

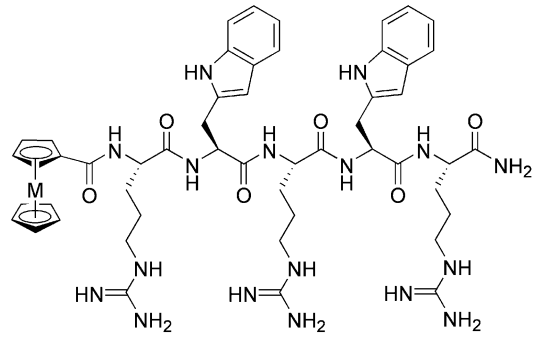


Scheme 19. Synthesis of ferrocene-containing tripeptides by use of a ferrocene building block and solid-phase peptide synthesis.

was attached by formation of an amide bond with the free N-terminal amino group of the solid support. The ferrocene moiety is stable towards deprotection reagents and to resin cleavage, although the ferrocenoyl peptides are only stable when phenol rather than water is used in the cleavage mixtures. The activity of the resulting metallocene-pentapeptide conjugate [Fe(Cp)(C_5H_4)–C(O)–WRWRW–NH_2] (**93**, Figure 4) was indeed increased and was even better than that of the naturally occurring 20 amino acid pilosulin, which was used as a positive control.



Scheme 20. Solid-phase peptide synthesis of metallocene-peptide bioconjugates.



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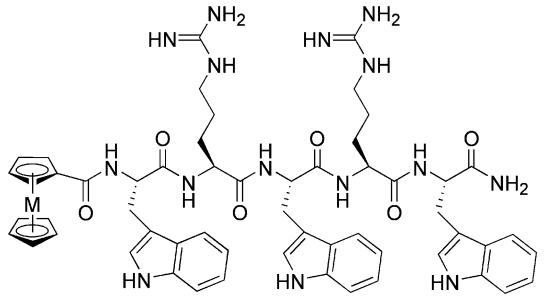


M = Co+, Fe 93

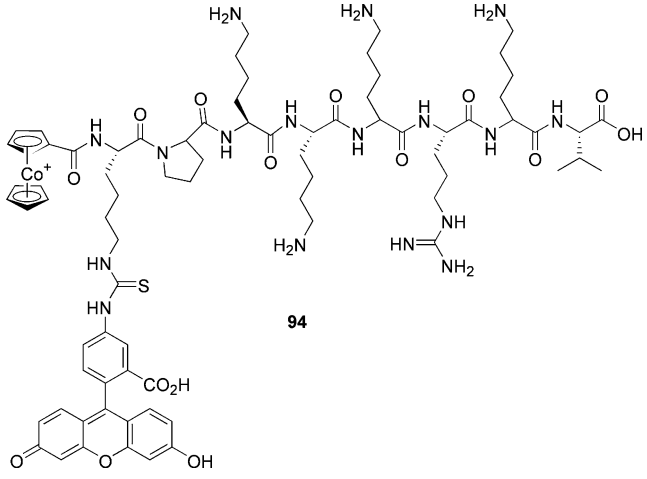
Figure 4. Metallocene-pentapeptide conjugates **92** and **93**.

4. Cobalt and Rhodium (Group 9) Metal Complex–Peptide Conjugates

4.1 Metallocene (Cobaltocenium) Conjugate

While much work has been devoted to ferrocene bioconjugates,[35,43] the closely related cobaltocenium group has received considerably less attention, although the cobaltcenium cation has a much higher redox potential and better chemical stability than ferrocene. Its unique electrochemical properties have, however, been exploited in enzyme biosensors and DNA detection,[44] and also in a more recent study on the cellular uptake and directed nuclear delivery of a cobaltocenium–NLS peptide bioconjugate.[45] The lipophilic nature of the ferrocenyl moiety acts as a mimic for the bulky Trp residue, whereas the positively charged cobaltocenium moiety is isostructural with the neutral ferrocene, thus allowing an assessment of additional positive charge, and acting as a bulky Arg mimetic.[46] Capping of the N termini of Arg- and Trp-containing hexapeptide sequences results in net losses of one unit of positive charge in the case of the ferrocenoyl bioconjugates, but the cobaltocenium analogues retain the overall charges of the peptides, which is favourable for their antibacterial activities.

Metzler-Nolte and co-workers reported the first nonradioactive organometallic–peptide conjugate[47] that specifically delivers the organometallic species into the nucleus of a cell. Solid-phase peptide synthesis was used to prepare the cobaltocenium conjugate of a nuclear localization signal peptide. The cobaltocenium–NLS conjugate significantly accumulates in the nuclei of HepG2 cells. The heptapeptide H-Pro-Lys-Lys-Lys-Arg-Lys-Val-OH[48] was chosen as the antigen NLS, which serves as an "address label" for proteins and indicates their destination as the cell nucleus. In addition, this heptapeptide enables the active transport of a variety of substrates through the nuclear pore complex.[49] An additional protected lysine residue was introduced at the N terminus of the NLS peptide using Wang resin as solid support. Fluorescein isothiocyanate (FITC) was used as a label to visualize the metal conjugate inside the cells. For this purpose, the N-terminal lysine residue was modified with a Mtt protecting group. After coupling of the cobaltocenium moiety to the peptide, the N-terminal Fmoc protecting group was cleaved, and cobaltocenium carboxylic acid was coupled by use of TBTU. Cleavage from the resin and removal of all amino acid side chains were accomplished with concentrated trifluoroacetic acid, yielding the cobaltocenium–NLS peptide bioconjugate **94** (Figure 5).



94

Figure 5. Cobaltocenium–NLS peptide bioconjugate **94**.

4.2 Phenanthrenequinone Diimine (Phi) Rhodium Conjugate

Barton and co-workers have focused on the development of peptide conjugates of rhodium(III) complexes as models for sequence-selective DNA binding proteins.[50] To this end, a family of rhodium–peptide complexes (Figure 6) was synthesized by coupling short oligopeptides to the intercalating $[Rh(phi)_2(phen')]^{3+}$ (phi = phenanthrenequinone diimine; phen = phenanthroline) moiety to explore whether the side chain functionalities of small peptides may be used to augment metal complex recognition.[51] To summarize this work, DNA site-specificity depends on the peptide side chain functional groups. Moreover, the phi complexes of rhodium cleave DNA upon photoactivation.

Barton and co-workers used two complementary solid-phase peptide synthesis strategies for the covalent attachment of phi complexes of rhodium(III) complexes to specific sites on synthetic peptides.[52] All the natural amino acids except for methionine were used in the synthesis, and

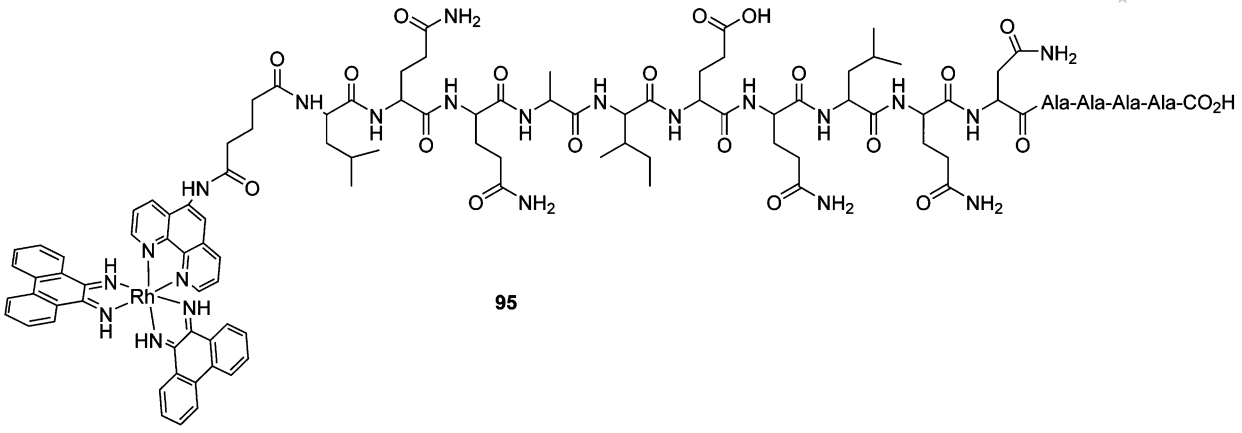
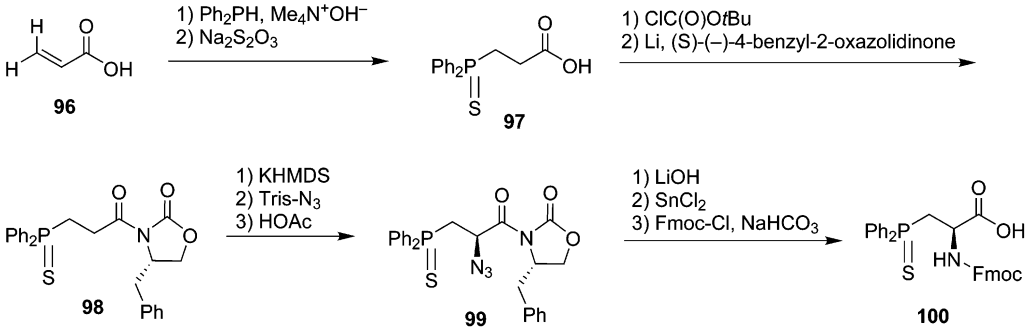


Figure 6. Phenanthrenequinone diimine rhodium peptide conjugate.

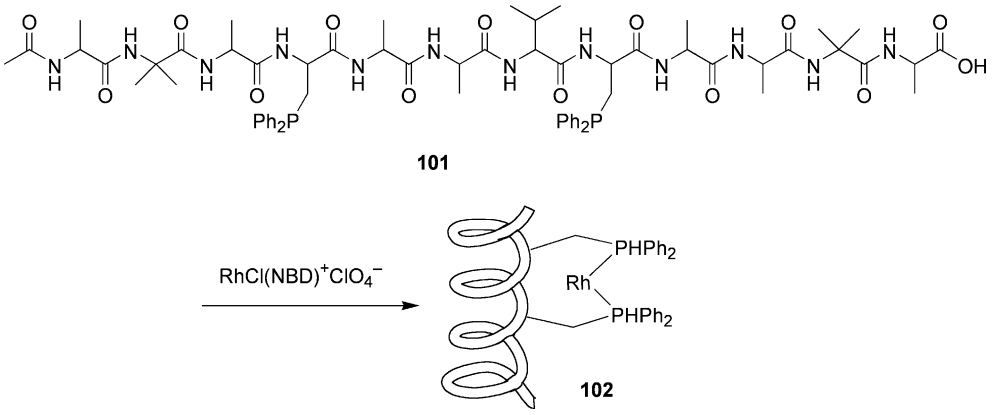
peptides ranging from 5 to 30 amino acids were successfully coupled to the rhodium complex by standard solid-phase synthesis procedures. The metal–peptide conjugates were synthesized either by the coordination method or by direct coupling. In the coordination strategy the chelating ligand is first coupled onto the amino terminus of the peptide on the resin. Then, the resin-bound peptide containing the chelating ligand is treated with $[Rh(phi)_2(DMF)_2](OTf)_3$, in a manner similar to that used for the synthesis of the parent rhodium complex. In the direct coupling strategy, the coordinatively saturated metal complex is assembled first. The functionalized metal complex and the terminal amine of the peptide bound to the resin are then condensed in a way that is analogous to the addition of another residue to the growing peptide chain. Several sets of conditions for the synthesis were examined, in which peptides were constructed by both Fmoc and *t*Boc methodologies and with manual as well as automated solid-phase techniques. Furthermore, a range of coupling agents was examined with both strategies. To summarize, in the case of the coordination method, several different coupling reagents were used with similar success. These reagents include DCC/HOBt, DSC, TBTU and TSTU. With the direct coupling method, Barton et al. observed that the presence of the metal centre makes the coupling reaction less efficient. The metal–peptide complexes are more difficult to cleave from the resin than the peptide alone. Several types of linkage to the resins, such as MBHA, PAM and PEG-PAM, were also examined, but variation in the linker does not affect the yield of the cleaved product. The presence of the metal complex, however, does significantly decrease the overall yield; furthermore it tends to inhibit the coupling reaction, since coordination on the resin is of lower efficiency than the coordination of the metal complex alone in solution. In conclusion, both strategies offer distinct advantages over solution-phase methods, in that functionalization of side chains is precluded. Thus, selective attachment of the metal centre to a specific residue or to the N terminus can be reliably accomplished.

4.3 (Diphenylphosphanyl)serine (Pps) Rhodium Conjugate

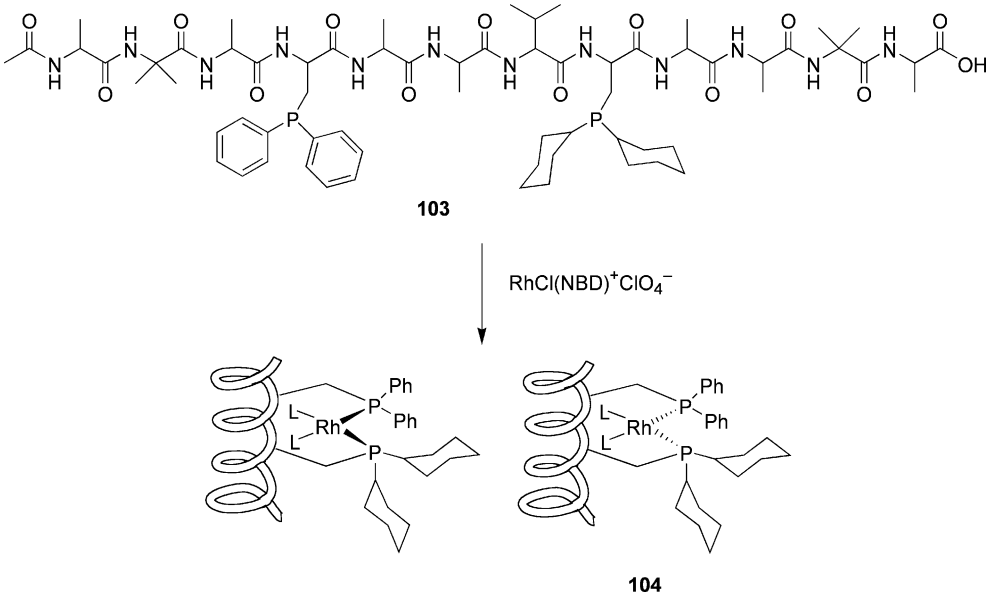
Gilbertson et al. have reported important examples of resin-bound peptide-based phosphane transition metal complexes over the last decade.[53] Rhodium was used to prepare the first peptide–phosphane–metal complexes. For the incorporation of a phosphane-containing amino acid building block it was necessary to prevent the undesirable formation of phosphane oxide. To overcome this problem, a temporary conversion of the phosphane into the phosphane sulfide[54] gave rise to an amino acid that could be used in standard coupling procedures. The best route to the required amino acid involved the use of Evans' chiral oxazol-



Scheme 21. Synthesis of a phosphane-containing Fmoc-protected amino acid building block for use in solid-phase peptide synthesis.



Scheme 22. Complexation of the bis(phosphane) ligand **101** with a rhodium salt.



Scheme 23. Mixed bidentate Pps,Cps-based rhodium conjugates.

idinone chemistry (Scheme 21). Addition of diphenylphosphane to acrylic acid proceeded smoothly with tetramethylammonium hydroxide as a base. Treatment with sodium thiosulfate converted the phosphane into the phosphane sulfide **97**. Acid **97** is then converted into the amino acid by formation of the oxazolidinone **98**. Cleavage of the chiral auxiliary and reduction of the azide **98** with tin(II) chloride gives amino acid **99**, which was finally converted into the Fmoc-protected amino acid **100** ready for peptide synthesis.

Once the desired peptide was assembled, the phosphane was regenerated by desulfurization with Raney nickel.[55] The phosphane-containing amino acids were incorporated in *i*, *i* + 4 positions to stabilize helix formation and thus to be able to chelate one metal ion between them (Scheme 22). The peptide conjugate was synthesized by standard Fmoc solid-phase peptide synthesis on Wang resin, and the (diphenylphosphanyl)serine (Pps) was incorporated as a dipeptide with alanine [Fmoc-Pps(sulfide)-Ala-OPfp].[56] The resulting bis(phosphane) ligand **101** was complexed with rhodium by stirring with $RhCl(NBD)^+ClO_4^-$ (NBD = norbonadiene).

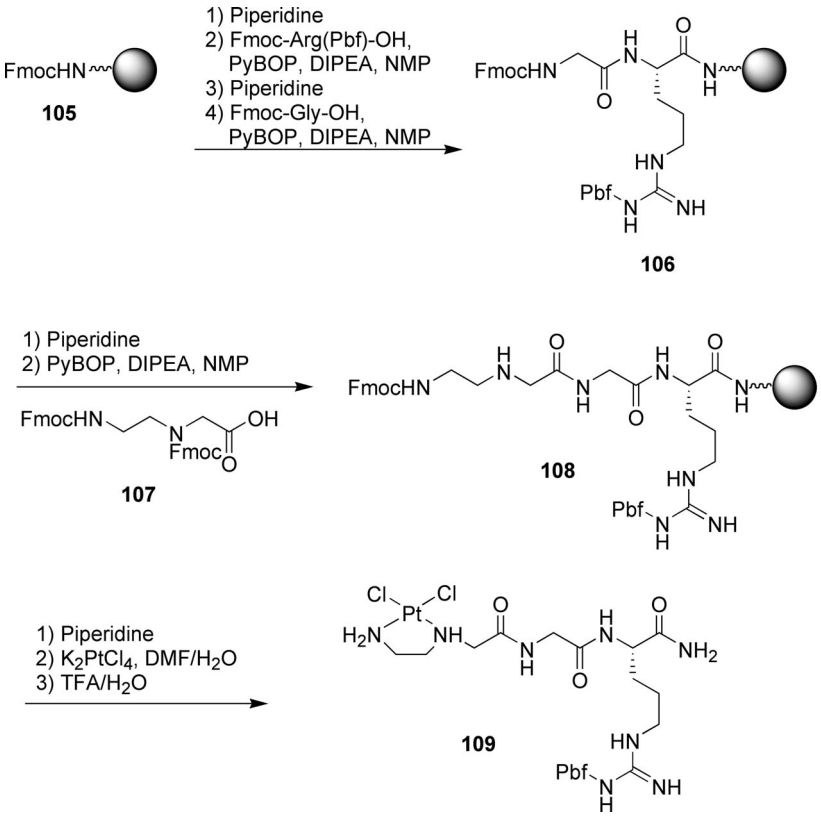
4.4 Mixed Bidentate Pps,Cps-Based Rhodium Conjugate

The (diphenylphosphanyl)serine (Pps) group was later incorporated into the 12-residue peptide **103** along with a (dicyclohexylphosphanyl)serine (Cps) unit (Scheme 23).[57] The synthesis of the peptide conjugate, as well as the rhodium complexation, was carried out as described before, but on polystyrene resin.

5. Nickel, Palladium and Platinum (Group 10) Metal Complex–Peptide Conjugates

5.1 Ethylenediamine Platinum Conjugate

In 2000 Reedijk et al. reported the first synthesis of a trimeric arginine-containing peptide-dichloroplatinum(II)



Scheme 24. Solid-phase synthesis of platinum complex **109**.

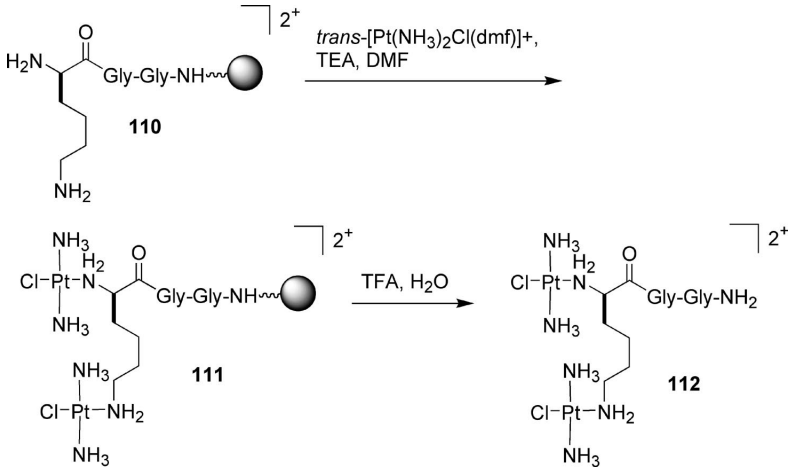
complex with potential antitumor activity by solid-phase synthesis.[58] An ethylenediamine moiety, serving as a platinum-chelating ligand, was tethered to a resin-bound arginine-glycine dipeptide. The solid-phase peptide synthesis was performed on Rink amide resin with commercially available protected amino acids Fmoc-Arg(Pbf)-OH and Fmoc-Gly-OH by a standard Fmoc protocol.[59] Fmoc-protected *N*-2-aminoethyl-glycine derivative **107** was then condensed with the dipeptide **106**, followed by platination of the ethylenediamine moiety, subsequent deprotection and release from the solid support (Scheme 24). Preliminary resin-cleavage experiments with TFA/H_2O/(TIS) led to metallic platinum and free ligand, probably due to the reduction of the coordinated $PtCl_2$ moiety by the scavenger TIS. However, nearly quantitative complexation was achieved by treatment with excess K_2PtCl_4 in DMF/H_2O followed by a resin cleavage with TFA/H_2O.

Later, Reedijk and co-workers examined the scope and generality of the solid-phase platination approach by preparing a six by six array of individual dichloroplatinum peptide analogues.[60] The parallel solid-phase peptide synthesis of a dichloroplatinum-peptide array was performed on Rink amide resin with six natural amino acids on an automated synthesizer. Unfortunately, these platinum peptide complexes showed no potential as cytotoxic agents, but only demonstrated the utility of solid-phase peptide synthesis for the preparation of platinum drugs. However, in a subsequent publication,[61] Reedijk et al. did report on some cytotoxic platinum tripeptide complexes, although the highest activity, which was measured for the tripeptide conjugate containing the Gly-Gly dipeptide, was still lower than that of cisplatin.

5.2 Dinuclear $N_{\alpha,\varepsilon}$-L-Lysine Platinum Conjugate

As an extension of these studies, Reedijk et al. described the first solid-phase peptide synthesis of dinuclear lysine-bridged platinum(II) complexes.[62] Platination of the lysine was achieved with a fivefold excess of activated transplatin to give the immobilized compound **111** (Scheme 25). To avoid strongly acidic cleavage conditions, in view of the moderate stability of the immobilized platinum complex **111**, Rink amide MBHA was used in combination with the 2-chlorotrityl linker, which allow mild cleavage conditions. Both linkers were suitable for the solid-phase peptide synthesis of dinuclear *trans*-platinum complexes. Biological testing of the platinum complexes showed their potential as anticancer agents. However, in comparison with cisplatin, compound **112** displayed a 60-fold decrease in activity.

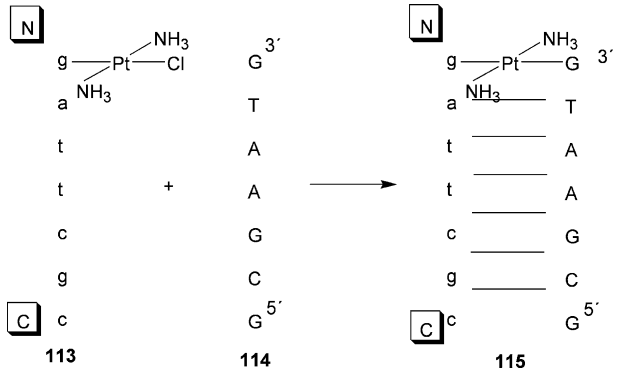
Metal complexes of suitable geometry and coordination properties are promising cross-linking agents.[63] One application of metal complex cross-linking is to increase the affinity of an antisense oligonucleotide to its target.[64] Lip-



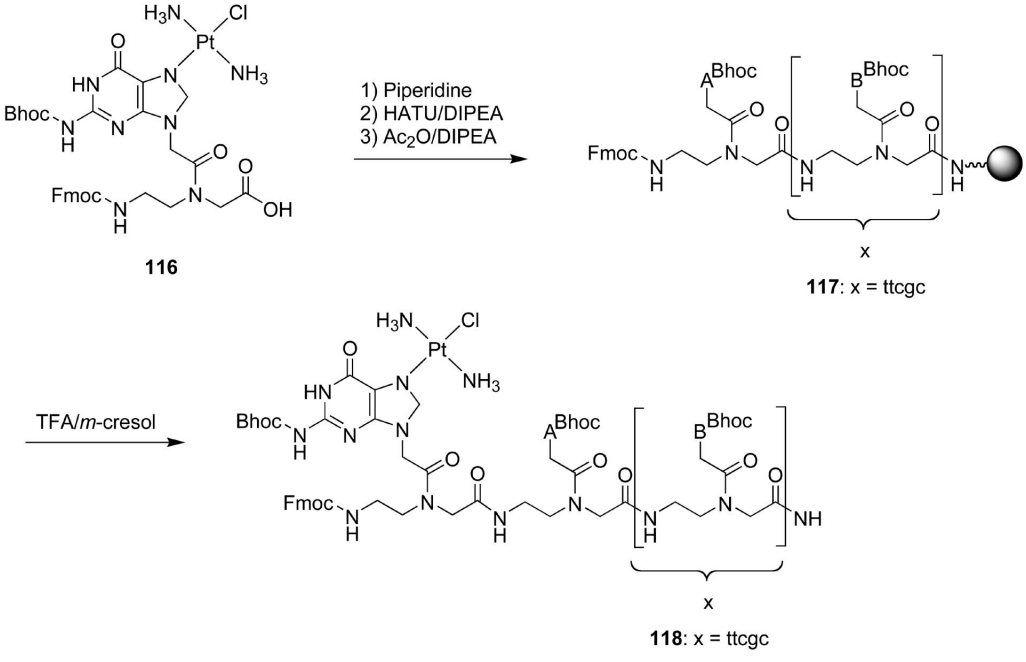
Scheme 25. Solid-phase synthesis of platinum complex **112** by metallation on solid support.

pert et al. used this strategy in a model cross-linking reaction between the monofunctional *trans*-Pt-modified PNA oligomer *trans*-[$(NH_3)_2$Pt(g-*N*7-attcgc)Cl]$^+$ (**113**) and its complementary deoxyoligonucleotide 5′d(GCGAATG) (**114**) (Scheme 26).[65] The *trans*-Pt^{II}-modified building block **116** was synthesized through the reaction between *trans*-[$Pt(NH_3)_2Cl(DMF)]BF_4$ and Fmoc/Bhoc-PNA G.

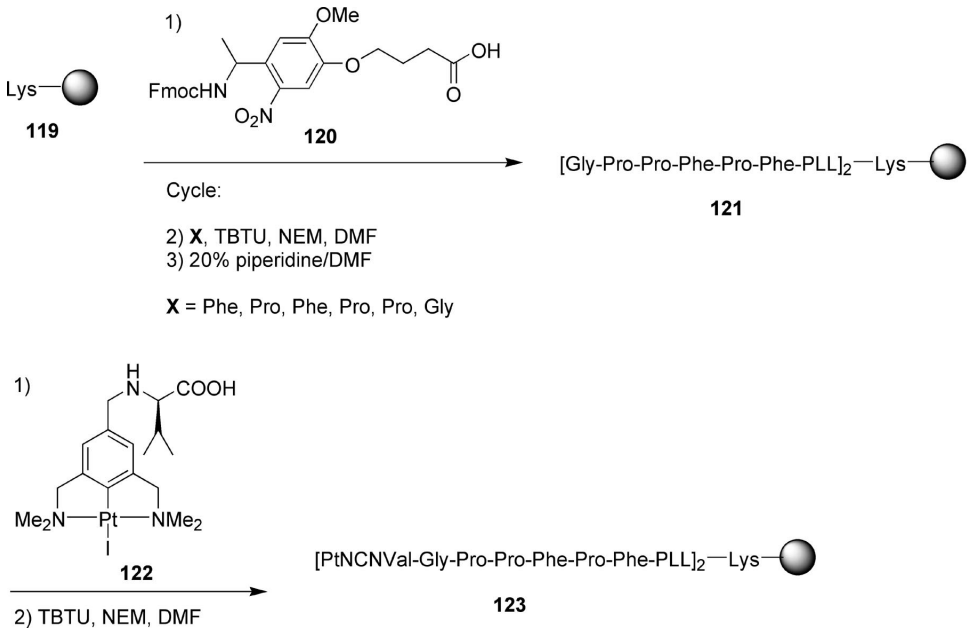
Building block **116** was then coupled to the Rink amide-bound fully protected PNA oligomers with the aid of the coupling reagent HATU. Removal of the Bhoc protecting groups along with the release from the solid support was effected with TFA/*m*-cresol (Scheme 27). In summary, this methodology allows the preparation of monofunctional *trans*-Pt^{II}-modified mixed pu/pym PNA oligomers, which have been shown to cross-link sequence-specifically with a target oligonucleotide.



Scheme 26. Cross-linking reaction between PNA **113** and DNA **114**.



Scheme 27. Solid-phase synthesis of a monofunctional *trans*-Pt^{II}-modified PNA oligomer.



Scheme 28. Solid-phase synthesis of a platinum-biomarker-containing peptide by use of metal-containing amino acid **122**.

5.3 Tetradentate Monoanionic "Pincer" NCN {[$C_6H_2(CH_2NMe_2)_2$-2,6-R-4]$^-$} Platinum Conjugate

Van Koten et al. reported a robust organoplatinum(II) biomarker that can be incorporated into peptides by standard solid-phase coupling techniques.[66] The biomarker-containing peptides can be identified by addition of an aqueous KI_3 solution, producing visually detectable coloured resin beads. For the almost instantaneous change of colour from colourless to deep purple, capping of only 6% of the available amine termini of the resin-bound peptide is sufficient. Furthermore, this colouration process is reversible by washing with DMF/Et_3N or DMF/morpholine solutions. $PEGA_{1900}$ resin [a copolymer of bis(2-aminopropyl)-poly(ethylene glycol)/acrylamide] was chosen as the solid support since it combines good characteristics for organic synthesis and screening in aqueous buffer solution, which is required for a biomarker-function in solid-phase screening assays. A lysine residue was first coupled to the resin, to double its loading capacity. The peptide sequence Gly–Pro–Pro–Phe–Pro–Phe was synthesized on a photolabile linker,[67] by syringe technology[68] with Fmoc/OPfp-derivatized amino acids, which were activated with Dhbt-OH. Finally, the *N*-protected platinum(II) biomarker **122** was attached to the N terminus of the resin-bound peptide **121** with TBTU and NEM activation (Scheme 28).

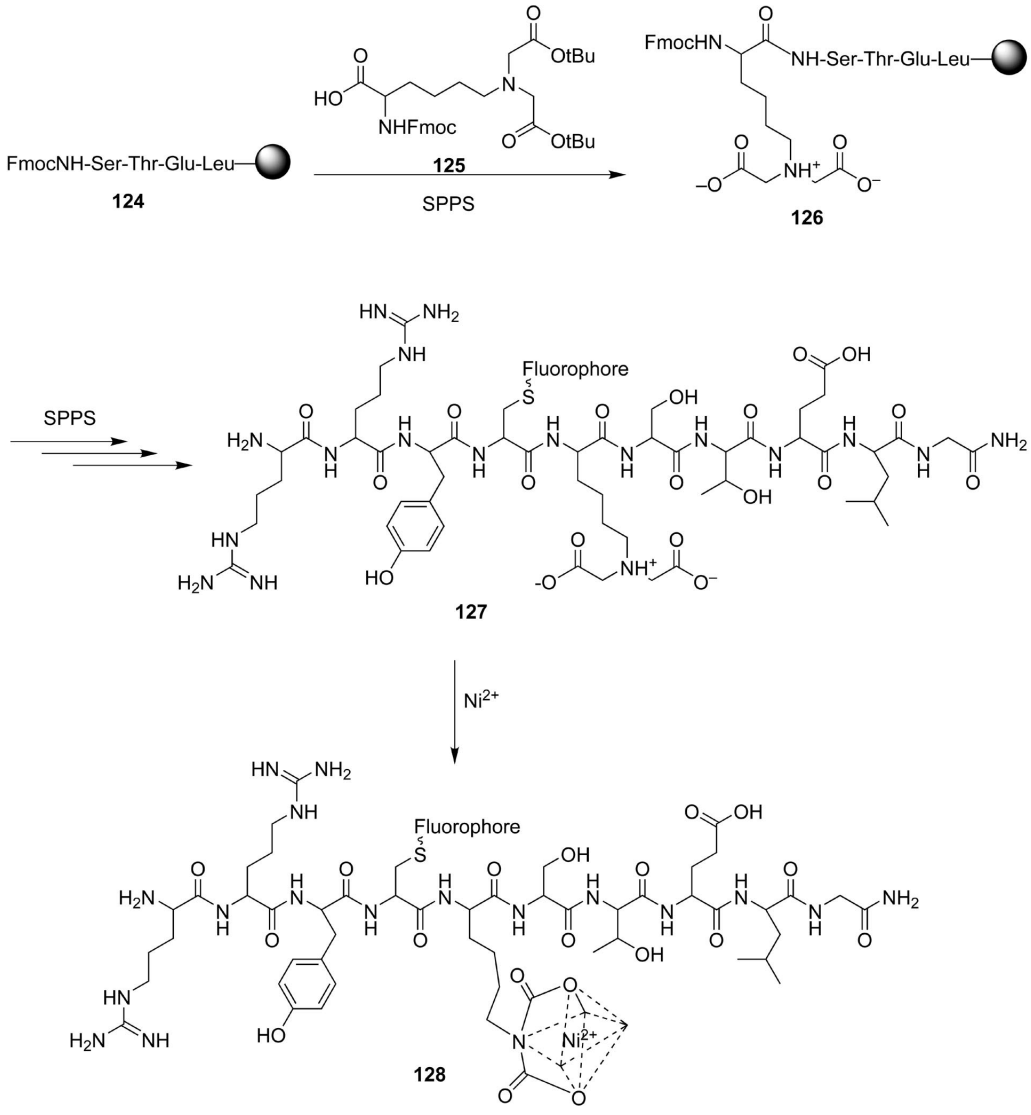
5.4 Iminodiacetic Acid (IDA) Nickel Conjugate

Metallopeptides of the general form $Ni^{II}AA_1$–AA_2–His[69] are used in biochemical analysis of protein–nucleic acid and protein–protein interactions.[70] Long and co-workers prepared two libraries derived from AA_1–AA_2–His sequences in which the first and the second positions of the peptide ligand were varied.[71] Standard *t*Boc protocols on methylbenzydrylamine (MBHA) resin were used, including all possible combinations of 18 natural α-amino acids excluding Cys and Trp to prevent disulfide formation and partial DNA intercalation[72] of these residues. The optimized metallopeptide Ni^{II}-Pro–Lys–His was found to cleave DNA an order of magnitude more efficiently than Ni^{II}-Gly-Gly–His.

Tampé and co-workers synthesized a metal-chelating amino acid building block for synthetic receptors.[73] Such synthetic receptors bearing an IDA-chelate were employed as metal ion sensors and as receptors for histidine-tagged proteins. Standard solid-phase peptide synthesis was used to incorporate the SAAC **125** into a polypeptide (Scheme 29). The peptide conjugate was further labelled with fluorescein at a cysteine residue[74] to signal metal ion binding. After release of the IDA-peptide **127** from the resin, it was treated with Ni^{2+} and several experiments were performed, demonstrating strong binding to imidazole.

5.5 Bidentate Phosphane Palladium Conjugates

Palladium(II) allyl complexes were prepared by Meldal and co-workers from resin-bound ligands to demonstrate their catalytic properties.[75] The palladium complexes **133**–**135** and **138** were synthesized on solid support by use of Fmoc-protected amino acids and Fmoc-protected amino aldehydes. Phosphane moieties were introduced by phosphanylmethylation of the free amines as the final solid-phase synthetic step, prior to complexation with palladium. $PEGA_{1900}$ resin[76] was selected because of its excellent swelling properties in organic solvents, as well as in water. After the $PEGA_{1900}$ resin had been functionalized with Fmoc-glycine by TBTU activation and subsequent Fmoc-



Scheme 29. Solid-phase peptide synthesis of fluorescein-labelled IDA peptide **127** and subsequent metallation in solution to give nickel complex **128**.

deprotection with piperidine, the HMBA linker was introduced with the aid of TBTU. The HMBA linker can be efficiently cleaved under mild conditions and is also suitable for on-bead NMR analysis, since it possesses no stereocenter making the analysis more difficult. The first amino acid Fmoc-phenylalanine was attached to the HMBA linker by MSNT activation in dichloromethane (Scheme 30). For all the other couplings TBTU was sufficient. The resulting peptide-based bidentate phosphane palladium conjugates **132** and **137** were shown to be suitable for palladium catalysis of asymmetric allylic substitution reactions.

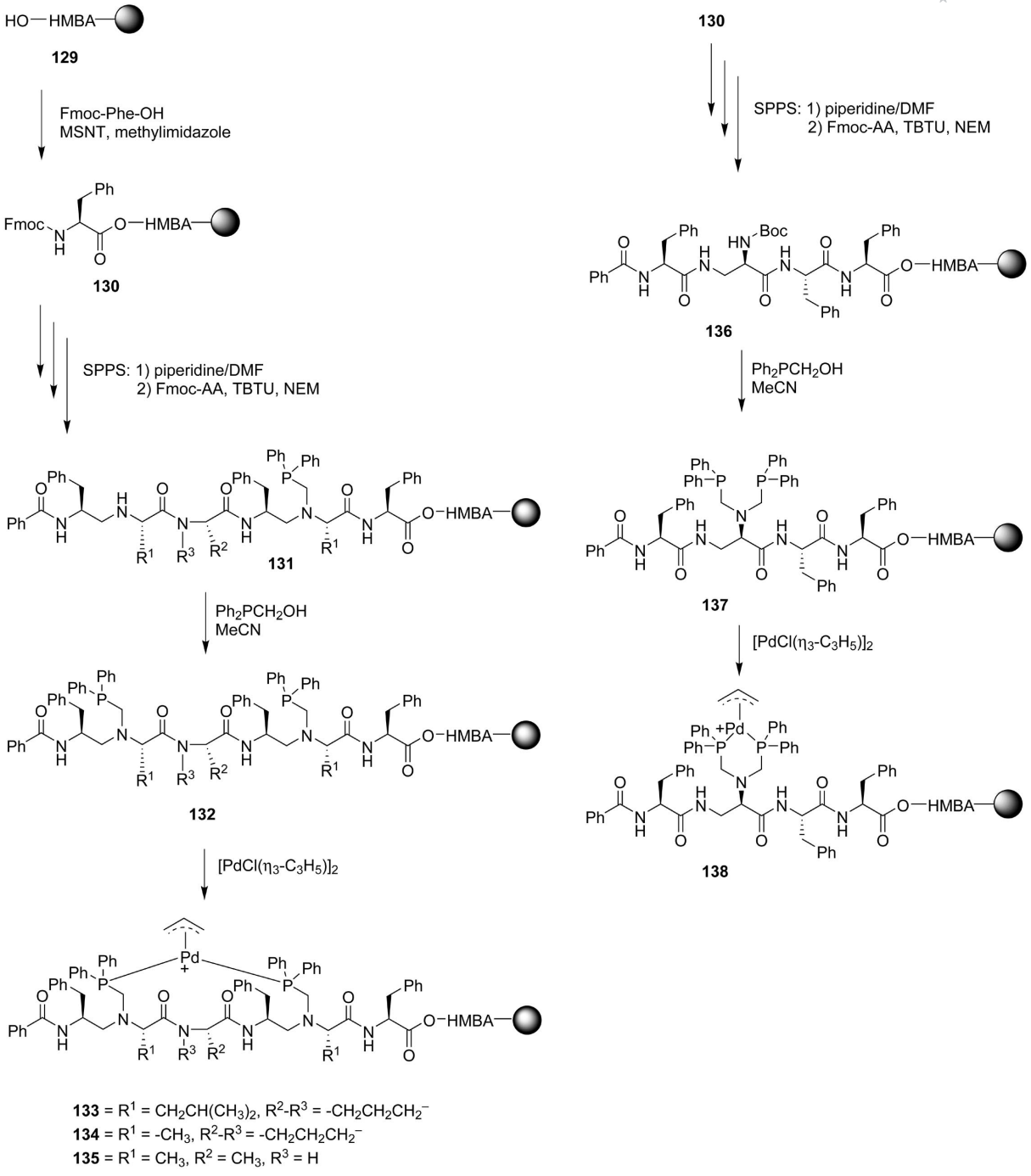
5.6 Bidentate P,S-Based Palladium Conjugates

Recently, bidentate mixed heteroatom ligands have proven to be very successful for asymmetric organic synthesis.[77] One class of such ligands are P,S-ligands,[78] which have been successfully applied in palladium-catalysed allylic substitution reactions.[79] Meldal et al.[80] expanded their methodology for the solid-phase synthesis of peptide-based bidentate phosphane ligands described above to the solid-phase peptide synthesis of P,S-bidentate chelating palladium(II) complexes, exploiting the readily available chiral pool of cysteine derivatives (Scheme 31).

6. Copper (Group 11) Metal Complex–Peptide Conjugates

6.1 IDA Copper Conjugate

Peptides with metal complexes in their side chains and peptide–metal complex conjugates have been used to enhance or control binding affinities and peptide conformations.[81] To extend the scope of solid-phase synthesis of peptide–metal complex conjugates, König et al.[82] reported their preparation from modified amino acids bearing metal

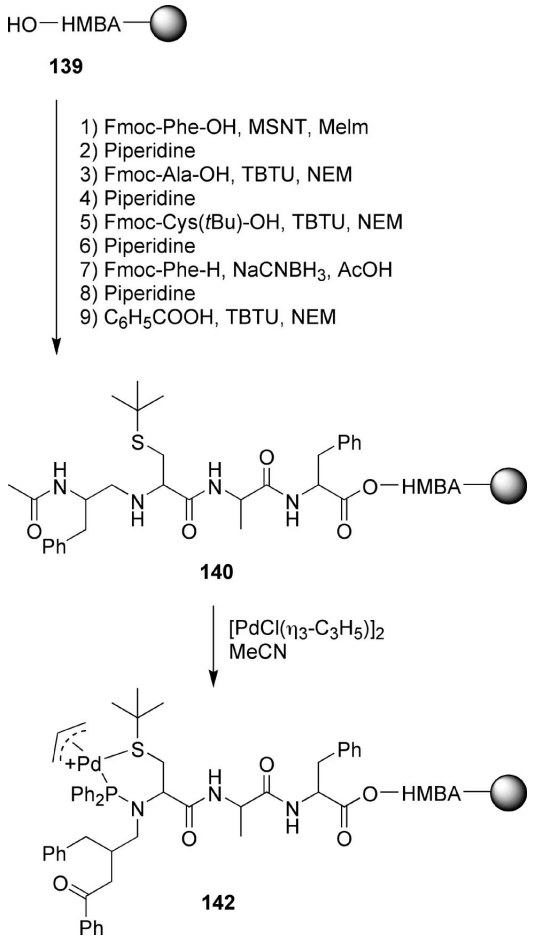


Scheme 30. Formation of palladium(II) allyl complexes on solid support.

complex ligands or metal complexes in their side chains. The IDA motif, known for its ability to bind imidazole residues and N-terminal His, was chosen and converted into its copper complex as a SAAC. This modified amino acid **143** was incorporated into a peptide sequence by standard solid-phase peptide synthesis (Scheme 32). The use of HMBA-AM resin allowed nucleophilic cleavage of the copper peptide conjugate from the resin without decomplexation.

6.2 Bis(2-picolyl)amine (bpa) Metal Conjugate

As discussed above, radioactive metals have been successfully and extensively applied to radioimaging (e.g., with ^{99m}Tc complexes). Control of cellular uptake and metal ion localization is thus a challenge for medicinal inorganic chemistry. However, this concept is not widely applied to non-radioactive metals.



Scheme 31. Palladium catalyst derived from a solid-phase synthesized peptide scaffold and metallation on solid support.

Copper, on the other hand, plays an important role in cell-regulating processes, but in certain cells there is not one single free copper ion.[83] Any Cu^{2+} ion is sequestered by so-called Cu chaperones, proteins that also serve to deliver the metal to specific Cu enzymes.[84] For such systems, Metzler-Nolte et al. proposed to use bioconjugates of metal-chelating ligands, linked to physiologically active peptides.[85] The preparation of metal–bpa complexes linked to amino acids and a cellular localization signal peptide – namely, a nuclear localization signal[86] (nls) – was reported. The nls peptide used by Metzler-Nolte et al. in their work is a heptapeptide with the primary sequence H-Pro-Lys-Lys-Lys-Arg-Lys-Phe-OH and serves as a tag to proteins, indicating their destinations in the nuclei of cells.[87] The nls-bpa bioconjugate **148** (Figure 7) was prepared by Fmoc solid-phase peptide synthesis on Rink amide resin with an acid-labile linker, and acid-labile side chain protecting groups for amino acids Lys (Boc) and Arg (Pbf) were used. The peptide synthesis cycle consisted of Fmoc removal with piperidine and TBTU coupling. Metal complexation was carried out in aqueous solution with $Cu(NO_3)_2$. The formation of the complex **148-Cu** was immediately apparent from the deep blue colour of the solution due to a blue shift of the Cu d–d transition in the Cu(bpa) complex. The metal–peptide conjugates were suggested as artificial metallochaperones, because they have the potential to deliver metal ions to specific compartments in the cell as determined by the peptide moieties.

7. Zinc (Group 12) Metal Complex–Peptide Conjugate

7.1 Bpa Zinc Conjugate

Metzler et al. also showed that Zn^{2+} binds to their nls peptide conjugate **148**. The Cu^{II} and Zn^{II} complexes were characterized, including by X-ray structure analyses, and the results indicated similar structural features in the transition metal complexes.

Kraemer and co-workers prepared conjugates of peptide nucleic acids (PNAs) and metal-binding ligands by solid-phase synthesis.[88] The ligands were attached to the PNAs through linkers of different length for optimization of metal complex–DNA interaction. Synthesis of conjugates was accomplished through sequential coupling/deprotection steps with the required number of Fmoc-Gly-OH building blocks to the terminal amino group of the Rink resin-bound PNA. Amination with bis(2-picolyl)amine, PNA deprotection and cleavage gave conjugates with 2-picolylamine. Equimolar concentrations of bioavailable metal ions Ni^{2+}, Zn^{2+} and Cu^{2+} were used in the complexation step, and the affinities of the metal–bpa conjugates towards DNA (Figure 8) were shown to be strongly dependent upon the nature of the metal, in the order Ni^{2+}, $Zn^{2+} > Cu^{2+}$.

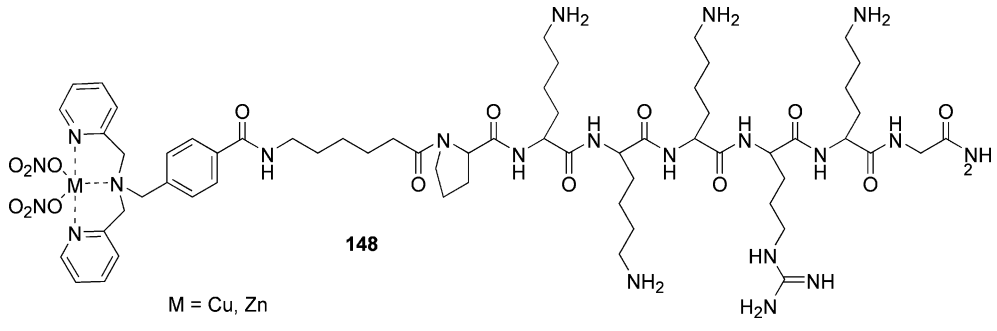
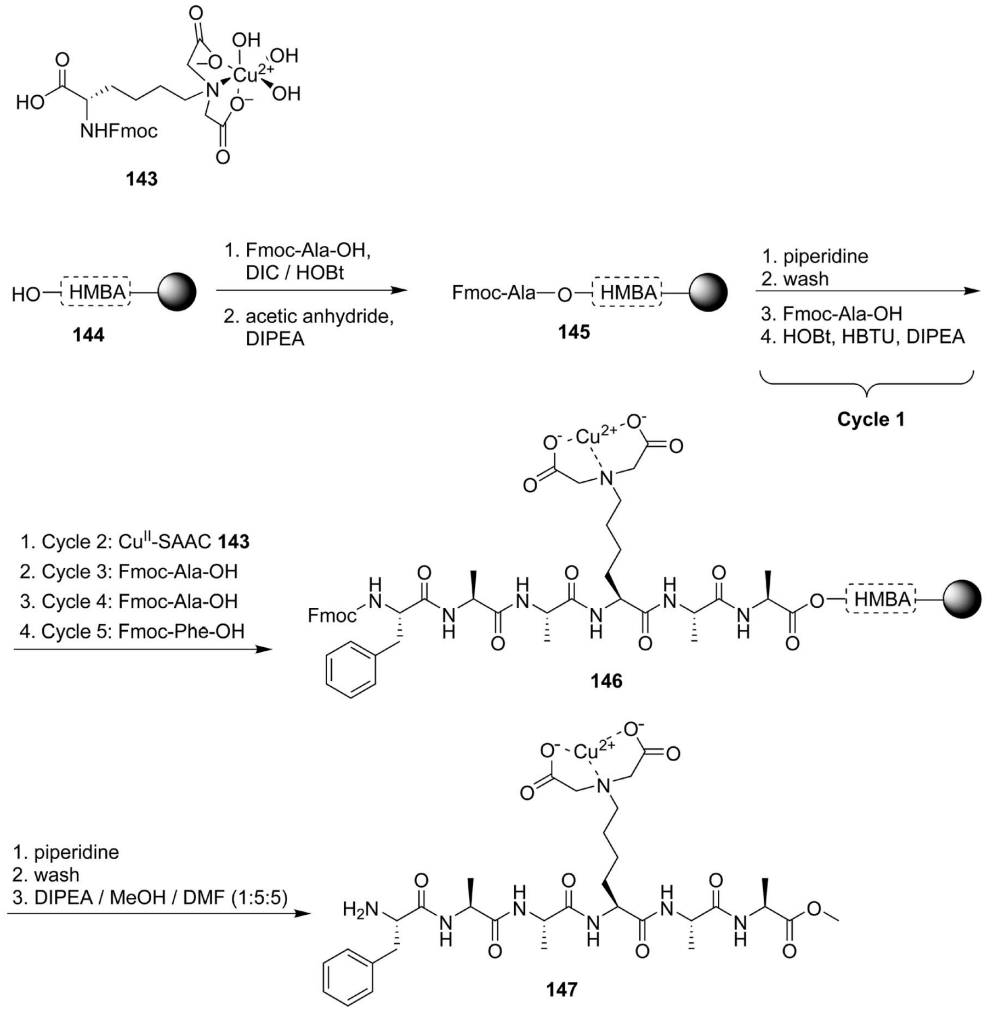


Figure 7. Structures of metal bioconjugates **148-M**. M = Cu^{II} or Zn^{II}.



Scheme 32. Incorporation of Cu^{II}-IDA SAAC into a peptide sequence.

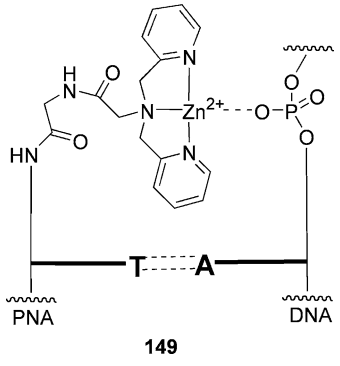


Figure 8. A proposed approach for metal-dependent binding of PNA probes to oligonucleotide targets.

König et al. reported solid-phase peptide synthesis protocols in which the positions and numbers of SAACs and their metal complexes may be varied.[89] Peptide–metal complex conjugates were obtained either by incorporation of the metal-coordinated SAAC followed by mild nucleophilic resin-cleavage, or by complexation in metal salt solution after cleavage from the resin.

A bpa-containing peptide (Scheme 33) and a dinuclear peptide receptor (Scheme 34) based on the bpa chelate were synthesized on Rink amide resin by solid-phase peptide synthesis. Fmoc-protected aliphatic amino acids and SAAC **150** (Figure 9) were coupled with HBTU, HOBt and DIPEA in NMP/DMF by a conventional fritted syringe technique. After cleavage from the resin, the peptide conjugates were treated with $Zn(NO_3)_2$ in an aqueous solution.

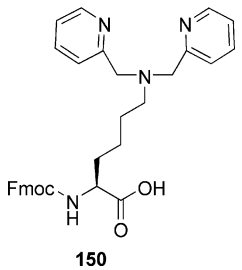
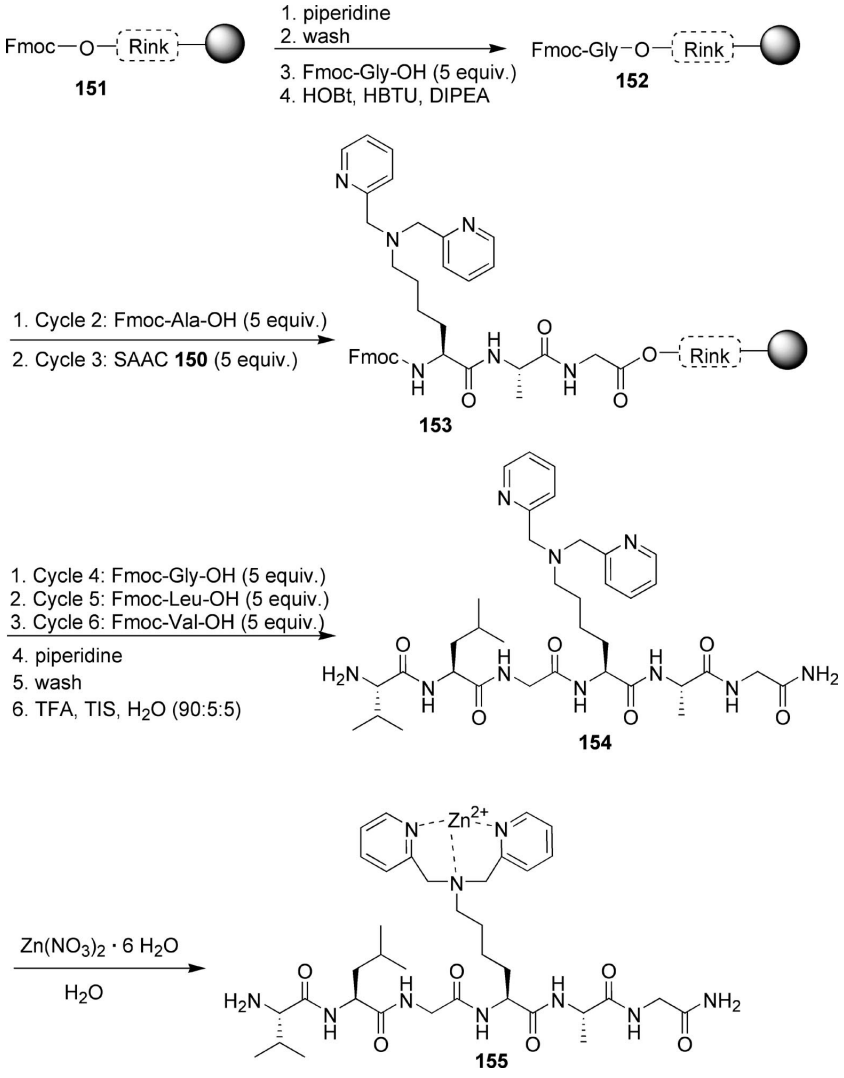


Figure 9. Bpa SAAC **150**.

To expand the versatile solid-phase approach, the already metal-coordinated bpa-derived Fmoc-amino acid **159** (Figure 10) was incorporated into the peptide chain.



Scheme 33. Solid-phase peptide synthesis of peptide conjugate **154** and subsequent metallation to provide peptide metal complex **155** in solution.

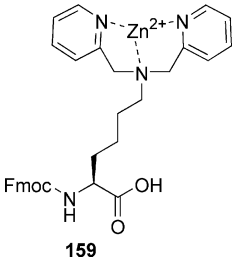


Figure 10. Zn^{II}-bpa SAAC **159**.

To avoid the loss of metal ions under acidic conditions, which are necessary to cleave from Rink amide resin, HMBA-AM was used as resin, as it allows nucleophilic cleavage of the peptide (Scheme 35).

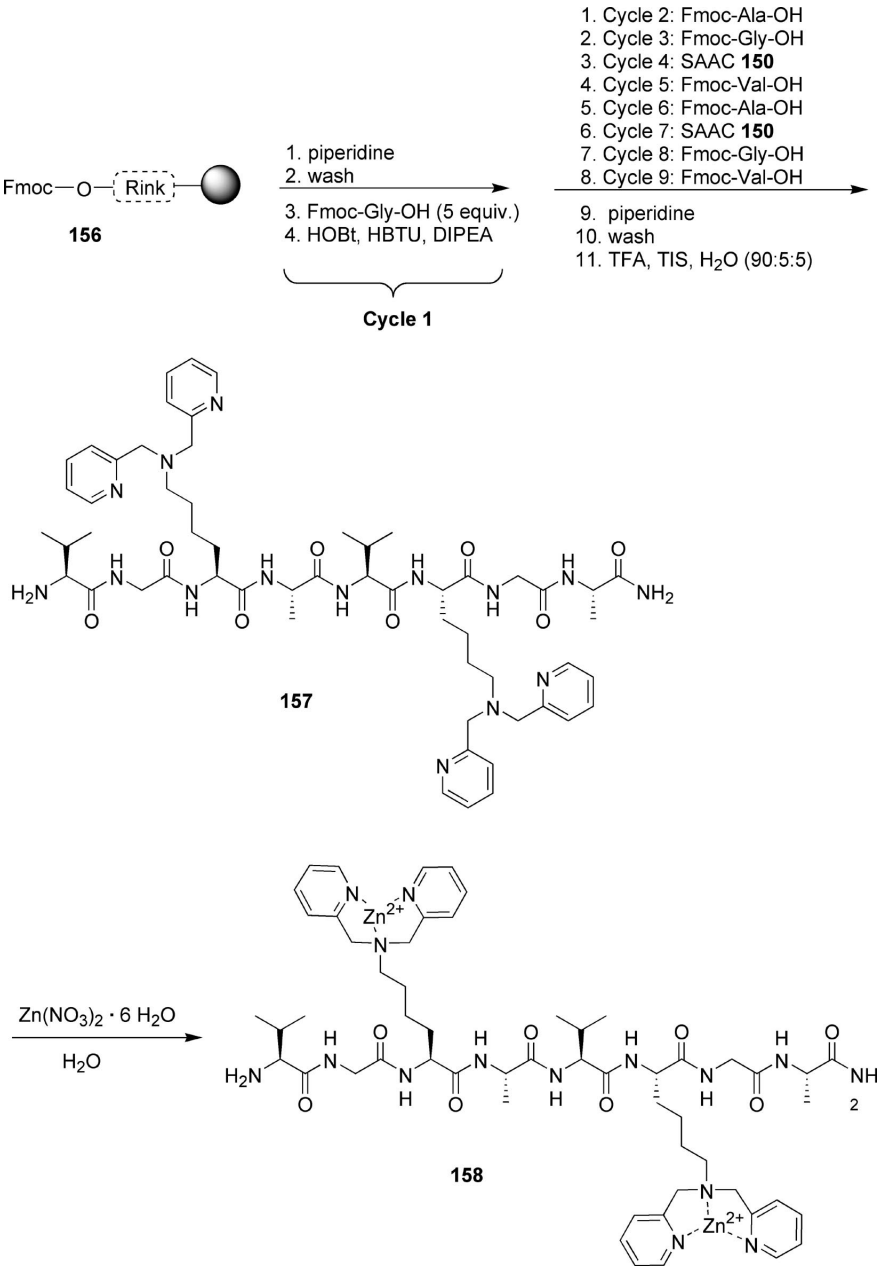
7.2 Bis-bpa Zinc Conjugate

Bis(Zn^{II} chloride)-SAAC **164** has also been successfully used in solid-phase peptide synthesis. Cleavage with a DIPEA/MeOH/DMF solution gave the metallated peptide bis-bpa zinc complex **167** (Scheme 36).

7.3 Bis(1,4,7,10-tetraazacyclododecane) Bis(cyclene) Zinc Conjugate

The solid-phase synthesis of metal-complex-containing peptides bearing cyclene moieties has been performed.[90] The amino acid complex **173** was prepared from the previously reported triazene-bis(cyclene)[91] **168** by treatment with α-amino Z-protected L-Lys–OBn (Scheme 37). Nucleophilic aromatic substitution gave compound **169**, and the benzyl protecting groups were simultaneously removed by hydrogenolysis with 10% palladium on charcoal as catalyst. The complexation of the Fmoc-protected cyclene ligand with Zn^{II} required careful control of the reaction conditions.

After Boc removal with HCl-saturated ether, the complexation of the hydrochloride salt was achieved with a Zn salt in a buffered solution (Hepes buffer, pH 8). Preliminary



Scheme 34. Solid-phase peptide synthesis of bis-bps ligand **157** and subsequent metallation to provide dinuclear peptide metal complex **158** in solution.

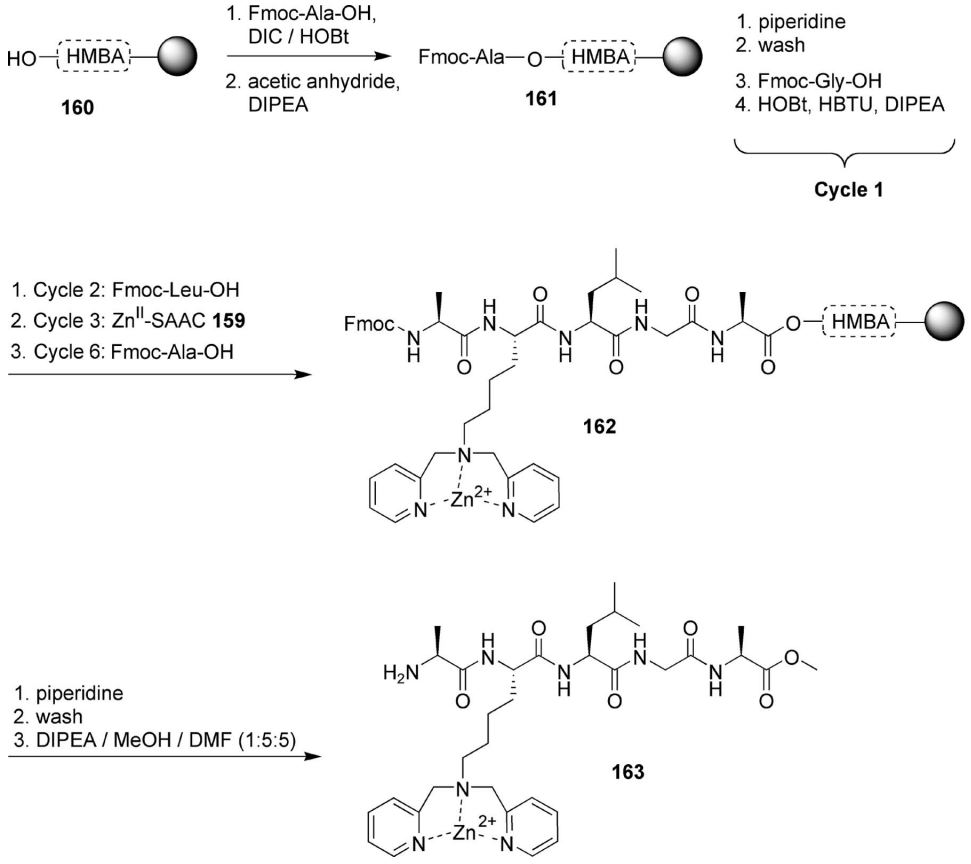
attempts to couple amino acid **171** to aliphatic amino acids using HBTU, TBTU and DIPEA as coupling reagents failed. The more efficient reagent HOAt[92] was used instead of HOBt together with the onium salt HATU. DIPEA was exchanged for collidine,[93] a more suitable base for the HOAt reagent. With HOAt/HATU/collidine, the coupling of **171** in two coupling cycles gave dipeptide **177** (Scheme 38).

A more extended peptide **180** was obtained on the Fmoc–Ala-loaded SASRIN resin **178** under the same coupling conditions (Scheme 39). In the following steps, the Boc groups were cleaved with HCl-saturated ether and the neutralized compound **181** was subsequently treated with $Zn(ClO_4)_2$ salt to provide the peptide complex **182**.

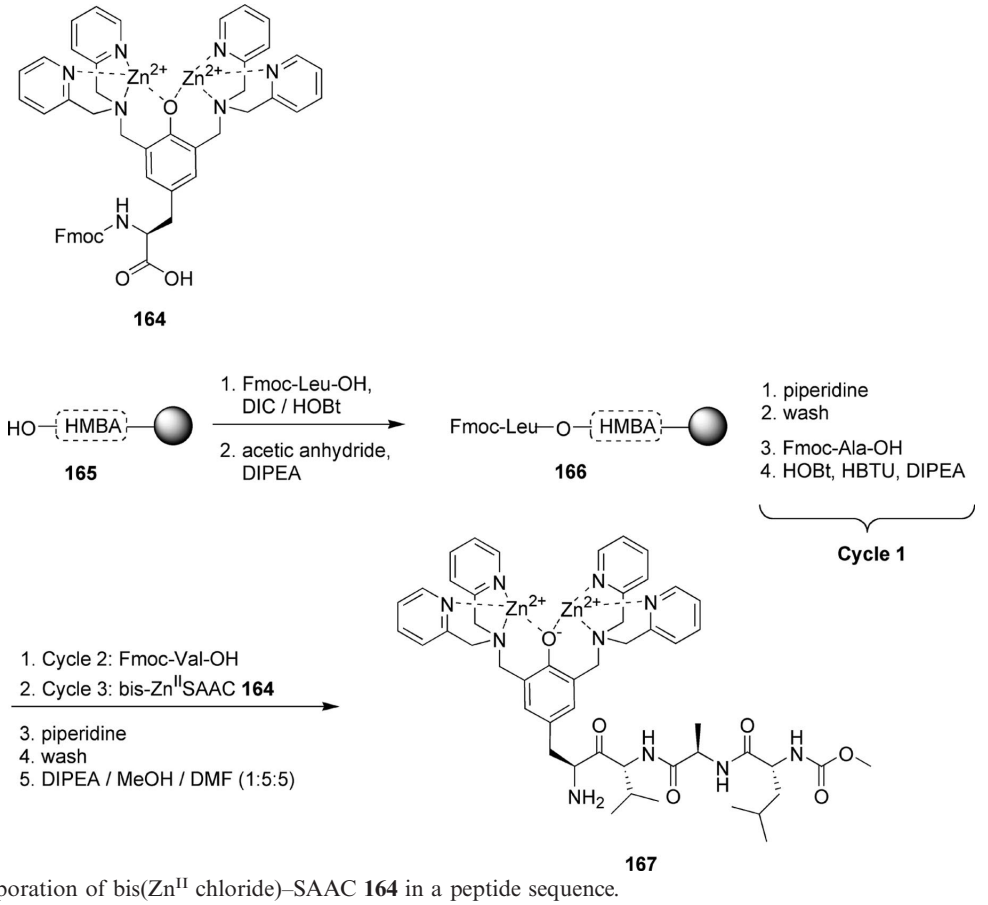
8. Samarium, Europium, Terbium and Gadolinium (Lanthanides) Metal Complex–Peptide Conjugates

8.1 *N*-(Isothiocyanatobenzyl)diethylenetriamine-*N*,*N*′,*N*′′,*N*′′′-tetrakis(acetic acid) Metal Conjugate

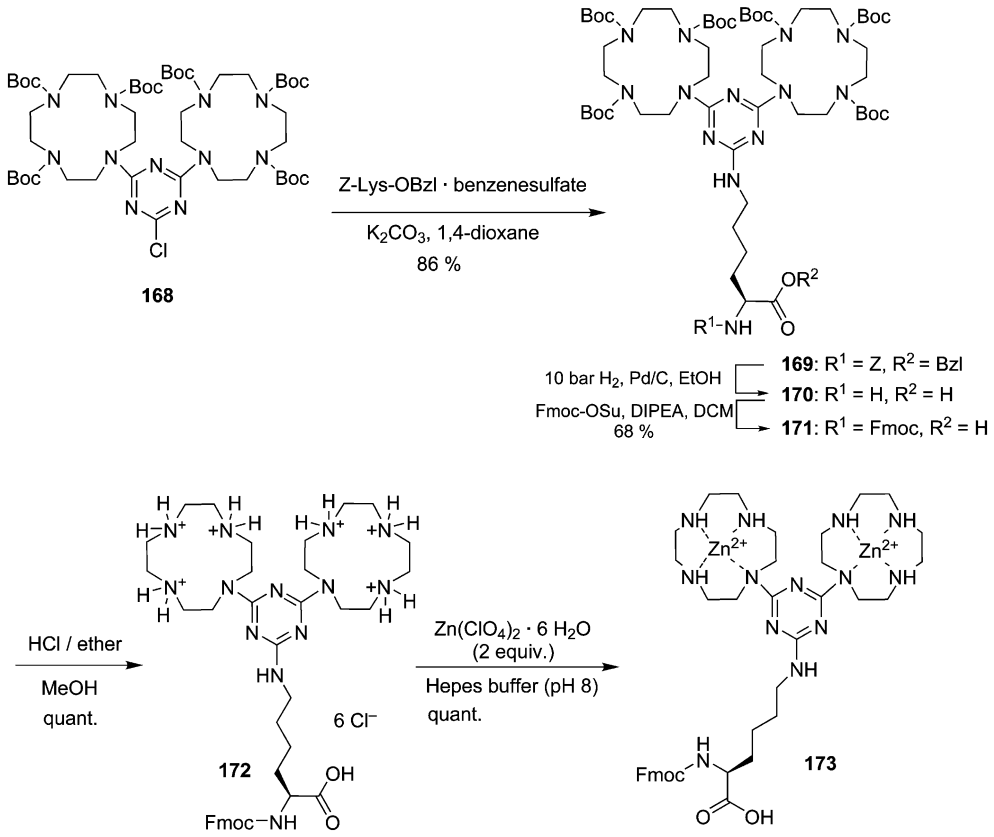
The chelates of certain lanthanides, such as Eu^{3+}, Tb^{3+}, Sm^{3+} and Dy^{3+}, have unique fluorescence properties (e.g., large Stokes shifts, sharp emission peaks and exceptionally long decay times).[94] These properties are exploited in time-resolved fluorimetry[95] (TRF). Oligopeptide conjugates were synthesized and used in a TRF-quenching assay (based on caspase-3, an enzyme that plays a key role in



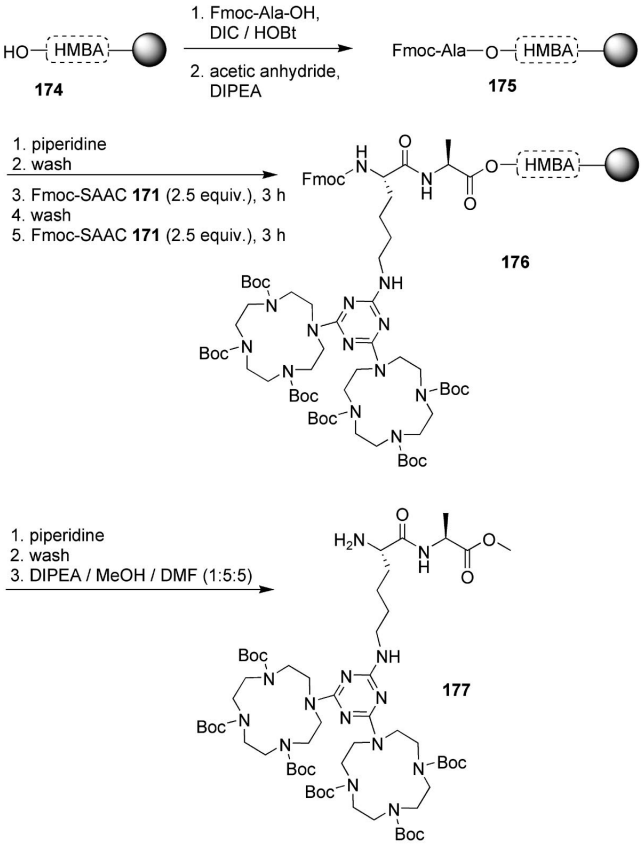
Scheme 35. Direct solid-phase peptide synthesis of metal–peptide conjugate **163** on HMBA-AM resin with metal-containing amino acid **159**.



Scheme 36. Incorporation of bis(Zn^{II} chloride)–SAAC **164** in a peptide sequence.



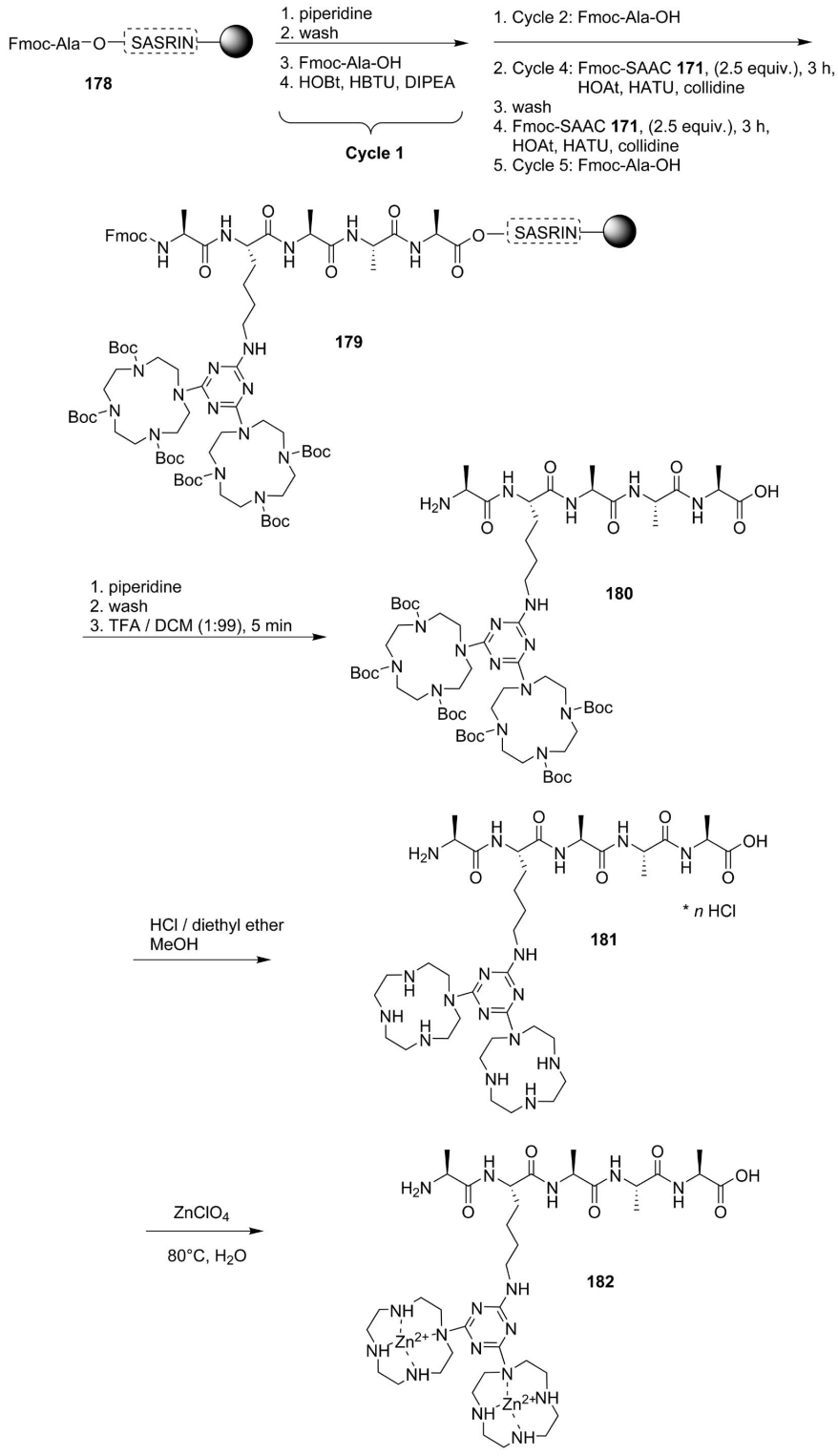
Scheme 37. Preparation of Fmoc-protected amino acid **171** and the bis(Zn^{II}-cyclene) SAAC **173**.



Scheme 38. Solid-phase synthesis of dipeptide conjugate **177**.

programmed cell death, or apoptosis) and in a receptor binding assay (based on motilin, a polypeptide hormone secreted by Mo cells of the small intestine that increases the migrating myoelectric complex component of gastrointestinal motility and stimulates the production of pepsin).[96] Hovinen et al. described the synthesis of oligopeptide building blocks that allow for the introduction of lanthanide(III) chelates into synthetic oligopeptides by standard automated solid-phase peptide synthesis. The applicability of building block **183**[97] for oligopeptide derivatization was demonstrated with peptide sequences of motilin, substance-P, neurokinin-A and caspase-3 synthesized by Fmoc solid-phase peptide synthesis (Scheme 40). After the building block **183** had been coupled to the amino terminus of the coding sequence with use of a prolonged reaction time, but otherwise standard HBTU/HOBt conditions, the oligopeptide was released from the resin. Treatment of the deblocked oligomer with europium(III) citrate converted the conjugate into the corresponding europium peptide chelate **185**.

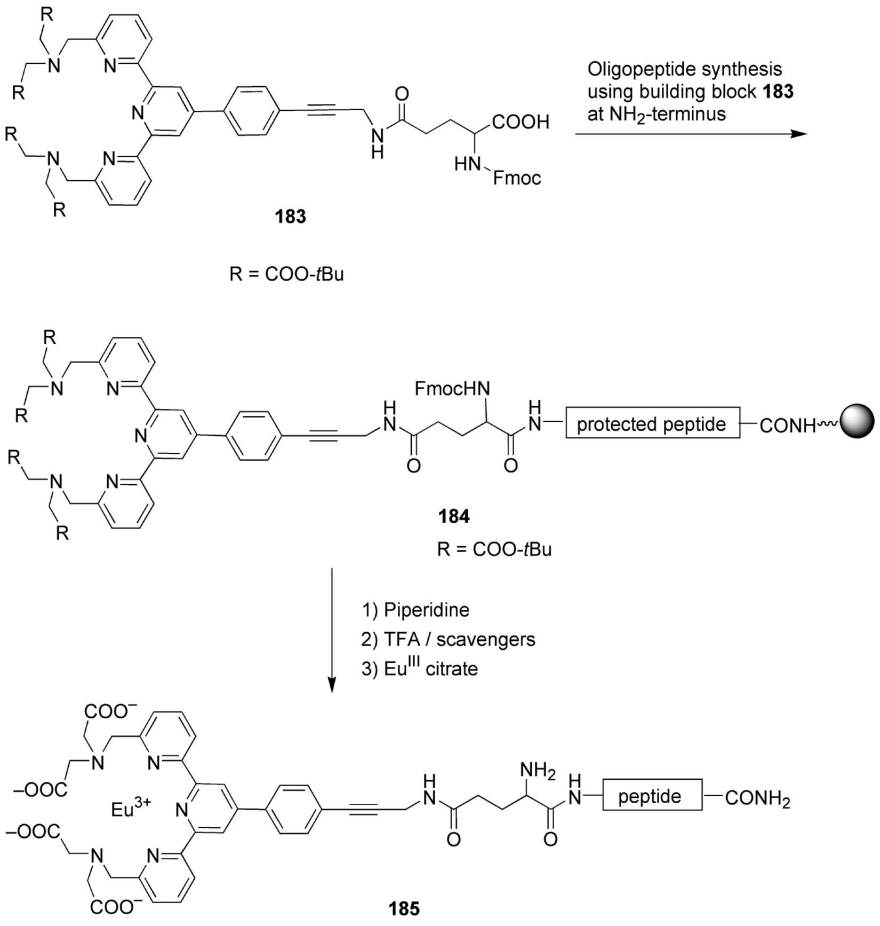
Karvinen et al. have paved the way to a multiparametric caspase assay by characterizing the fluorescence properties of a series of lanthanide (Ln^{3+}) chelates (Scheme 41) incorporated into peptides and testing their functionality in a caspase-3 assay.[98] Caspases are a group of cysteine proteases involved in apoptosis[99] and inflammatory reactions.[100] As the caspases and their substrates are a well characterized[101] and interesting group of enzymes as potential drug targets,[102] Karvinen et al. have chosen them as a model system for the development of a multiparametric



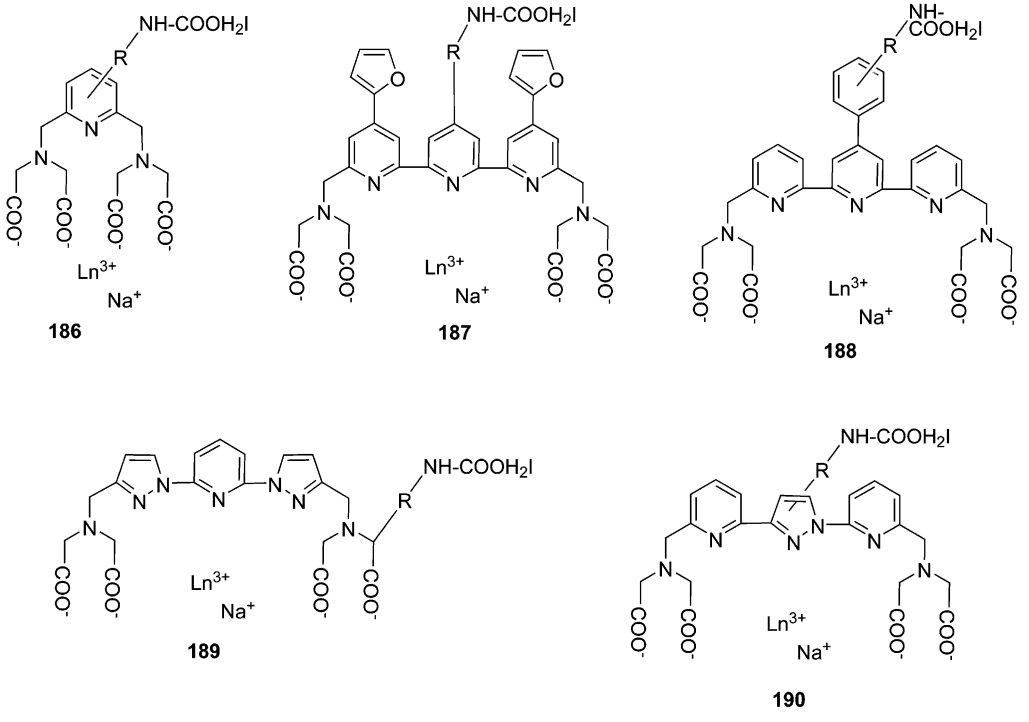
Scheme 39. Solid-phase synthesis of peptide **180** and peptide complex **182**.

homogeneous time-resolved fluorescence quenching assay (TR-FQA). The principle of the enzymatic assay is shown in Figure 11.

The homogeneous multiparametric assay was capable of measuring the activities of three different caspases from one well through the use of specific substrates labelled with europium, samarium and terbium chelates (Scheme 41). Although the quenching efficiencies were significantly lower than those observed earlier,[103] some of the chelates tested during this work proved to be extremely functional in TR-FQ assays, and the technique might be adaptable to DNA assays.



Scheme 40. Introduction of a luminescent europium(III) chelate by solid-phase oligopeptide synthesis with N-terminal **183** and subsequent complex formation in solution.



Scheme 41. Simplified structures of the tested lanthanide chelates.

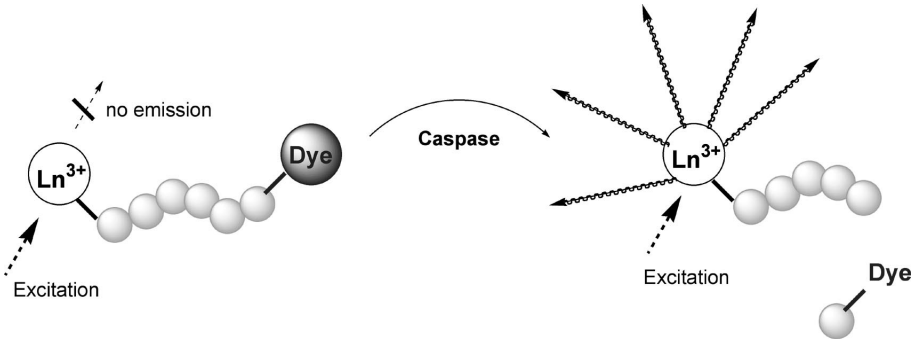
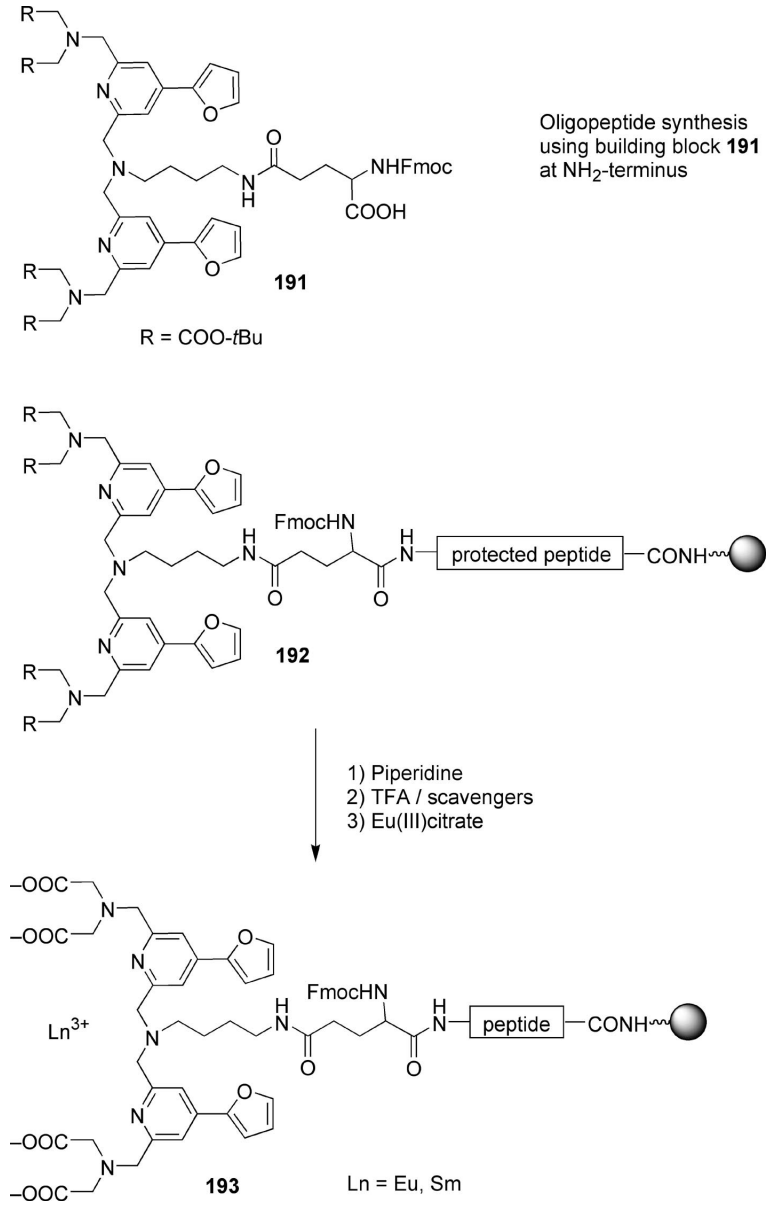


Figure 11. Principle of the TR-FQA caspase assay. The assay is based on recovery of the Ln^{3+} fluorescence after removal of the quencher by protease activity. QSY-7 ε-amino-labelled lysine, inserted into a caspace recognition sequence, was used as quencher for all lanthanide chelates.

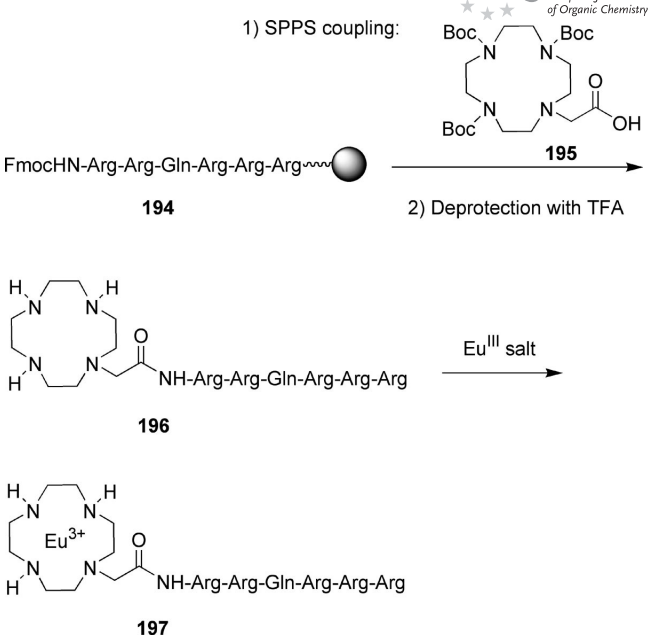


Scheme 42. Introduction of luminescent lanthanide(III) chelates into oligopeptides using building block **191**.

Hovinen et al. modified the synthesis of the building block **187**, allowing the introduction of photoluminescent europium(III) and samarium(III) chelates into synthetic oligopeptides on solid-phase by Fmoc chemistry. Oligopeptide synthesis and introduction of the Fmoc-protected building block **191** into the growing peptide chain was performed as reported earlier.[104] Upon completion of the oligopeptide synthesis, the conjugates were converted into the corresponding lanthanide(III) chelates by treatment with the appropriate lanthanide(III) salts (Scheme 42).

8.2 1,4,7,10-Tetraazacyclododecane (Cyclene) Europium Conjugate

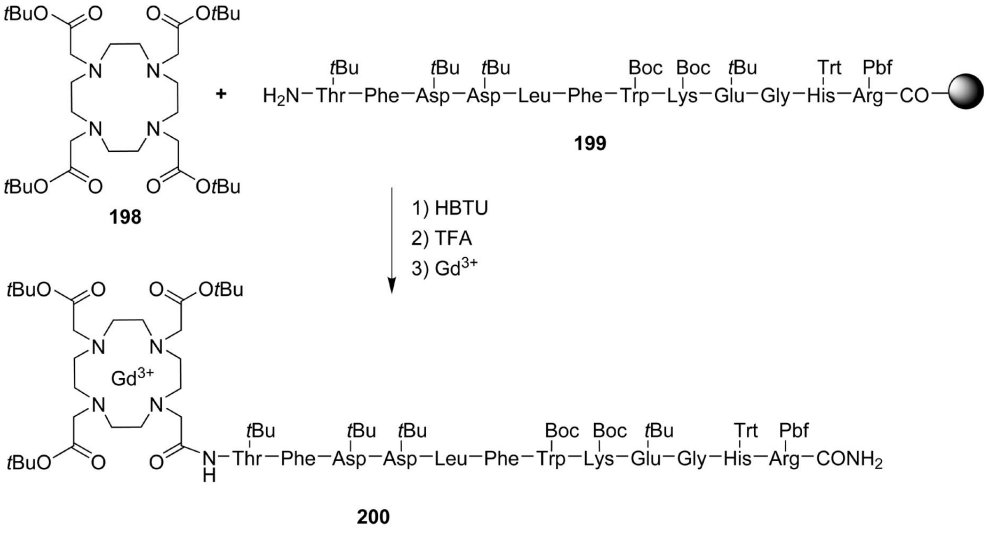
The metal-coordinating ligand cyclene was attached to the arginine-rich region of the TAT protein (a transactivator of HIV-1 infection, responsible for the replication and expression of HIV-1), and the lanthanide complexes of the ligand–peptide conjugate were investigated in hydrolysis-cleavage experiments.[105] TAR-RNA of HIV-1 was chosen as the target for the hydrolysis studies, as it is recognized by the HIV-1 regulatory TAT.[106] The peptide–cyclene conjugate nonamer **196** with the attached cyclene moiety was synthesized by standard solid-phase peptide synthesis. In the last coupling step, the Boc-protected cycleneacetic acid **195**[107] was coupled to the N terminus of the nonapeptide **194**. After subsequent cleavage from the resin under standard TFA conditions, the peptide **196** was incubated with the Eu^{III} salt (Scheme 43). Surprisingly, the nonamer–cyclene conjugate **196**, without Eu^{III}, gave selective and efficient cleavage at neutral pH and room temperature, and the authors report that those cleavage reactions are more efficient in the absence of Eu^{III} than in its presence.



Scheme 43. Synthesis of peptide–cyclene conjugates by solid-phase synthesis and europium complex formation in solution.

8.3 1,4,7,10-Tetraazacyclododecane-*N*,*N*′,*N*′′,*N*′′′-tetraacetic Acid (DOTA) Gadolinium Conjugate

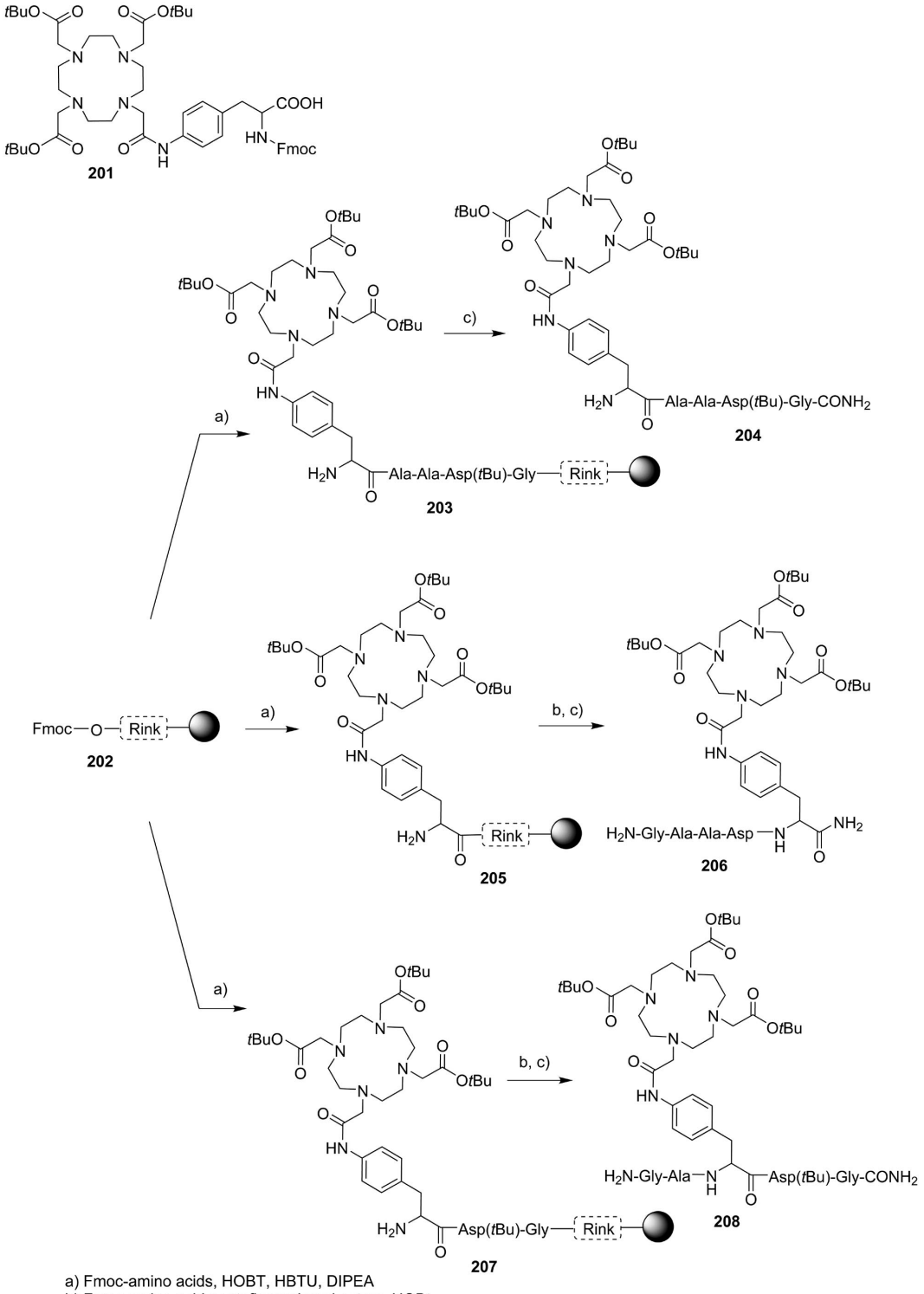
Several types of ligands, including DOTA, DTPA, NOTA and TETA,[108] have been attached to peptides. DOTA is of particular interest, since this macrocyclic ligand forms complexes with exceptionally high binding affinities and kinetic stabilities with a variety of metal ions.[109] Sherry et al. prepared Gd^{3+}-G80BP (*B*inding *P*eptide) by solid-phase peptide synthesis (Scheme 44) and demon-



Scheme 44. Solid-phase peptide synthesis of Gd^{3+}-G80BP **200**.

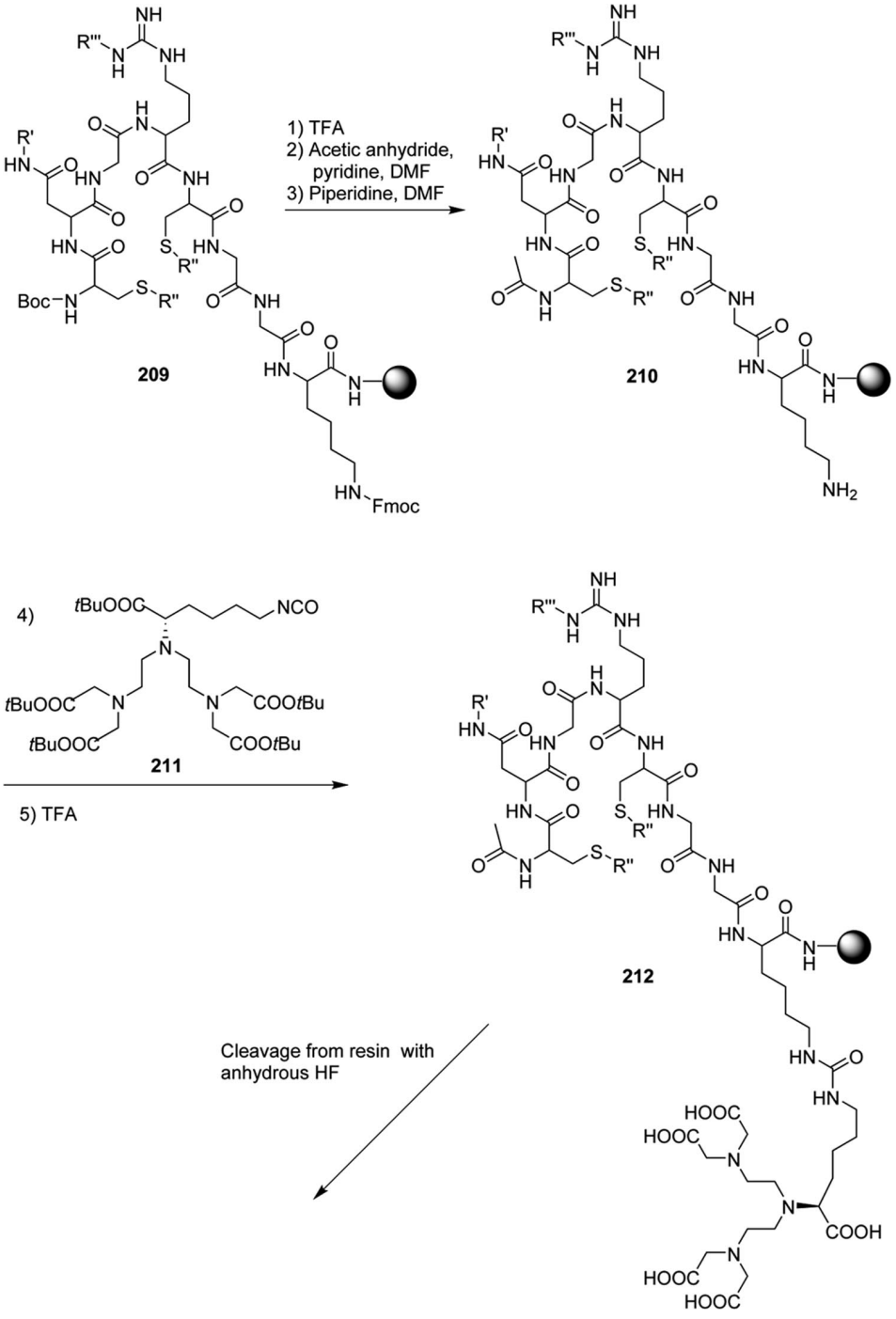
strated that magnetic resonance imaging (MRI) can detect the binding event of a Gd^{3+}-DOTA-labelled peptide (Gd^{3+}-G80BP) to its target protein Gal-80[110] (Gal-80 is a protein involved in regulation of galactose metabolism).[111]

In a later work, Sherry et al. modified the Gal-80 binding peptide TFDDLFWKEGHR by introducing a DOTA-chelating group at three different residues (Scheme 45).[112] Conjugation of DOTA to the N terminus of the resin-bound peptide was accomplished with DOTA-tris(*t*Bu) ester by standard Fmoc solid-phase peptide synthesis. Attempts to add subsequent amino acids beyond DOTA, even with either HATU or TFFH as coupling agents (these

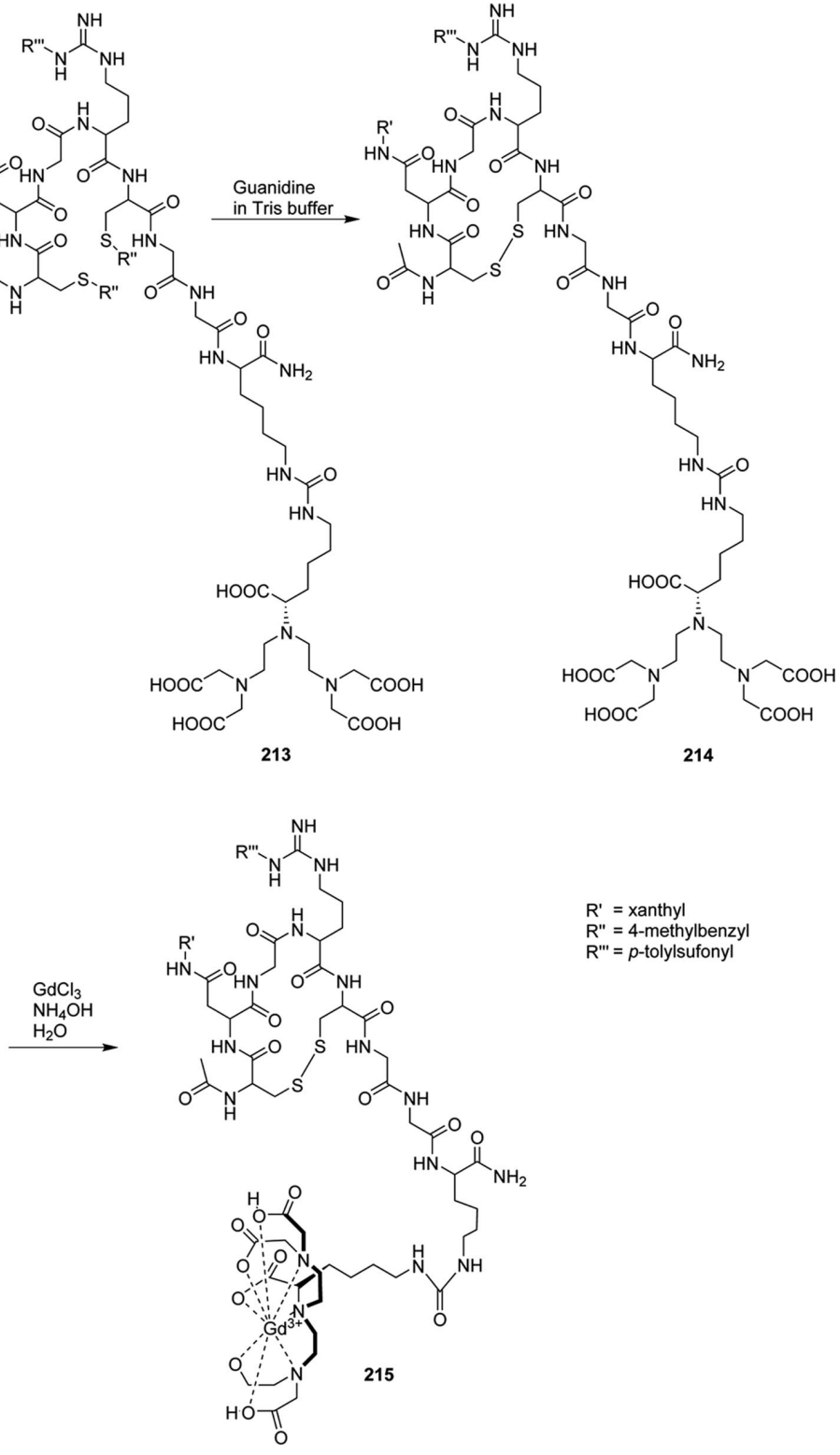


Scheme 45. Solid-phase peptide synthesis of three pentapeptides with variable DOTA positions.

coupling agents are reported to have superior coupling capabilities to HBTU), were not successful. A report by Lewis et al. showed that Fmoc–DOTA-lysine can be introduced into a peptide sequence by using a XAL-PEG-PS resin.[113] XAL-PEG-PS solid supports are prepared by grafting soluble polar PEG chains onto microporous polystyrene-co-divinylbenzene and have been shown to be superior to conventional resins for the synthesis of hydrophobic peptides.[114] However, Sherry et al. used the more reactive activated amino acid Fmoc-pentafluorophenyl ester in their work to couple the remaining amino acids successfully to the *endo*-DOTA peptides. After addition of Gd^{3+} to each peptide–DOTA conjugate, competitive binding experiments showed that the *exo*-peptide labelled with Gd^{3+}–DOTA at the N terminus had a reasonable affinity for Gal-80, while those peptides labelled with Gd^{3+}–DOTA at *endo* positions within the peptide sequence had no detectable binding affinity for Gal-80.



Scheme 46. Synthesis of cNGR–Gd^{III}DTPA complex **215** by solid-phase synthesis of the ligand and subsequent gadolinium complex formation in solution.



Scheme 46. *Continued*

8.4 Diethylenetriaminepentaacetic Acid (DTPA) Gadolinium Conjugate

Gadolinium complexes of DTPA are widely employed as contrast agents in medicinal imaging.[115] The effectiveness of Gd^{III}DTPA-based contrast agents can be improved by incorporating target-specific oligopeptides to induce accumulation of MRI probes in the tissue of interest.[116] A cyclic peptide containing the Cys–Asn–Gly–Arg–Cys

(CNGRC) sequence (cNGR) was identified as a targeting unit for the aminopeptidase CD13, which is overexpressed on endothelial cells during angiogenesis.[117] Hackeng and Meijer et al.[118] designed a cNGR–Gd^{III}DTPA complex **215** composed of the cNGR targeting domain and a Gd^{III}-DTPA complex for imaging of angiogenesis (Scheme 46). The gadolinium chelate was introduced at the ε-amine of the lysine side chain of the peptide **210**. For this purpose, an isocyanate-functionalized lysine-based DTPA pentaester **211** was coupled to the resin-bound peptide **210**. Solid-phase peptide synthesis and HBTU activation for Boc chemistry on a MBHA resin[119] was applied to synthesize side chain-protected BocCNGRCGGK(Fmoc)–MBHA **209** containing the target-specific NGR sequence. The conversion of the amine-functionalized DTPA[120] into the corresponding isocyanate **211** was achieved with di-*tert*-butyl tricarbonate, a versatile reagent for the quantitative conversion of primary amines into isocyanates under mild reaction conditions.[121] The DTPA-functionalized oligopeptide **212** was obtained by treatment of the lysine side chain ε-amine group with an excess of isocyanate-functionalized DTPA analogue **211**. After quantitative formation of the disulfide bridge by oxidation, the gadolinium complex **215** was prepared by addition of gadolinium chloride to a solution of peptide conjugate **214** in water.

Conclusions

The discussed examples of metal complex–peptide conjugates synthesized on solid phase show that a wide variety of different structures are already accessible by the developed methods. Procedures in many cases differ from standard SPPS protocols in order to address the special requirements of ligand and complex stability. Both general strategies – the synthesis of peptide–ligand conjugates and complexation with excess metal ions on solid support or the incorporation of an amino acid complex in the growing immobilized peptide chain – have their specific advantages and limitations. While complexation of peptide–ligand conjugates is synthetically easier in many cases, it does not allow the specific preparation of bi- or oligonuclear complexes with different metal ions. This is in principle possible with artificial metal complex amino acids, if they are kinetically and thermodynamically sufficiently stable and introduced in the right order. However, all reaction conditions of the subsequent peptide synthesis, including deprotection and cleavage steps, must be compatible with the stability of the complex. With further advancements of the methodology the preparation of peptide–metal complex conjugates by automated solid-phase synthesis will surely become more common. However, the special conditions necessary for the formation of various metal complex types and their individual stability profiles will always call for specific protocols.

Abbreviations

Bhoc – *N*-Benzhydryloxycarbonyl

Boc – *tert*-Butoxycarbonyl

BOP – (Benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate

ByPOP – (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate

DCC – *N*,*N*′-Dicyclohexylcarbodiimide

Dde – 1-(4,4-Dimethyl-2,6-dioxocyclohexyldiene)ethyl

Dhbt-OH – 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine

DIC – *N*,*N*′-Diisopropylcarbodiimide

DIPEA – *N*,*N*-Diisopropylethylamine

DMAP – 4-(Dimethylamino)pyridine

DMF – Dimethylformamide

DNA – Deoxyribonucleic acid

DSC – *N*,*N*-Disuccinimidyl carbonate

EDTA – Ethylenediaminetetraacetic acid

FITC – Fluorescein isothiocyanate

HATU – 2-(1*H*-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium

HBTU – 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HMBA – Hydroxymethylbenzoic acid

HMBA-AM – 4-Hydroxymethylbenzoic acid AM

HOAt – 1-Hydroxy-7-azabenzotriazole

HOBt – 1-Hydroxybenzotriazole

MBHA – 4-Methylbenzhydrylamine

MeOH – Methanol

MSNT – 1-(Mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole

Mtt – 4-Methyltrityl

NBD – 4-Halo-7-nitrobenzo-2-oxa-1,3-diazole

NEM – *N*-Ethylmorpholine

NHS – *N*-Hydroxysuccinimide

NMP – *N*-Methylpyrrolidone

OPfp – Pentafluorophenyl ester

PAL linker – 5-(Aminomethyl-3,5-dimethoxyphenoxy)pentanoic acid

PAM resin – [*p*-(Hydroxymethyl)phenyl]acetamidomethyl polystyrene

Pbf – 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl

PEG – Polyethylene glycol

PNA – Peptide nucleic acid

Pu – Purine

Pym – Pyrimidine

SAAC – Single amino acid chelate

SASRIN – Super acid-sensitive resin

SPPS – Solid-phase peptide synthesis

TBTU – 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate

TFA – Trifluoroacetic acid

TFFH – *N*,*N*,*N*′,*N*′-Tetramethylfluoroformamidinium hexafluorophosphate

TIS – Triisopropylsilane

TMS – Trimethylsilane

TSTU – *N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(succinimidyl)uronium tetrafluoroborate

XAL – Xanthenyloxyalkylamide

Acknowledgments

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