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Solid-supported synthesis of oligomeric bioconjugates

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Contents

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Abbreviations: AcHmb, 2-acetyloxy-4-methoxybenzyl; Acm, acetamidomethyl; Alloc, allyloxycarbonyl; AMBA, 4-(acyloxymethyl)benzylidene acetal; BAL, backbone amide linker; BHA, benzhydrylamine; Bhoc, benzhydrylcarbonyl; BOP, (benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate; Bpy, 2:2′-Bipyridine; Bz, benzoyl; BSA, bis(trimethylsilyl)acetamide; CPG, controlled pore glass; DABCYL, 4-(4′-dimethylaminophenylazo)benzoic acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, dicyclohexylcarbodiimide; Dde, 1-(4,4-dimethyl-2,6-dioxacyclohex-1-ylidene)ethyl; Dhbt-OH, 3,4-dihydro-1-hydroxy-4-oxo-1,2,3-benzotriazole; DIC, diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMAP, 4-dimethylaminopyridine; DMSO, dimethyldulfoxide; DMTr, 4,4′-dimethoxytrityl; DOTA, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; en, 1,2-ethylenediamine; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium haxafluorophosphate; HMBA, 4-hydroxymethylbenzoic acid; HMPA, 4-hydroxymethylphenoxyacetic acid; HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)butanoic acid; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HYCRAM, hydroxycrotonoylaminomethyl; IAEDANS, 5-(2′-iodoacetamidoethyl)aminonaphthalene sulfonic acid; LCAA, long chain aminoalkyl; MBHA, methylbenzhydrylamine; MMTr, 4-methoxytrityl; Msc, 2-(methylsulfonyl)ethoxycarbonyl; Mpt-MA, dimethylphosphinothioic mixed anhydride; NHS, *N*-hydroxysuccinimide; NMP, 1-methyl-2-pyrrolidinone; Npe, 4-nitrophenylethyl; ODN, oligodeoxyribonucleotide; ORN, oligoribonucleotide; PAL, 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid; PAM, phenylacetamidomethyl; PEG, polyethyleneglycol; PEGA, polyethyleneglycol-poly(*N*,*N*-dimethylacrylamide); Pfp, pentafluorophenyl; PfPyU, *N*,*N*-*N*′,*N*′-bis(tetramethylene)-*O*-pentafluorophenyluronium hexafluorophosphate; phen′, 5-amidoglutaryl-1,10-phenanthroline; Phi, 9,10-phenantrene quinone diimine; PS, polystyrene; Px, 9-phenylxanthen-9-yl; PyBOP, benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate; Rink, acid, 4-(α-hydroxy-2,4-dimethoxybenzyl)phenoxyacetic acid; Rink, amide, 4-(α-amino-2,4-dimethoxybenzyl)phenoxyacetic acid; TBDMS, *tert*-butyldimethylsilyl; TBTU, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; Teoc, 2,2,2-trichloroethoxycarbonyl; Tf, trifluoromethanesulfonyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Tr, trityl; TSTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate.

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1. Introduction

Oligomeric bioconjugates, i.e. oligonucleotides, peptides or oligosaccharides bearing unnatural organic structures or constituents of other biopolymers, have during the past two decades found an increasing number of applications as research tools for molecular and cell biology. Conjugate groups are aimed at providing the oligomeric biomolecules with novel properties, such as fluorescence emission, catalytic activity, altered hydrophobicity or bioaffinity, resistance towards biodegradation or ability to carry metal ions, to mention only a few. While the early syntheses of oligomeric bioconjugates have mainly been carried out in solution, an increasing number of such conjugates are nowadays prepared either entirely on a solid support or the conjugate group is introduced upon cleavage of the oligomer from the support. Since the oligomer itself is in any case assembled on a support, it is only natural to also perform the conjugation on the same support. The major advantage of a solid-supported synthesis compared to conjugation in solution is a less laborious purification. Most of the side products may be removed by simple washing when the conjugate still is anchored to the support and, after release into solution, only one chromatographic purification is usually needed. When the conjugation is carried out in solution, the biopolymer must be purified both before and after conjugation. This advantage becomes obvious on preparing conjugates that consist of segments of two different types of biopolymers, such as peptide conjugates of oligonucleotides. When two different protocols are applied consecutively on a single support, no purification of the prefabricated peptide and oligonucleotide is needed. Another attractive feature is the possibility for exploitation of a fully automatic machine-assisted synthesis, which allows the convenient preparation of conjugate libraries by parallel synthesis and, hence, the development of various microarray techniques.

Among biopolymers, only oligonucleotides and peptides are routinely synthesised on a solid support, while such methods for the preparation of oligosaccharides are still at an early phase of development. Accordingly, the present paper is limited to a review of the methods described for the solid-supported synthesis of organic conjugates of oligonucleotides and peptides, peptide conjugates of oligonucleotides, and glycoconjugates of peptides.

2. Oligonucleotide conjugates

2.1. General

Conjugate groups are attached to oligonucleotides for many different purposes. Reporter groups, such as fluorescent dyes or spin labels, allow sensitive detection of oligonucleotides. Chemically reactive groups result in sequence-selective cross-linking or cleavage of a base moiety or phosphodiester linkage. Intercalators and groove-binding agents stabilise double helical structures, hydrophobic groups increase cellular uptake and bioaffinity groups, mainly biotin, allow non-covalent immobilisation to a streptavidin-coated surface. Accordingly, convenient synthetic methods for the preparation of simple organic conjugates of oligonucleotides are of increasing importance.

Two strategies are generally used for the synthesis of oligonucleotides on a solid support, viz. the phosphoramidite and the H-phosphonate strategy (Fig. 1). With both approaches, the chain is elongated by coupling the entering monomeric building block, viz. a 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) or 3′-(*H*-phosphonate) of an appropriately protected nucleoside, to the 5′-hydroxy group of the growing oligonucleotide chain anchored to an amino-functionalised support via a 3′-succinate linker. The most commonly used supports are aminoalkylated glass, such as LCAA-CPG and 3-aminopropyl-CPG, or aminoalkylated polystyrene. The 5′-hydroxy function of the building blocks is usually protected with an acid-labile group and the base moiety with a base-labile acyl group. The most commonly used activators for the phosphoramidite and H-phosphonate coupling are tetrazole and pivaloyl chloride, respectively. The most striking difference between the two strategies is that, on applying the phosphoramidite strategy, the initial product, the phosphite ester, is oxidised to a phosphate ester immediately after each coupling, while the H-phosphonate diester linkages are oxidised to phosphate esters in a single step after completion of the chain assembly. The chain is cleaved from the support and the base and phosphate moieties are deprotected by ammonolysis. These two protocols are used to obtain both oligodeoxyribonucleotides (ODNs)[1] and oligoribonucleotides (ORNs).[2] On preparing ORNs, the additional functionality, viz. the 2′-hydroxy group, is protected as a silyl ether that withstands both acidic and basic conditions and is removed by fluoride ion after the ammonolysis.

2.2. Conjugation to the 5′-terminus

Since the oligonucleotide chain is normally assembled in the 3′→5′ direction, attachment of a conjugate group to the 5′-hydroxy group is quite straightforward. Introduction of the conjugate group as a prefabricated phosphoramidite or H-phosphonate reagent by an additional coupling cycle at the end of the chain assembly has received increasing popularity during the last few years. Fluorescent dyes,[3] metal chelates,[4,5] photochemical crosslinking agents,[6] aromatic redox probes,[7] bile acids,[8] minor groove-binding agents,[9] intercalators,[10] dienes[11] and biotin[12] have all been tethered in this manner as phosphoramidites, and metal chelates,[13] lipids,[14,15] porphyrins[16] and sapphyrin[17] as H-phosphonates, to the 5′-terminus of ODNs. While this approach usually ensures efficient conjugation, it suffers

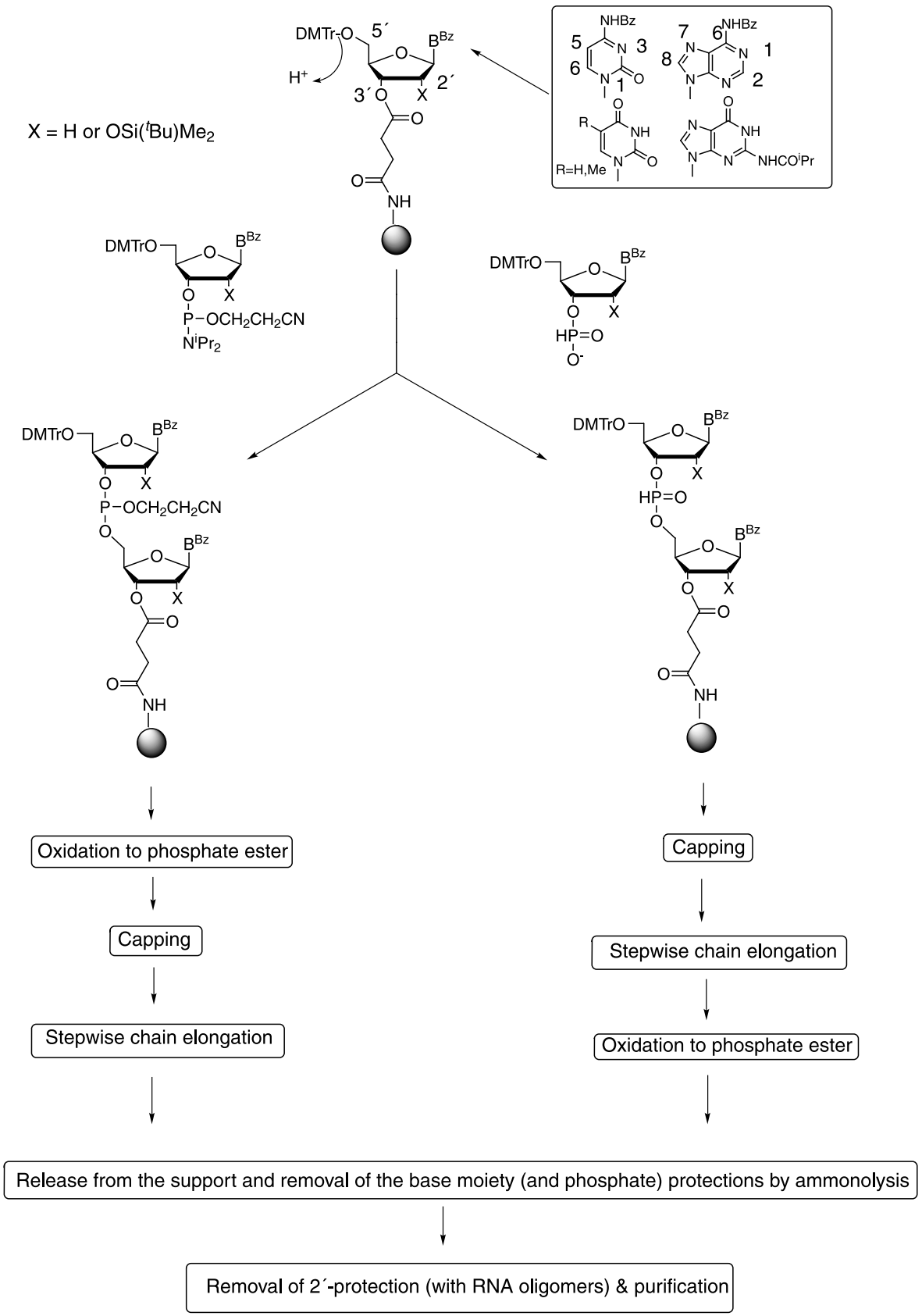

Figure 1. The principle of solid-supported oligonucleotide synthesis by the phosphoramidite and the H-phosphonate strategy.

from the shortcoming that each molecule required for conjugation must be separately converted to a phosphitylated building block.

A more versatile strategy for the solid-supported 5′-conjugation involves the insertion of a masked nucleophilic or electrophilic group into the 5′-end of the ODN chain by normal phosphoramidite or H-phosphonate coupling. The inserted functional group is deprotected in such a manner that the ODN chain remains anchored to the support and the actual conjugation is carried out on the support. Among nucleophilic groups, the primary amino function has been most extensively used for this purpose. An amino tail is easily introduced as a phosphoramidite or an H-phosphonate reagent. Since the amino group protection ought to be removed without the loss of the base moiety protection or cleavage from the support, building blocks masked with an acid-labile protecting group are applicable. These include, for example, *N*-DMTr-6-aminohexyl, *N*-MMTr-6-aminohexyl and *N*-Px-6-aminohexyl phosphoramidites and H-phosphonates.[18] Alternatively, the 5′-hydroxy group of the support-bound ODN may either be phosphonylated with *H*-pyrophosphonate and condensed with an aminoalcohol or phosphonylated with diphenyl *H*-phosphonate and transesterified with an aminoalcohol.[19] The amino tail may also be attached without a phosphoester bridge by activating the 5′-hydroxy group on-support with carbonyldiimidazole and reacting it subsequently with an α,ω-alkanediamine.[20] The alkyl carbamate linkage obtained withstands the ammonolytic base moiety deprotection and cleavage from the support.

Several examples of conjugation to the 5′-terminal amino group of a solid-supported ODN have been published. Carboxylic acids have been coupled with the aid of conventional activators of peptide synthesis. Accordingly, rhodamine has been conjugated by HBTU/EtN^iPr_2 activation in a mixture of DMF and MeCN,[21] various diimidazole carboxylic acids by PyBOP/HOBt/EtN^iPr_2 in DMF,[22] *meso*-tetracarboxyporphine by EDC/DMAP in pyridine[23] and the Δ-{Rh(phi)$_2$[4-Me-4′-(4-carboxybutyl)-bpy]} complex by TSTU/EtN^iPr_2 activation in a mixture of MeOH, CH_2Cl_2 and MeCN.[24] In all of these examples, the conjugate group is sufficiently stable to withstand the ammonolytic cleavage and deprotection. Conjugates of base-labile compounds, such as folic, retinoic and arachidonic acid, have been similarly obtained by EDC/DMAP/Et_3N activation in DMF using more readily removable *N*-pent-4-enoyl protecting groups at the base moieties.[25] Alternatively, carboxylic acids may be coupled to amino tails as active esters. Minor groove binders have been reacted as a Pfp-ester in DMSO in the presence of Et_3N,[26] and carboxyfluorescein as an NHS-ester, converted in situ to a highly reactive 4-dimethylaminopyridinium derivative.[27] Isothiocyanates of fluorescent dyes have been efficiently attached to 5′-terminal aliphatic amino groups with the aid of DBU.[19]

An aminooxy group is known to be a more powerful nucleophile than an amino group, in particular as an attack on a carbonyl carbon is concerned. That is why it is somewhat surprising that aminooxy-functionalised ODNs have not been extensively exploited in solid-supported conjugation. A 5′-terminal aminooxy group has been introduced as an 11-phthalimidooxy-3,6,9-trioxaundecyl[28] or *N*-trityl-6-aminooxyhexyl[29] phosphoramidite. The phthaloyl protection has been removed with hydrazinium acetate in pyridine and the trityl protection with acid, i.e. under conditions that do not cleave the normal succinyl linker. Aldehydes readily react with the exposed aminooxy group, yielding fully protected oxime conjugates that may be cleaved from the support and deprotected by normal ammonolysis.[28] 5′-Hydrazide conjugates have been obtained by an attack of hydrazine on an ethoxycarbonyl tail introduced as a phosphoramidite reagent.[30]

The mercapto group is another nucleophilic function extensively utilised in conjugation. Several phosphoramidite[31–35] and H-phosphonate[18] reagents for the introduction of a 5′-mercaptoalkyl tether have been described. The resulting mercapto-functionalised ODNs have, however, been used for conjugation only in solution.

An approach complementary to that described above involves derivatisation of the 5′-terminus of a support-bound ODN with an electrophilic group, to which conjugate groups bearing amino or mercapto functions may be attached. Phosphoramidite reagents derived from 2-(2,2,2-trifluoroethoxy)-2-oxoethanol, 2-(2-chloroethoxy)-2-oxoethanol (**1**),[36] 2-benzylthio-2-oxoethanol (**2**),[37] 5′-methoxyoxalamido-5′-deoxythymidine (**3**)[38] and *N*-chloroacetyl-6-aminohexanol (**4**)[39] have been used to introduce such groups. Figure 2 shows the structures of the electrophilic tails and those of the conjugates obtained by treatment with amines or mercaptans. The amide, amine and sulfide bonds formed upon conjugation are all sufficiently stable to withstand normal ammonolytic cleavage and deprotection of the ODN conjugate.

An aldehyde group is another electrophilic group introduced to the 5′-end of support-bound ODNs. A benzoyl-[40] or 4-methoxybenzylidene-[41] protected 5,6-dihydroxyhexyl tail has been coupled as a phosphoramidite reagent and the aldehyde function is created by periodate ion oxidation of the deprotected diol. The subsequent conjugation by reductive amination[40] or oxime formation[41] has, however, been carried out in solution. A 5′-carboxylic acid function has been introduced as a 2-chlorotrityl-protected phosphoramidite reagent and primary amines have then been attached by peptide coupling.[42]

Weakly nucleophilic compounds, such as alcohols, may be tethered to the 5′-terminus of a support-bound ODN by phosphitylating the 5′-hydroxy function first with a phosphorochloridite, $^iPr_2NP(OCH_2CH_2CN)Cl$, and subjecting the resulting 5′-phosphoramidite to tetrazole-promoted alcoholysis to a phosphite triester. Oxidation to a phosphate triester and removal of the 2-cyanoethyl group by β-elimination give a stable phosphodiester-linked conjugate.[43] 5′-Glycosylated ODNs have been obtained by glycosidation of the 5′-hydroxy group of the support-bound base- protected ODN with a fully benzoylated glycosyl trichloroacetimidate in CH_2Cl_2 using Me_3SiOTf as an activator.[44] 2-(2-DMTrO-ethanesulfonyl)ethyl[45] and 3-DMTrO-2,2-bis(ethoxycarbonyl)propyl[46,47] phosphoramidite reagents allow convenient 5′-phosphorylation.

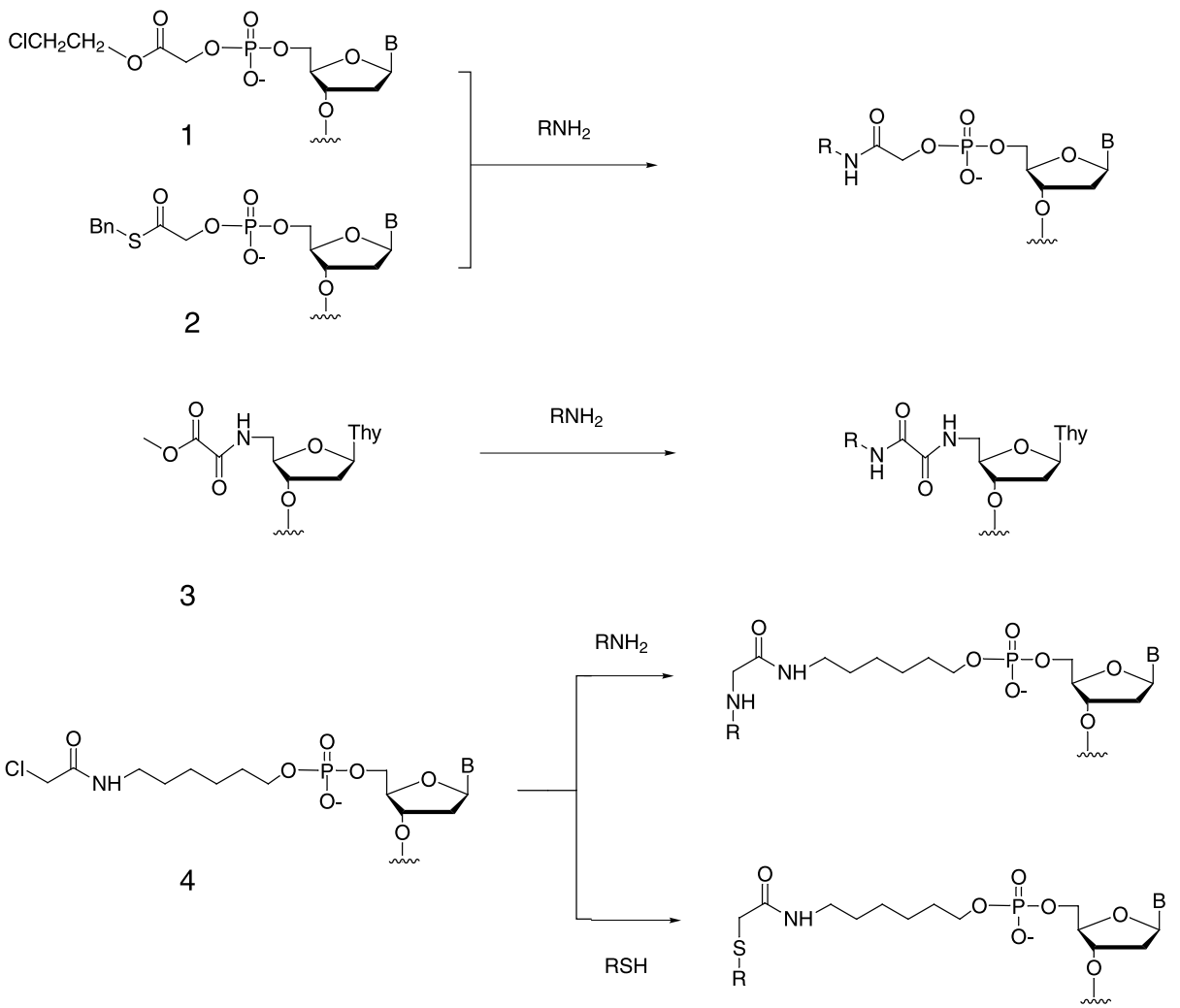

Figure 2. Electrophilic tails of support-bound ODNs and the conjugates obtained by treatment with nucleophilic reagents.[36–39]

After detritylation, the carbon skeleton is cleaved upon ammonolysis by β-elimination or retro aldol condensation, respectively, leaving the phosphate group bound to the 5′-hydroxy group. Support-bound ODNs have been converted to their 5′-triphosphates by consecutive phosphitylation with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, reaction with tributylammonium pyrophosphate and oxidation with iodine.[48]

Efforts towards the preparation of 5′-capped ORNs, i.e. ORNs bearing a 5′-terminal 7-methylguanosine linked to the penultimate nucleoside via a 5′,5′-triphosphate bridge, deserve special attention, owing to the evident importance of such conjugates as research tools for molecular and cell biology. As the first successful example of such a synthesis on a solid support, an N^2,N^2,7-trimethylguanosine-capped trinucleotide block of U1 snRNA (**9**) has been prepared (Fig. 3).[49] The first nucleoside is immobilised by reacting its 3′-phosphorochloridite (**5**) with the amino groups of a highly crosslinked polystyrene support. Accordingly, upon oxidation, an acid-labile phosphoramidate linkage is obtained. The chain is then elongated by conventional phosphoramidite chemistry and the 5′-terminal hydroxy function is phosphorylated with a phosphoramidite reagent derived from bis(2-hydroxyethyl)sulfone (**6**). The 5′-phosphate obtained is phosphorylated further to pyrophosphate with reagent **7**. The cap nucleoside is finally introduced as a 2′,3′-phenylboranylated 5′-phosphoroimidazolide (**8**). The conjugate is released from the support with 80% acetic acid and deprotected by consecutive treatments with aqueous hydrogen chloride and alkaline phosphatase.

2.3. Conjugation to the 3′-terminus

3′-Conjugates of oligonucleotides are more complicated to prepare than the 5′-conjugates. As discussed above, the chain assembly proceeds from the 3′- to 5′-terminus and, hence, the part of linker that, after the cleavage, remains bonded to the ODN chain must bear a separate orthogonally protected functional group for the attachment of the conjugate group. Figure 4 shows the structures of some branched handles exploited. Within these structures, the Fmoc group has commonly been used for protecting the amino function, but it has also been argued that this group is not entirely compatible with the ODN synthesis and the phthaloyl group has been recommended for this purpose.[50] Several different ODN 3′-conjugates, including 5(6)-carboxyfluorescein, biotin and short peptide conjugates, have

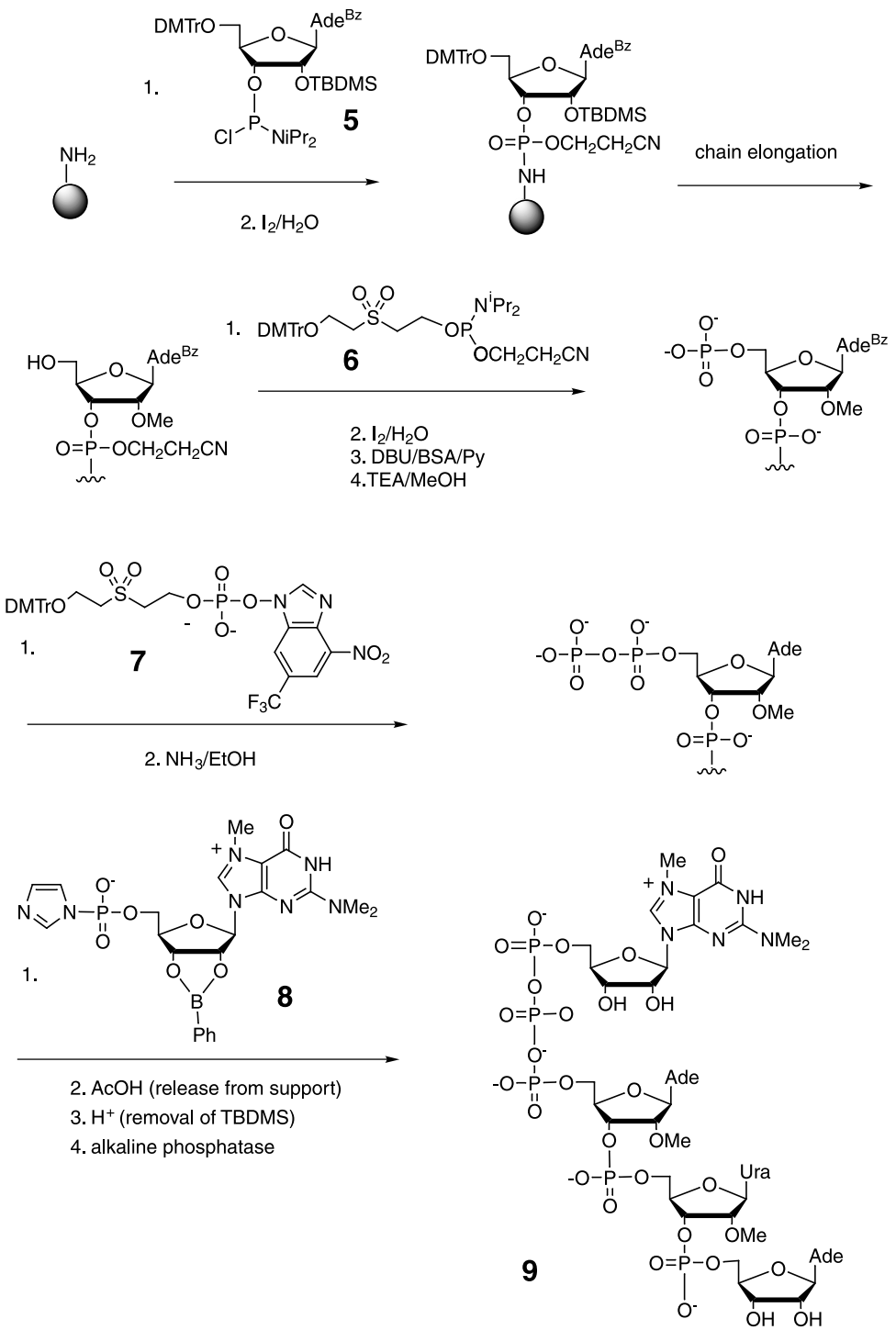

Figure 3. Preparation of 5′-capped ORNs on a solid support.[49]

been prepared on the linkers **10** and **11** in Figure 4.[51] The Fmoc protection is removed with piperidine and the desired carboxylic acid is coupled to the exposed amino group. Subsequently, the hydroxy function is detritylated with acid and the ODN is assembled on this group. The conjugate is released from the support by normal ammonolysis. It is worth noting that this linker is enantiopure, since the only chiral starting material employed in its synthesis is homoserine, and it has been used as the L-enantiomer. An N^{α}-Fmoc-N^{ε}-MMTr-lysine linker esterified to a hydroxy-derivatised PEG-coated PS (**12**) has been used to prepare tetraphenylporphyrin conjugates.[52] Removal of the acid-labile MMTr group allows acylation of the conjugate group as a carboxylic acid to the ε-amino group, while the ODN chain is assembled on the α-amino group after removal of the base-labile Fmoc protection. The preparation of 3′-conjugates derived from a naphthalene diimide intercalator serves as an example of an approach that involves, in addition to the conjugation, a structural transformation of the conjugate group on a support.[53] The reaction of naphthalene 1,4,5,8-tetra-carboxylic acid dianhydride (**14**) with the amino group of the linker **13** esterified to succinylated CPG and subsequent

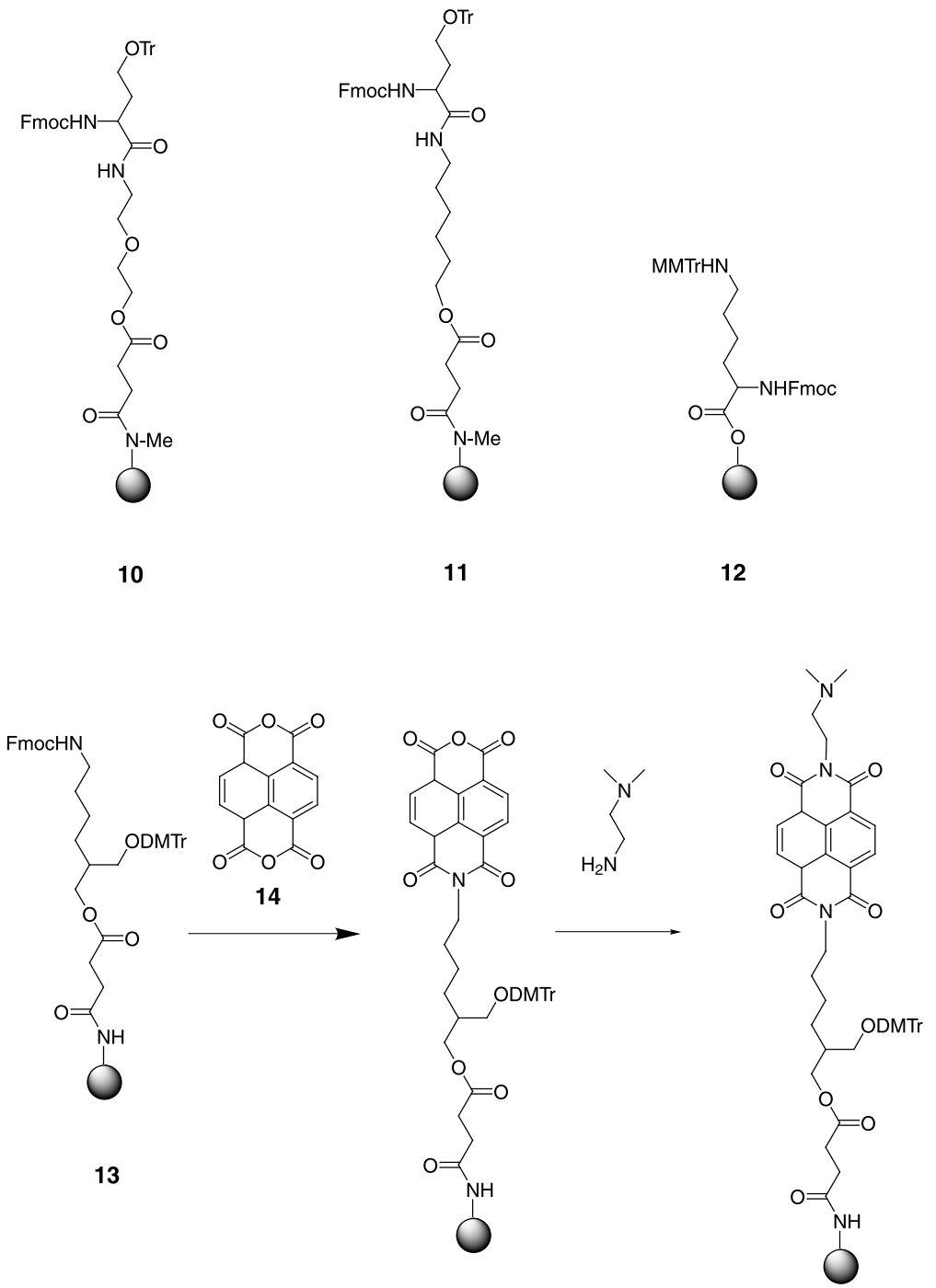

Figure 4. Linkers used for the synthesis of 3′-conjugates of ODNs, the conjugate group being attached on the support,[51–53] and the preparation of a naphthalene diimide conjugate group on one of the supports.[53]

treatment with an external primary amine gives the desired diimide structure. The ODN chain is then assembled on the detritylated hydroxy function.

More often, conjugate groups have been tethered to the 3′-end of an ODN chain by making use of appropriately modified linkers in such a manner that only the ODN synthesis, not the attachment of the conjugate group, takes place on the support. An early example is offered by the preparation of an anthraquinone conjugate.[54] 2-[Bis(2-hydroxyethyl)amino]anthraquinone is esterified via one of the hydroxy groups to a succinylated support, while the other hydroxy function is used for the chain assembly (**15** in Figure 5). Acridine and cholesterol conjugates have, in turn, been prepared on a linker obtained by the reduction of L-hydroxyproline to the corresponding alcohol and acylation of the secondary nitrogen atom with 9-acridine carboxylic acid or cholesterol chloroformate (**16**).[55,56] Dimethoxytritylation of the primary hydroxy function and esterification of the secondary hydroxy to succinylated LCAA-CPG then gives the support. 2-(4-Aminobutyl)-1,3-propanediol linkers bearing biotin, fluorescein and substituted acridine carboxylic acids coupled to the amino group (**17**),[57] a 1-*O*-(3-aminopropyl)glycerol linker (**18**)

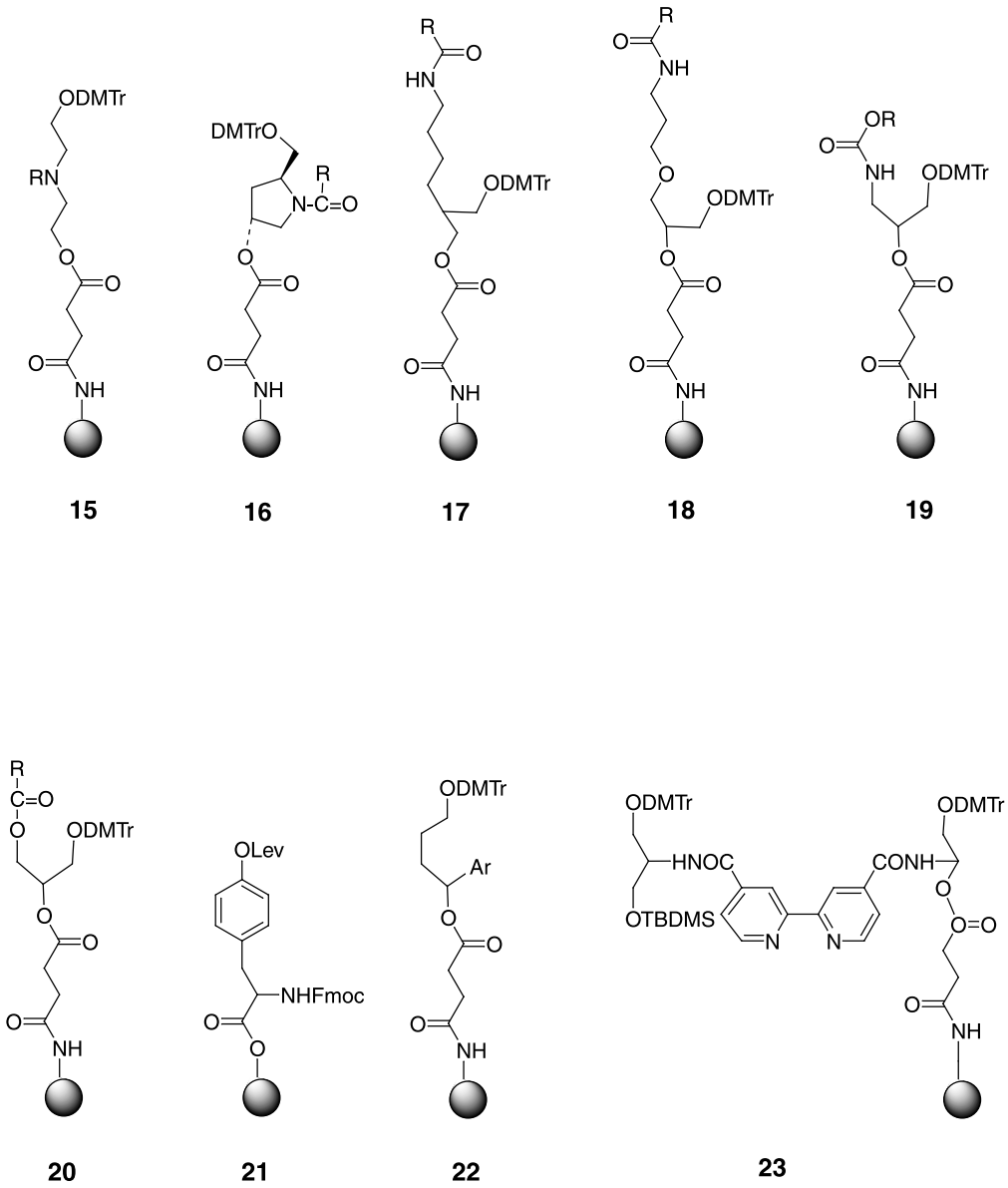

Figure 5. Linkers used for the synthesis of 3′-conjugates of ODNs, the conjugate group being attached to the linker prior to immobilisation on the support.[55–69] R is the conjugate group, Ar a pyrenyl group.

bearing fluorescein[58] and adamantane[59] carboxylic acids and a 2,3-dihydroxypropaneamine linker (**19**) bearing cholesterol[60] and dihydropyrroloindole[61] carboxylic acids offer additional examples. 3′-(1-*O*-Hexadecylglycerol),[62] 3′-phosphoryltyrosine[63] and 3′-(3-hydroxy-4-pyrenylbutyl)[64] conjugates have been prepared on the supports **20**–**22**, respectively. 3′-Glycosyl ODNs have been obtained by esterifying an appropriately protected methyl glycoside through one of its secondary hydroxy functions to a succinylated support and assembling the ODN chain on the primary hydroxy function.[65] 3′,5′-Bisglycosylated ODNs have been prepared by inserting a glucose-derived phosphoramidite as the last building block.[66]

ODNs exhibiting an intrachain 3′-3′ inversion are expected to find applications as agents capable of recognising a $(\text{purine})_m(\text{pyrimidine})_n$ sequence in DNA through a triple helix formation. A possible way to prepare such ODNs is to assemble the two segments of the chain on a common 3′-conjugate group. A 2,2′-bipyridine linker, **23**,[67] and an N^4,N^4-tetramethylene-bridged 5′-*O*-DMTr-2′-deoxycytidine dimer esterified to a succinylated support[68] have been used for this purpose.

3′(2′)-Conjugates of ODNs have also been synthesised by tethering the desired conjugate group to the 3′(2′)-oxygen atom of an appropriately protected ribonucleoside in solution and immobilising this terminal nucleoside through the 2′(3′)-hydroxy group to a succinylated support. This approach has, for example, been utilised to obtain 3′-(puromycin-5-yl)phosphate,[69] 3′-*O*-pyrenylmethyl,[70] 2′-*O*-cholesteryl[71] and 2′-*O*-acylamido[72] conjugates of ODNs. ODNs bearing a fluorescein dye at the base moiety of the 3′-terminal nucleoside have been obtained by esterifying a fluorescently tagged nucleoside to a succinylated LCAA-CPG support and assembling the chain on this support.[73]

Linkers containing a tether that withstands the ODN chain

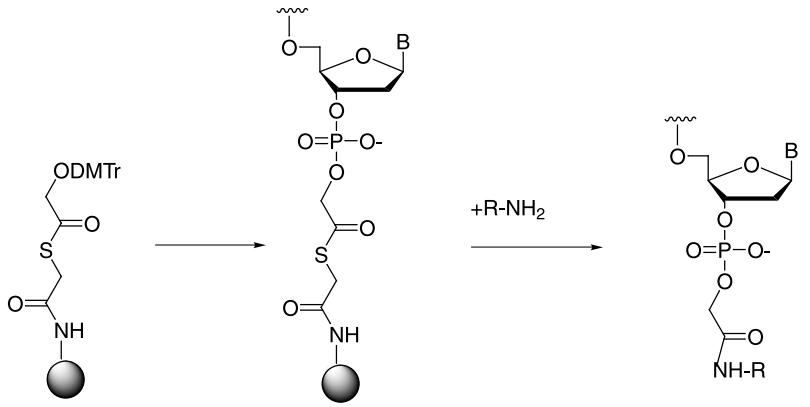

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Figure 6. Introduction of 3′-conjugate groups by cleavage of a thioester linker.[37]

assembly, but which may be cleaved by the entering conjugate group which simultaneously becomes bound to the ODN, offer an alternative for the use of branched linkers. A thioester bond is suitable for this purpose. ODNs may, for example, be assembled on the thioester linker **24** depicted in Figure 6. Upon cleavage with amines, the amine remains bonded to the ODN through an amide bond. Histamine, for example, has been tethered in this manner to the 3′-end of ODNs.[37] A number of linkers containing a normal ester bond have been used for the same purpose, but the cleavage of oxygen esters with nitrogen nucleophiles is considerably slower than that of thioesters.[74] Another interesting alternative to obtain the 3′-conjugates is afforded by the immobilisation of 5′-*O*-DMTr-2′-deoxycytidine 3′-(2-chlorophenyl)phosphate via the 4-amino group to a solid-supported succinyl linker.[75] The conjugate group bearing a hydroxy function is attached by DCC-promoted esterification to the phosphodiester group and the ODN chain is assembled on the 5′-hydroxy function after removal of the DMTr protection.

Serious attempts have also been made to assemble ODNs from 3′-*O*-DMTr-protected 2′-deoxyribonucleoside 5′-phosphoramidites in an inverse direction, which leaves the 3′-terminal hydroxy function free for derivatisation. 3′-Cholesterol and vitamin E have, for example, been attached as the phosphoramidites by an additional coupling cycle.[76] Amino-β-cyclodextrin has, in turn, been attached to the 4-nitrophenylcarbonate-activated 3′-hydroxy function via a carbamate linkage.[59]

ODNs functionalised with an amino or a mercapto group at the 3′-terminus are used extensively as starting materials for conjugation in solution and, hence, their preparation on a solid support is of interest. The linkers used to introduce a 3′-aminoalkyl tail fall into three major categories. First, linkers that contain a side-chain amino function protected with a base-labile group.[77] Secondly, linkers containing an exceptionally labile amide bond, either a backbone phthalimido linkage,[78] or a normal amide bond, the cleavage of which is assisted by a neighbouring carboxy group.[79] Thirdly, linkers containing a carbamate linkage, the rupture of which may be triggered by a reaction elsewhere along the linker: (i) by reductive cleavage of a disulfide bond two carbons from the carbamate oxygen atom, resulting in the departure of episulfide and carbon dioxide,[80,81] (ii) by β-elimination at a 2-(2-nitrophenyl)-ethoxycarbonyl or 9-fluorenylmethoxycarbonyl fragment of the linker,[82] or (iii) by photochemical cleavage of a 2-nitro-5-methoxybenzyl group bonded to the carbamate oxygen.[83] 3′-Mercaptoalkyl-tethered ODNs are conveniently obtained on disulfide linkers.[84,85] 3′-Carboxyalkyl-tethered ODNs have been prepared on a photolabile 4-(ω-hydroxyacyloxymethyl)-2-methoxy-5-nitrophenoxy linker,[86] on a Pd-labile 12-hydroxy-8-oxa-9-oxo-5-dodecenoyl linker[87] and on ω-hydroxyacyloxy linkers using ω-aminocarboxylic acids as the cleaving agents.[88] ODNs bearing an aminoalkyl tail at the 3′-terminus and a mercaptoalkyl group at the 5′-terminus have been obtained by assembling the ODN on a phthalimido-based linker and introducing an acetyl-protected ω-mercaptoalkyl phosphoramidite to the 5′-end as the last building block.[89] Release from the support is achieved by cleavage of the phthaloyl group with a mixture of 2,2′-dithiopyridine and concentrated aqueous ammonia in the presence of phenol and methanol. Accordingly, the released ODN bears an unprotected 3′-terminal aminohexyl tether and a 5′-terminal dithiopyridylalkyl tether.

Several solid phase methods have been described for the preparation of ODN 3′-phosphates (Fig. 7). Chain assembly on a 5-methoxy-2-nitrobenzyl-[90] (**25**) or a 2-nitrobenzyl-[91] (**26**) derived linker followed by photochemical cleavage yields ODN 3′-phosphates. Chain assembly on a disulfide linker (**27**) and subsequent reductive cleavage from the support, in turn, yields a 3′-terminal 2-mercaptoethyl-phosphate, which, at high pH, is rather rapidly converted to the 3′-phosphate by cleavage of episulfide.[92,93] It is also possible to immobilise the 3′-terminal nucleoside as a 3′-(2-cyanoethylphosphorochloridite) directly onto an amino-alkylated support and oxidise the resulting support-bound phosphoramidite to phosphoramidate.[94] The release from the support as an ODN 3′-phosphate is effected by ammonolysis to a phosphoramidate diester, which is susceptible to acid-catalysed hydrolysis. There are also linkers that allow the synthesis according to an unmodified protocol, including release by conventional ammonolysis. Ammonolysis of the succinyl ester bond of the linker **28**, for example, releases the ODN as a 3′-phosphate, since the 4-hydroxybenzyl group is rapidly eliminated from the phosphate group as 4-methylenecyclohexa-2,5-dienone.[95]

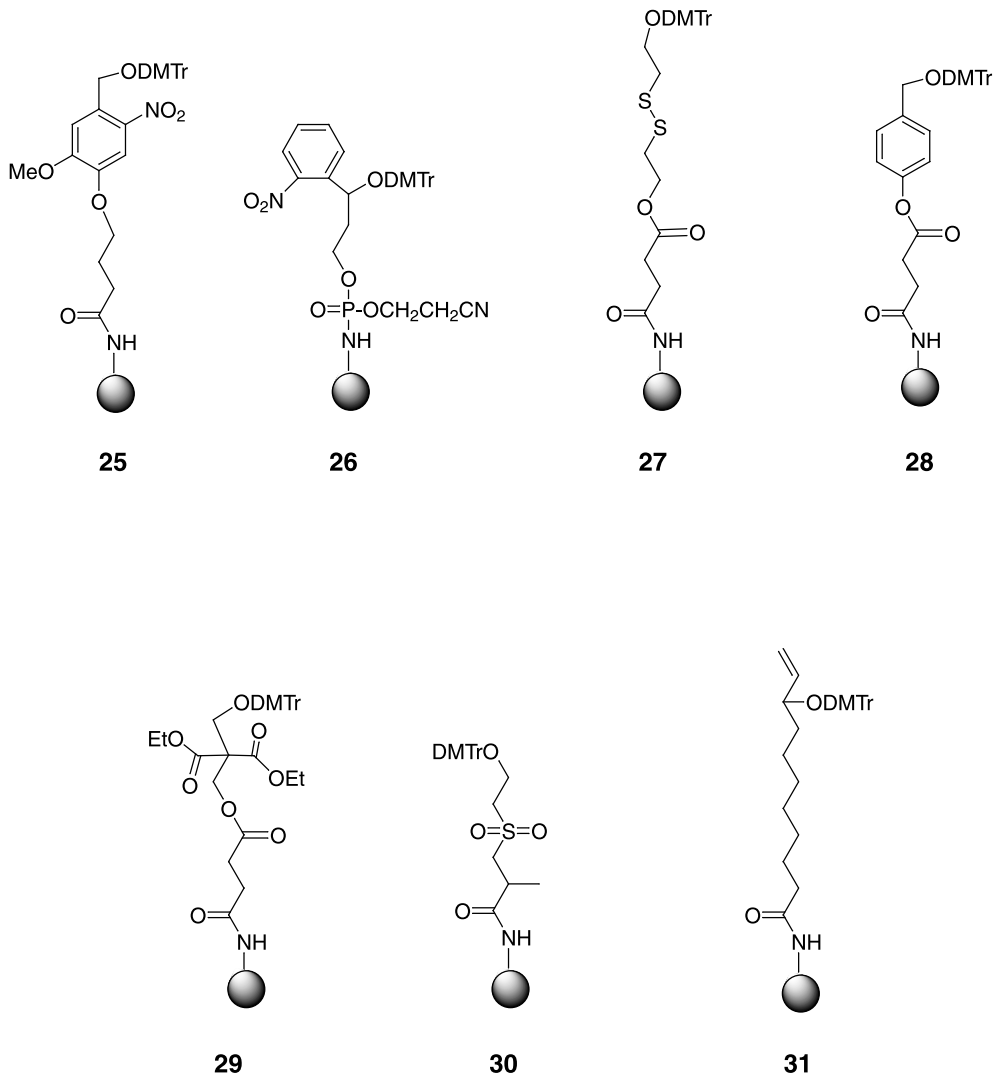


Figure 7. Linkers employed for the preparation of ODN 3′-phosphates.[90–99]

A 2,2-bis(ethoxycarbonyl)-1,3-propanediol handle tethered to an aminoalkyl support via a succinyl[96] (**29**) or malonyl linker[97] behaves similarly. Ammonolytic cleavage of the ester bond first releases the ODN as a 3′-[3-hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate, which, by retro aldol condensation, is almost instantaneously converted to a free 3′-phosphate. The sulfone linker **30** releases the ODN 3′-phosphate by β-elimination upon ammonolysis.[98] ORN 3′-phosphates have been prepared on a Pd-labile allylic linker (**31**).[99]

2.4. Conjugation to nucleoside sugar and base moieties

The most straightforward method for the preparation of ODNs bearing a conjugate group at a desired base or sugar moiety is undoubtedly the incorporation of a preconjugated nucleoside into the ODN as a 5′-*O*-DMTr-protected 3′-phosphoramidite building block. In some cases, exceptionally labile base moiety protections have to be used and the conventional succinyl linker has to be replaced with a more base-labile tether, or with a linker which may be cleaved under orthogonal conditions. To mention a few examples, 2′-deoxyribonucleoside building blocks having metal ion chelators,[100] groove-binding polyamines,[101] duplex-stabilising amino sugars,[102] fluorescent markers[103] or spin labels[104] tethered to their base moiety have been incorporated into ODNs. 2′-*O*-Tethered 5′-*O*-DMTr-ribonucleoside 3′-phosphoramidites have been used to incorporate ferrocenyl groups[105] and additional sugar moieties.[106]

In order to avoid the laborious preparation of nucleoside conjugates and their transformation to phosphoramidite reagents, two versatile strategies for the solid-supported preparation of ODNs bearing a conjugate group at a given nucleoside unit have been established, one of which, the convertible nucleoside approach, consists of insertion of a nucleoside bearing a good leaving group into a desired position of the ODN chain and post-synthetic displacement of this group with the entering conjugate group on the support. The second approach involves the insertion of a modified nucleoside bearing a masked functional group into the ODN chain, deprotection of this functionality under orthogonal conditions and covalent attachment of the conjugate group while the otherwise fully protected oligomer still is anchored to the solid support. The functional group, or the displaceable leaving group, is usually tethered to *C*5 of a pyrimidine base or to *C*8 of a

purine base when the conjugate group is aimed at being situated in the major groove of a double helix and to *O*2′ of the sugar moiety when a structural modification in the minor groove is desired.

The stepwise displacement of two different leaving groups by two different nucleophiles offers a recent example of the convertible nucleoside method.[107] 5-Methoxycarbonylmethyl- and 5-cyanomethoxycarbonylmethyl-2′-deoxyuridines have been inserted in the desired positions of an ODN chain and the different susceptibility of these two ester functions to nucleophilic attack of amines has been exploited to introduce two different conjugate groups. ODNs bearing bulky groups at N^2 of a guanine base have been obtained by inserting O^2-Tf-O^6-Npe-2′-deoxyxanthosine into the chain and displacing the triflate group with an amine nucleophile on the support.[108] Similarly, ODNs containing a 2′-*O*-(ω-bromoalkyl)ribonucleoside have been subjected to nucleophilic displacement on the support.[109] In addition to simple nucleophilic displacements, reactions catalysed by metal ion species have been exploited in the convertible nucleoside approach. Ferrocene conjugates have been obtained by introducing 8-Br-N^6-Bz-2′-deoxyadenosine in an ODN and carrying out Pd(0)-promoted Soagashira coupling with ferrocenyl propargylamide on the support.[110] The 5-iodo-2′-deoxyuridine residue has, in turn, been derivatised with a variety of terminal alkynes using $Pd(PPh_3)_4$ and CuI as a catalyst.[111,112]

The convertible nucleoside approach has been applied to the preparation of ODNs having an ethylene cross-link between the amino functions of two cytosine bases. A 4-(1,2,4-triazol-1-yl)pyrimidin-2-one base at the 5′-end of a support-bound ODN chain is reacted with the amino group of 5′-*O*-DMTr-3′-*O*-TBDMS-N^4-(2-aminoethyl)-2′-deoxycytidine to obtain the interchain ethylene bridge. The ODN chains are then elongated in the 3′→5′ direction with normal 3′-phosphoramidite building blocks and after removal of the TBDMS protection in the 5′→3′ direction with 5′-phosphoramidites.[113]

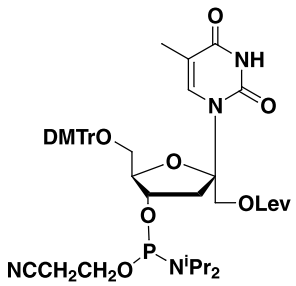
The convertible nucleoside approach has been applied to the functionalisation of ODNs at defined sites for subsequent use in solution. The bases used as the precursors include 6-(4-chlorophenoxy)purine and 6-chloropurine for N^6-tethered adenine,[114–116] 2-fluoro-O^6-Npe-inosine and 2-fluoroinosine for N^2-tethered guanine,[114–116] O^4-(4-chlorophenyl)- and O^4-(2,4,6-trimethylphenyl)- and O^4-(4-nitrophenyl)uracil for N^4-tethered cytosine,[114,115,117–119] 5-methyl-4-(1,2,4-triazol-1-yl)pyrimidin-2-one for *C*4-tethered thymine,[120] *N*3-nitrothymine for *N*3-tethered thymine[121] and 5-methoxycarbonyluracil for *C*5-tethered uracil.[122,123] ODNs containing a 2′-(2-methoxy-2-oxoethylthio)-2′-deoxyuridine residue have been converted to 2′-(2-alkylamino-2-oxoethylthio)-conjugates upon the cleavage from the support with amines.[124] 7-Vinyl-7-deazaguanine has been post-synthetically modified by a Diels–Alder reaction with maleimides.[125]

As mentioned above, conjugation to orthogonally protected functional groups offers a useful alternative for the convertible nucleoside approach. The use of a photolabile 3,4-dimethoxy-2-nitrobenzyloxycarbonyl group is a good example. The aliphatic amino or carboxy groups of 5-(ω-aminoalkyl)- and 5-(ω-carboxyalkyl)-2′-deoxyuridine,[126] 2′-amino-2′-deoxyuridine[127] and 2′-*O*-(aminobutyl)uridine[128] have been protected with this group and inserted as phosphoramidites at defined sites of a support-bound ODN chain. After photochemical deprotection, the exposed side-chain functionality has been subjected to PyBOP-mediated amide bond coupling with the conjugate group. A related approach involves the insertion of nucleoside phosphoramidites bearing an ω-phthalimidooxyalkyl group either at the base or the sugar moiety into ODNs, removal of the phthaloyl protection with hydrazinium acetate, which may be carried out on a normal succinyl linker, and reaction of the exposed aminooxy groups with aldehydes.[28] The oximes obtained withstand the standard ammonolysis. When the ODN chain is assembled on a disulfide linker, it may be fully deprotected while still anchored to the support.[129] The N^4-(6-aminohexyl) tail of a 2′-deoxycytidine residue may then be labelled by a DBU-promoted attack of the aliphatic amino group on isothiocyanates of fluorescent dyes without marked interference of the other amino groups.

ODNs containing an aldehydic function at any preselected position have been prepared by incorporation of 3-formylindole 2′-deoxyribonucleoside into the ODN as a phosphoramidite building block without any protection at the aldehyde group[130] or by post-synthetic oxidation of the ω-alkene tether of 8-(4-pentenyl)thioadenine with OsO_4 to the corresponding diol and further to aldehyde with the IO_4^- ion.[131] Alternatively, a 2′-deoxyguanosine building block bearing an acetyl-protected 3,4-dihydroxybutyl group at N^2,[132] or a uridine block bearing a benzoyl-protected 2,3-dihydroxypropyl group at *O*2′,[133] may be inserted, followed by oxidation of the diol group of the fully deprotected ODN with the IO_4^- ion. A cross-linking agent for DNA-processing enzymes has been obtained by inserting 1-(β-D-galactopyranosyl)thymine into their substrate ODN and oxidising the galactopyranosyl ring with IO_4^- to an acyclic dialdehyde.[134]

Besides chain assembly, phosphoramidite coupling may additionally be applied to the attachment of a desired conjugate group at any defined site within the sequence. For this purpose, 2′-deoxyuridine monomers bearing an Fmoc-protected hydroxyalkyl tether at C5 have been prepared.[135] The Fmoc protection is removed immediately after insertion of this monomer into the growing ODN chain and the exposed hydroxy function is reacted with a phosphoramidite reagent derived from the desired conjugate group. A closely related strategy utilises a 1′-*O*-levulinyl protected 3′-deoxypsicothymidine building block (**32**).[136] After the chain assembly, the levulinyl group is removed on-support with hydrazinium acetate and the exposed hydroxy group is reacted with a phosphoramidite reagent.

Instead of nucleosides, non-nucleosidic phosphoramidite reagents that allow chain elongation are often used to introduce intrachain conjugate groups. They are, typically, derived from propane-1,2- or 1,3-diols containing one or two side chains to tether reporter groups, intercalators, bioaffinity groups or metal chelates.[57,58,64,106,137–142] A



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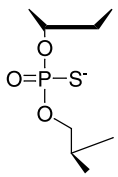

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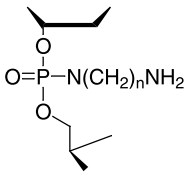

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similar methodology has been applied to insert various chemical fragments to form part of the sugar–phosphate backbone. Such structures include metal chelates,[143,144] azobenzene,[145] steroids[146] and disaccharides.[147]

2.5. Conjugation to internucleosidic linkages

Conjugate groups may also be attached to the internucleosidic linkages. When the 2-cyanoethyl protection is removed from a phosphotriester linkage with piperidine on a solid support and the resulting phosphodiester is activated with TsCl, an amine nucleophile can be used to introduce a conjugate group.[148] Introduction of a phosphorothioate (**33**) or a ω-aminoalkylphosphoramidate (**34**) linkage at the desired sites of the chain allows the post-synthetic attachment of thiol- and amino-specific conjugate groups, respectively.[149] Another approach involves the chain assembly by the phosphoramidite chemistry and the insertion of an H-phosphonate building block into the desired site of conjugation.[150] The conjugate group is introduced by oxidative amination of the *H*-phosphonate diester. A cholesteryl group has been tethered to the 3′-terminal internucleosidic phosphoramidate linkage in this manner.[151] ODNs bearing several histamine residues at their 5′-terminus have, in turn, been prepared by elongating the support-bound ODN chain with 4-DMTrO-butyl hydrogen phosphonate units and converting the H-phosphonate bonds to phosphoramidate linkages by oxidative amidation with histamine.[152] A similar chemistry has been applied to prepare ODNs having a polycationic 3′-terminal tail.[153] Double-labelling has been achieved by using a stable 2-(4-methoxybenzamido)ethyl-protection for a selected phosphorothioate linkage and a labile 2-(*N*-isopropyl-4-methoxybenzamido)ethyl-protection for the 3′-terminal thiophosphate and the phosphodiester bonds.[154] Accordingly, the phosphorothioate-protections may be removed in a stepwise manner.

3. Peptide conjugates

3.1. General

Two different strategies, namely Boc[155] and Fmoc[156] chemistry, are commonly applied to the solid-supported synthesis of peptides. The chain assembly is, in both cases, started by esterification of the first amino acid as an N-protected acid anhydride to the hydroxy functions of a solid support, which usually is a polystyrene-based resin (Fig. 8). With the Boc strategy, the α-amino function of the amino acid building blocks is protected with an acid-labile Boc group and the side chain functionalities with benzyl or benzyloxycarbonyl groups. After completion of the chain assembly, the side-chain protections are removed and the peptide is cleaved from the support by hydrogen fluoride treatment. On using the Fmoc chemistry, the α-amino group is protected with an Fmoc group, which is removed by β-elimination catalysed by weak organic bases, while the side-chain functional groups are protected with acid-labile groups, such as *t*-Bu, Tr, MMTr and Boc. Treatment with TFA removes the side-chain protections and releases the peptide in solution. The stepwise coupling of the N^{α}-protected amino acids to the support-bound α-amino group is, with both strategies, achieved by a variety of electrophilic activators that, when reacted with the carboxylic acid function, render the carbonyl carbon susceptible to nucleophilic attack. Usually, the activated carboxy group is first attacked by an auxiliary nucleophile, yielding an active ester that subsequently undergoes an acyl substitution reaction with the support-bound amino group. The generally used activators include carbodiimides, such as DCC and DIC, uronium salts, such as HBTU, TBTU and HATU, and phosphonium salts, such as BOP and PyBOP. As auxiliary nucleophiles, HOBt and its nitrogen analogue, HOAt, are the most common.

As indicated above, the peptide chain is usually anchored by an ester linkage to a support-bound hydroxy function. In particular, the Wang (**35**) and HMPA (**36**) linkers indicated in Figure 9 are widely used. They may be cleaved with 95% TFA, often in the presence of thioanisole or other scavengers. The PAM linker (**37**) is less, and the Rink acid linker (**38**) more, acid labile. The amide linkers, including MBHA (**39**), the Rink amide (**40**) and the PAL (**41**) linkers, are also cleaved with TFA. The BAL (**42**) and 2-chlorotrityl linkers (**44**) are sensitive to acids and the fluorenyl linker (**43**) to organic bases. The properties of the rest of the linkers in Figure 9 (**45**–**49**) are discussed below in relation to their conjugation on-support.

A prerequisite for a good conjugation strategy on a solid support is that either of the well-established protocols described above may be applied with a minimal number of changes. The variety of conjugate groups attached to peptides is smaller than that linked to oligonucleotides. In fact, the groups conjugated to peptides are usually either reporter groups or metal ion chelates, and, additionally, peptides having a side-chain hydroxy function phosphorylated are of considerable interest, owing to the central role of phosphoproteins in cell signalling. The preparation of these

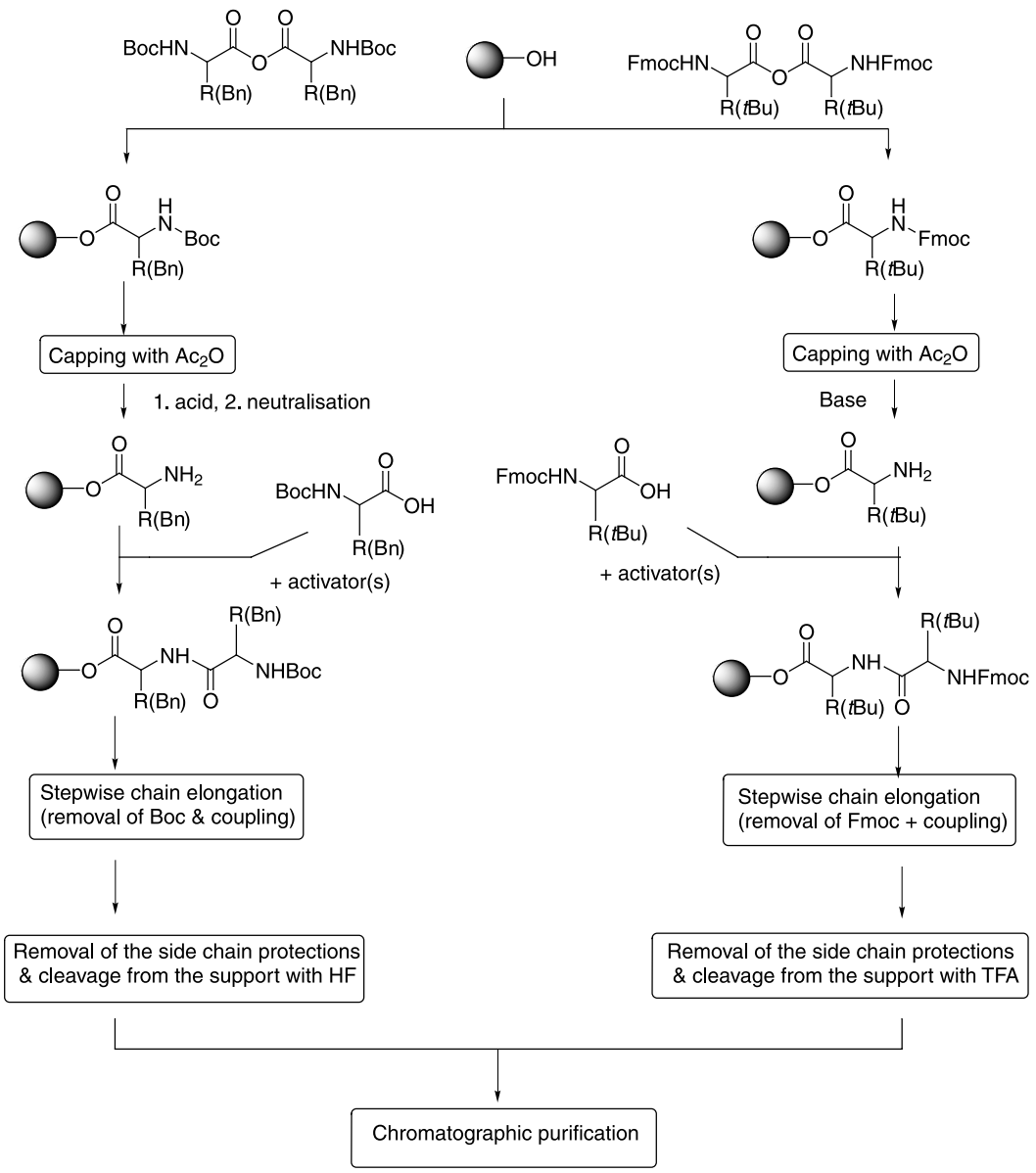


Figure 8. Solid-supported peptide synthesis by the Boc and Fmoc strategy.

peptide conjugates is discussed below, together with the solid-supported functionalisation of the peptides. Peptide–oligonucleotide conjugates and glycopeptides have received increasing interest during the last decade and the syntheses of these two families of conjugates are discussed in Sections 4 and 5, respectively.

3.2. N-Terminal and side-chain conjugation

Fluorescent dyes that tolerate the acidic conditions needed for the cleavage of the conjugate from the support have been attached to peptides on a solid support. Accordingly, fluorescein isothiocyanate has been reacted with the terminal α-amino group of a support-bound peptide in the presence of $^{i}Pr_2NEt$ and the resulting conjugate has been cleaved from the resin with HF.[157] It has been argued, however, that isothiocyanate when coupled to a terminal α-amino group results in a loss of the N-terminal amino acid by Edman degradation.[158] In order to avoid this, the isothiocyanates should be tethered to the peptide via a spacer and, hence, DIC/HOBt-promoted coupling of 4(5)-carboxyfluoresceine has been preferred over the use of the corresponding *N*-succinimidyl ester or isothiocyanate. Rhodamine has been coupled to the terminal α-amino group of a solid-supported peptide as an HBTU-activated carboxylic acid, acid chloride or isothiocyanate in the presence of $^{i}Pr_2NEt$.[159] Cleavage with TFA in the presence of scavengers has been observed to give the conjugates in a comparable yield. The other fluorescent markers conjugated on a solid support include dansyl chloride coupled to the terminal lysine ε-amino group in the presence of $^{i}Pr_2NEt$,[160] 7-amino-4-methylcoumarin-3-acetic acid coupled to the terminal α-amino group by DCC/HOBt activation[161] and Oregon Green 514 carboxylic acid coupled by TBTU/HOBt activation.[162]

Peptide nucleic acids, having the general structure **49**, have been fluorescently labelled to an amino-tethered nucleic acid base.[163] The chain is assembled by the Fmoc/HATU chemistry on a HYCRON-linker (**46**), which undergoes a

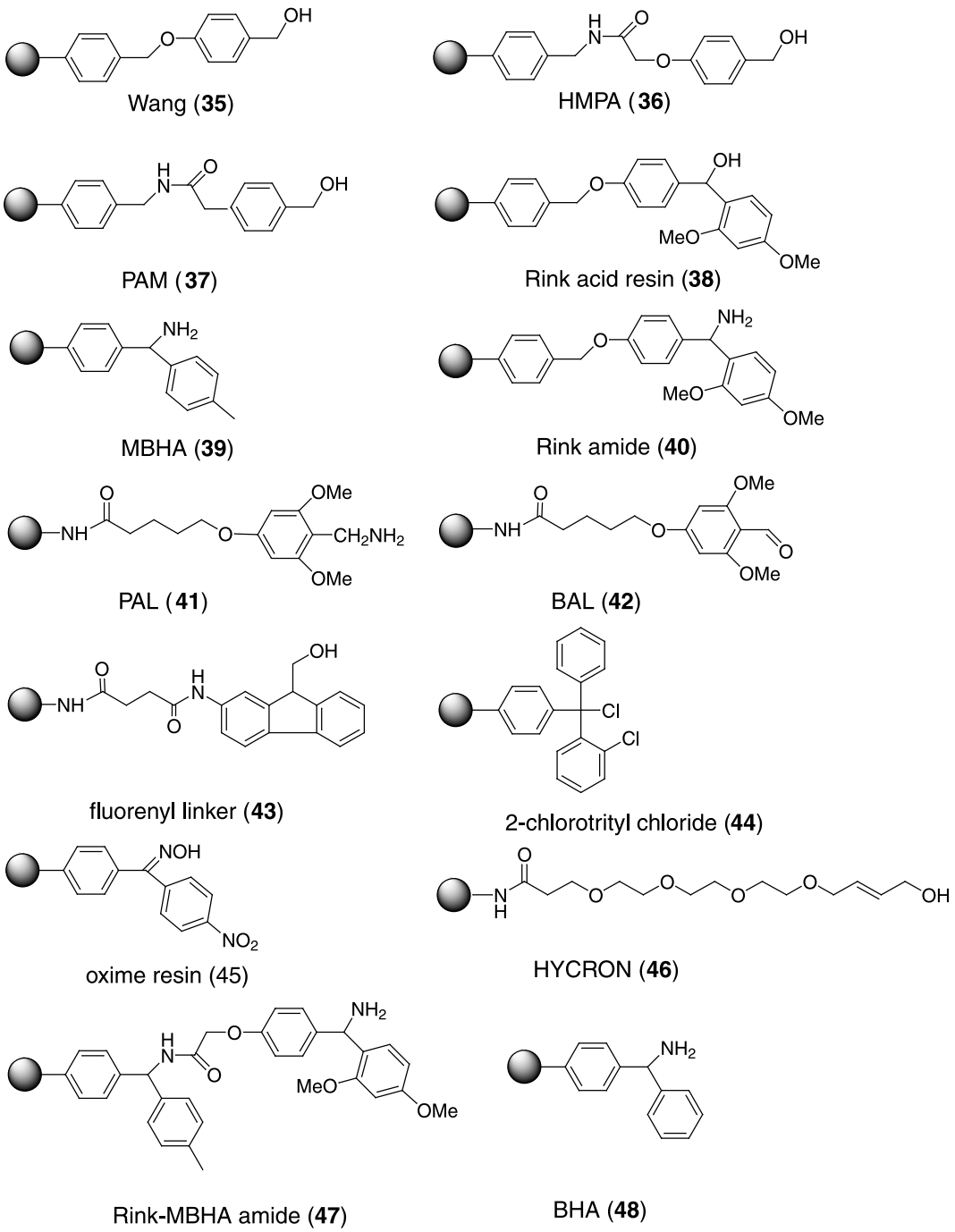


Figure 9. Linkers for solid-supported peptide synthesis.

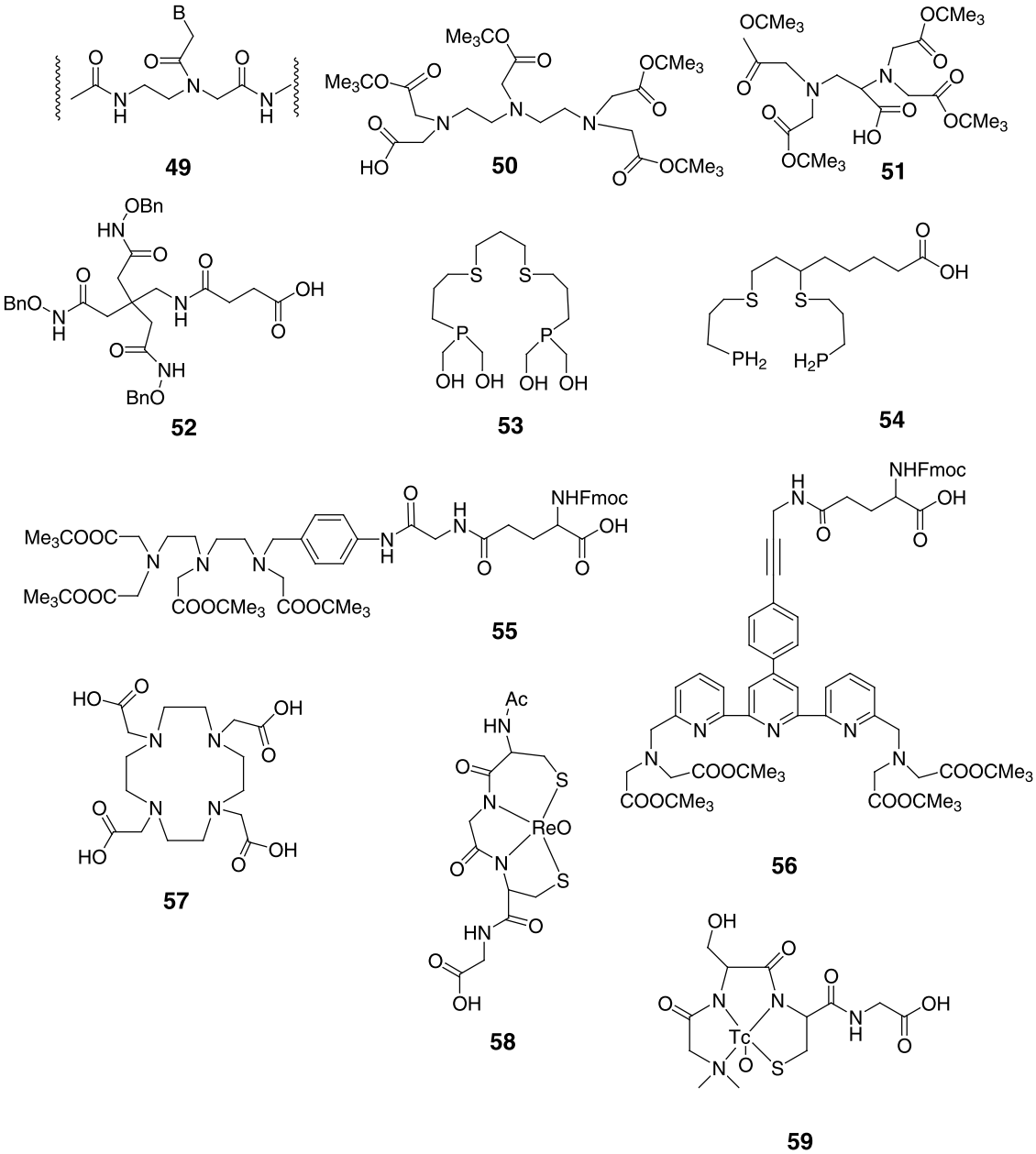
Pd(0)-promoted cleavage under mild conditions. At the site of conjugation, a building block derived from N^6-(*N*-Boc-6-aminohexyl)adenine is introduced. All the side-chain protections are removed with TFA/1,2-ethanethiol and labelling with DABCYL NHS ester is carried out. In addition to the amino tail, cysteine mercapto groups become labelled, but they may be easily deprotected with piperidine in DMF. The exposed mercapto functions are then reacted with IAEDANS, when double-labelled sequences are desired.

The convenient conjugation of metal ion-chelating agents to peptides is of considerable importance, owing to the numerous applications of peptides as carriers of radioactive markers, NMR-contrasting agents or catalytically active metal ions. Such conjugates are most frequently prepared by the standard amide coupling of an appropriately protected chelating agent as a carboxylic acid to an amino function of a support-bound peptide. Again, a prerequisite is that the conjugate group withstands the acid-promoted cleavage from the support. The commonly used complexing agent DTPA has, for example, been introduced as a tetra-*tert*-butyl ester (**50**) by DIC/HOBt coupling to the terminal α-amino group of a peptide assembled by the Fmoc chemistry on a 2-chlorotrityl chloride resin (**44**).[164] Cleavage from the support with TFA/thioanisole also

deprotects the chelating agent. EDTA functionalised by an additional carboxy group (**51**) has been attached similarly by DCC activation to a peptide assembled by the Boc chemistry on a PAM resin (**37**).[165] Trisuccin has been coupled in a benzyl-protected form (**52**) by DCC/HOBt activation to a Rink amide resin (**40**) -anchored peptide.[166] Cleavage with TFA in the presence of scavengers and subsequent hydrogenation gives the deprotected peptide conjugate. The P_2S_2 ligand (**53**) that efficiently binds the radioactive ^{99m}Tc and ^{188}Re isotopes has been coupled as HBTU-activated 6,8-bis(3-phosphanylpropylsulfanyl)octanoic acid (**54**) to the terminal backbone amino group of a peptide assembled by the Fmoc chemistry on a Rink amide resin (**40**).[167] Subsequent hydroxymethylation of the resin-bound conjugate with formaldehyde in ethanol and cleavage from the resin with a mixture of TFA and scavengers gives the conjugate. Amino acid building blocks allowing the solid-supported introduction of both non-fluorescent (**55**) and fluorescent (**56**) lanthanide ion-chelating ligands into peptides by normal HBTU/HOBt activation have been prepared.[168]

A useful stepwise method for the construction of the widely used DOTA–peptide conjugates (**57**) on a solid support has been reported.[169] The terminal amino group of a peptide assembled on a Rink amide resin (**40**) is first acylated with bromoacetyl bromide and the bromo substituent of the resulting *N*-bromoacetyl group is displaced with 1,4,7,10-tetraazacyclododecane. Treatment with *tert*-butyl bromoacetate then gives a *tert*-butyl-protected DOTA conjugate, which is finally removed from the resin by TFA/TIS treatment.

While the metal ion binding to the conjugate usually takes place post-synthetically in solution, as in the cases discussed above, some examples of a solid-supported conjugation of chelates already complexed with the metal ion have been reported. Rhenium oxide has been bound to a natural



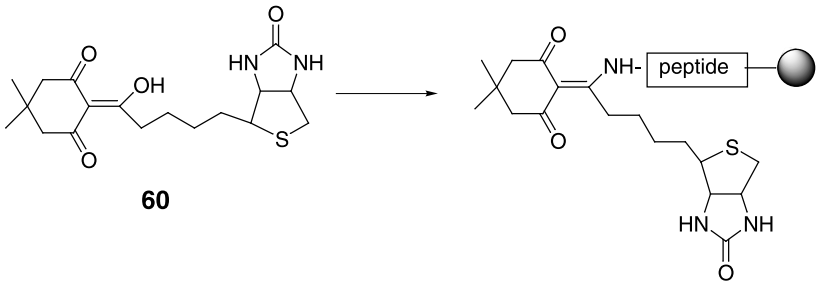
tetrapeptide, AcN-Cys-Gly-Cys-Gly-OH (**58**), and this was coupled to the terminal amino function of a support-bound peptide by the normal Fmoc/HBTU chemistry and cleaved with TFA/thioanisole/1,2-ethanedithiol.[170] Similarly, a prefabricated technetium chelate of Me_2N-Gly-Ser-Cys-Gly (**59**) has been attached as a Pfp-ester to an N-terminal 4-aminobutanoyl linker of a support-bound peptide.[171] A *cis*-dichloro(en)platinum(II) complex has been obtained by using Fmoc protected *N*-(2-aminoethyl)glycine as the N-terminal building block upon the chain assembly by the Fmoc/PyBOP chemistry and reacting the deprotected en tail with K_2PtCl_4.[172] The complex withstands cleavage from the support with TFA when sulfur-containing scavengers are not used. [Rh(phi)$_2$(phen$'$)] complex, where phen$'$ is the 5-(amidoglutaryl)-1,10-phenanthroline ligand, has been conjugated to a peptide by coupling the phen$'$ ligand first to the amino terminus of a solid-supported peptide and allowing a $[Rh(phi)_2(DMF)_2]^{3+}$ ion then to coordinate to the phenanthroline group.[173–175] The conjugate withstands standard acidic deprotection and cleavage conditions.

A useful method for the transient attachment of biotin to the amino terminus of peptides has been described.[176] 2-Biotinyldimedine (**60**) may be coupled to the terminal amino function of a solid-supported peptide by normal DCC/DMAP activation. The conjugate readily withstands cleavage from the support with TFA, but, when desired, it may be easily removed with hydrazine. *N*-Biotinyl-6-amino-1-oxohexyloxyacetic acid (**61**) may be used for the same purpose. Coupling by HBTU/HOBt activation gives the conjugate which is sufficiently stable to be cleaved from the support with TFA in the presence of scavengers, but can be removed by mild basic treatment, leaving the peptide in a glycollate-capped form.[177]

Peptides carrying at the desired sites redox-active conjugate groups, viz. anthraquinone, phenothiazine and $[Ru(bpy)_3]^{2+}$, have been assembled by the Boc chemistry on an HMBA resin, using BOP/HOBt for coupling.[178,179] The appropriate building blocks, **62**–**64**, have been obtained by acylating the ε-amino group of N^{α}-Boc-lysine with the carboxylic acid derivatives of the redox-active groups. 9-Anilinoacridine-4-carboxylic acid has been coupled as an NHS ester to the terminal amino group of support-bound peptides.[180]

A tetrapeptide, H-Cys-Val-Ileu-Ala-OH, bearing an *S*-farnesyl group and a PEG-tethered biotin at the N-terminal amino group (**65**) has been prepared on a benzophenone oxime resin (**45**).[181] The synthesis proceeds by stepwise conjugation of *N*-Boc-Ileu, *N*-Boc-Val and *S*-farnesyl-*N*-Tr-Cys by DCC/HOBt activation, acid-catalysed detritylation of the terminal amino group, acylation of the exposed group with the Pfp ester of the PEG-tethered biotin and cleavage of the conjugate from the resin using the methyl ester of alanine as a nucleophile. Two different types of oligopyrrole-tailed peptides have also been prepared on a solid support. The C-terminal conjugates (**66**) have been obtained by immobilising arginine through its guanidinium group to a Rink–MBHA amide resin (**47**), assembling the oligopyrrole from monomeric units by the Fmoc/HBTU/HOBt chemistry on its α-amino group and continuing the chain assembly with normal *N*-Fmoc amino acids.[182] Conjugates having the oligopyrrole tail bonded to the side-chain carboxy function of a glutamic acid residue (**67**) have been obtained by assembling the peptide by the normal Fmoc chemistry, but protecting the side-chain carboxy function of this particular glutamic acid as an allyl ester. This protection may be removed under orthogonal conditions and a prefabricated oligopyrrole tail is coupled to the exposed group by HATU coupling.[183]

A single exposed hydroxy group of a serine residue has been transformed to a bromo substituent with triphenylphosphine and carbon tetrabromide and the resin-bound bromo intermediate has been further converted to an *S*-phenylcysteine derivative without racemisation.[184] The TBDMS-protected hydroxy group of a homoserine residue has been converted to a chloro substituent with triphenylphosphine dichloride.[185] Bromo- and chloroalkyl groups may also be incorporated by using bromo- or chloroacetic acid as the last building block in the chain assembly.[186,187] Similarly,



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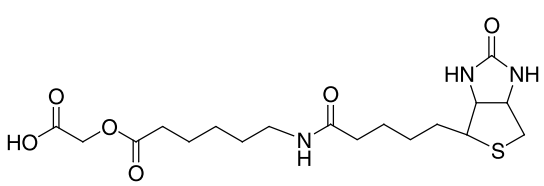

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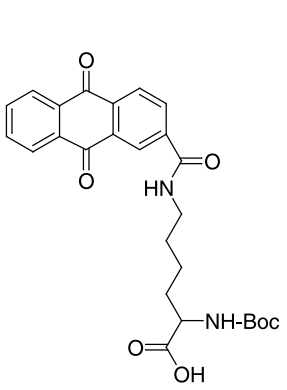

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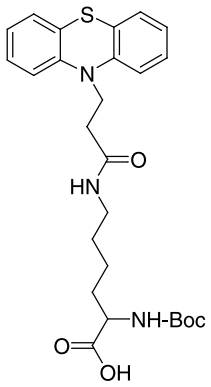

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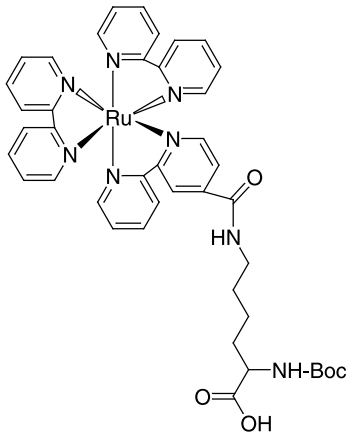

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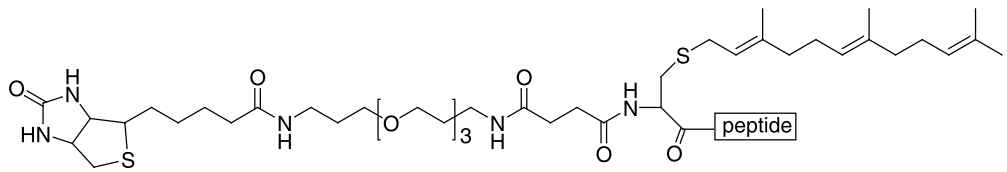

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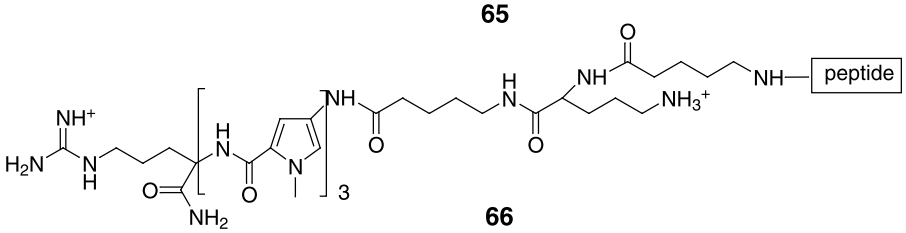

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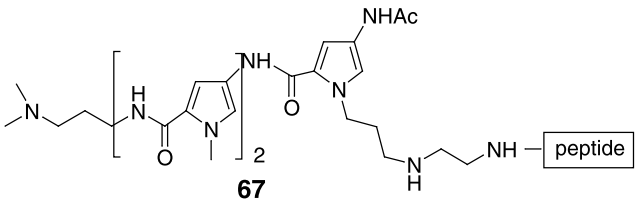

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Fmoc-protected 2-amino-4-bromobutanoic acid can be used to generate side-chain bromo-derivatised peptides on a solid support.[188] Solid-supported peptides bearing aliphatic bromo and chloro substituents are typically derivatised with mercaptans. A thioether-linked dendrimer of cyclic peptides, for example, has been prepared by reacting the four *N*-chloroacetyl groups of a solid-supported trilysyl core with cysteine-containing cyclic peptides.[189]

An N-terminal serine residue has been oxidised to a terminal oxoaldehyde with IO_4^- in H_2O.[190] This serves as a versatile starting material for several nucleophilic reactions, including the reductive amination, nitroaldol, Horner–Wadsworth–Emmons and Sakurai reactions.

Boc-protected aminooxyacetic acid has been used for the synthesis of peptides bearing an N-terminal aminooxy group, which readily binds aldehydes by oximation.[191] Fmoc-N^{β}-(*N*-Tr-aminooxyacetyl)-2,3-diaminopropionic acid, in turn, enables the introduction of a side-chain aminooxy function.[192] Hydrazinopeptides have been obtained by the electrophilic amination of amino groups with *N*-Boc-3-(4-cyanophenyl)oxaziridine on a solid support.[193,194] After completion of the chain assembly, the side chain protection is removed from a lysine residue and the exposed amino group is aminated. Solid-supported peptide amines have been converted to azides by a diazo transfer with triflyl azide in the presence of divalent copper ions.[195]

The α-carbon of the N-terminal amino acid residue may be alkylated on a solid support.[196,197] In the first step, the amino terminus is reacted with benzophenone. The resulting imine acidifies the α-hydrogen and, hence, facilitates the alkylation of this position. After alkylation, the α-amino group is regenerated by treatment with hydroxylamine, which then allows further peptide chain elongation.

Peptides bearing an aldehyde group at the N-terminus have been prepared by coupling an *N*-Boc protected perhydro-1,3-oxazidine-2-ylpropionic acid building block to the N-terminus and generating the aldehyde group by an acid treatment.[198] In order to avoid intramolecular reaction with the aldehyde group, the neighbouring amide nitrogens have to be kept protected. Side-chain aldehyde groups have been obtained on-support from asparagine- or glutamine-derived *N*-methoxy-*N*-methylamide precursors.[199] CuCl-promoted β-elimination of a hydroxy function activated with a water soluble carbodiimide has been shown to yield peptides containing α,β-didehydroamino acids.[200] [60]Fullerene linked to the side chain carboxy function of N^{α}-Fmoc-glutamate has been incorporated into a peptide sequence by the solid-phase Fmoc chemistry.[201] Peptides containing a sulfated tyrosine residue have been assembled by the Fmoc/PyBOP chemistry on a 2-chlorotrityl chloride linker (**44**).[202] This linker is highly acid labile and, hence, allows the cleavage of these acid-sensitive peptides from the resin with minimal loss. The tyrosine hydroxy function has been converted to an iodine substituent with bis(pyridine)-iodonium tetrafluoroborate.[203]

3.3. C-Terminal conjugation

Since the solid-supported peptide synthesis proceeds from the C- to N-terminus, conjugation to the C-terminus is achieved upon cleavage from the support or it is carried out post-synthetically in solution. Among the C-terminally modified peptides, the solid-supported thioesters are undoubtedly the most extensively used for conjugation. The support-bound peptide thioesters are conveniently obtained by Boc/HBTU/HOBt on an *N*-(mercaptoacetyl)-BHA resin (**48**)[204] or an *N*-(mercaptopropionyl)-PEGA resin[205] and the acid-labile side-chain protections may be removed with HF without cleaving the thioester bond. Cleavage of the fully deprotected peptide with various nucleophiles, such as alcohols, thiols, amines, hydrazines, hydroxylamines, hydride ion and Grignard reagents, gives peptide esters, thioesters, amides, hydrazides, hydroxamic acids, alcohols and ketones. More importantly, the support-bound peptide thioester has been shown to undergo an intermolecular native ligation,[206,207] i.e. an intermolecular nucleophilic acyl substitution of the thioester by the mercapto function of an N-terminal cysteine residue of another peptide, followed by rearrangement of the thioester

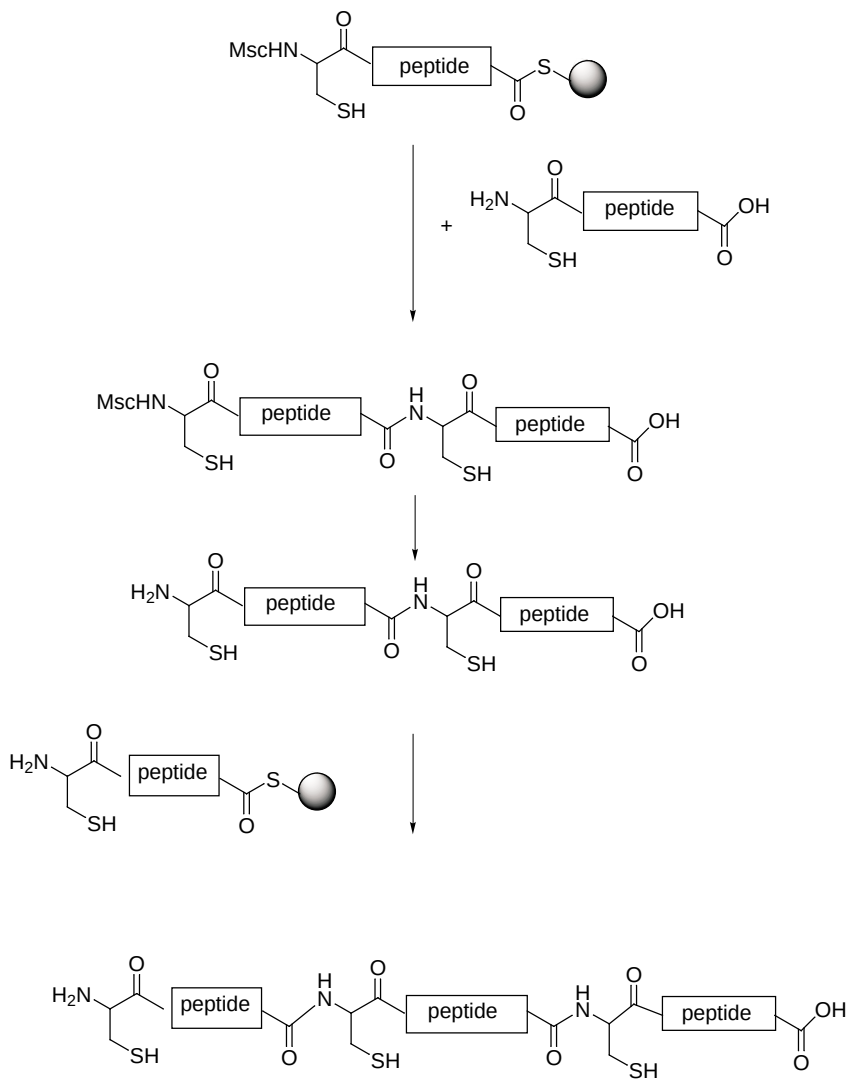


Figure 10. Native ligation on a solid support.[207]

bond formed, to give an amide bond.[208] Accordingly, large polypeptides may be assembled by stepwise ligation on a solid support. Figure 10 shows one possible strategy.[207] A solid-supported peptide thioester having the α-amino group of the N-terminal cysteine protected with a base-labile Msc group is first cleaved from the support, using the N-terminal amino group of the peptide to be ligated as a nucleophile. In the second step, the newly formed peptide is, after deprotection of the terminal amino group, used as a nucleophile to ligate to the next solid-supported peptide thioester. Another strategy involves several consecutive ligations on a single support, either by an attack of the terminal cysteine residue of the peptide to be ligated on the thioester function of an N-terminal immobilised peptide (Fig. 11a) or by an attack of the N-terminal cysteine of a support-bound peptide on the thioester group of a peptide in solution (Fig. 11b).[209] Upon completion of the ligation, the linker is cleaved by aqueous bases. Several consecutive ligations have also been carried out to a support-bound terminal cysteine using a safety catch amide linker (Fig. 12).[210] The linker is stable in its sulfoxide form under a wide range of conditions, but becomes acid labile upon reduction to the aryl sulfide.[211]

As indicated above, peptide thioesters are usually prepared by the Boc chemistry, since the thioester bond is cleaved by the piperidine treatment normally used in the Fmoc chemistry. It has, however, been reported that deblocking with a mixture of 1-methylpyrrolidine, hexamethyleneimine and HOBt in a mixture of NMP and DMSO allows the synthesis by the Fmoc chemistry.[212] Peptide thioesters have also been prepared[213] on an alkanesulfonamide linker.[214] Upon completion of the chain assembly, the sulfonamido nitrogen is alkylated with iodoacetonitrile or trimethylsilyldiazomethane, which converts the linker sensitive to nucleophiles. Accordingly, cleavage with an appropriate thiol in the presence of thiophenoxide ion yields peptide thioesters.

An older, but still useful, method for the C-terminal conjugation of peptides on a solid support is the cleavage of peptides from Kaiser's oxime linker (**45**) with nucleophiles.[215,216] The aminolysis of the oxime ester bond may be catalysed by carboxylic acids, and numerous peptide alkylamides have been obtained in this manner.[217,218] Peptide esters can additionally be used as the cleaving amines, since only a relatively small excess of the nucleophile is needed.[219] HOBt has also been used as a cleaving nucleophile,[220] and this allows the release of the peptide as an active ester, which may then be immediately attached to the terminal amino group of another support-bound peptide. Alternatively, peptide *N*-alkylamides may be prepared by assembling the peptide on a photo-labile 4-alkylaminomethyl-3-nitrobenzoyl linker.[221] Peptide hydroxamic acids have been obtained by cleavage of a support-bound peptide thioester with *O*-trimethylsilylhydroxylamine.[222]

The backbone amide linker (**42**) strategy, based on immobilisation of the C-terminal amino acid in a carboxy-protected form through the α-amino group by reductive amination to a benzaldehyde-derived linker and acylation of the penultimate amino acid to the secondary nitrogen obtained (Fig. 13), has been applied to synthesis of a variety of C-terminal-modified peptides.[223] When the terminal carboxy group is protected with an orthogonal protecting group, such as an allyl group, this group may be deprotected after the chain assembly on the linker-bound amino group, keeping the peptide still anchored to the resin. Accordingly, the C-terminus is available for further derivatisation on a support.[224–226] A similar situation is achieved by applying the method of internal resin capture (Fig. 14).[227,228] The key feature of this method is an N^{ε}-Alloc-protected lysine residue connecting an HMPB or a Rink linker to aminomethylpolystyrene. The peptides assembled on this linker are capped with an Alloc-protected 4-(carboxymethoxy)benzyloxycarbonyl group. After removal of the Alloc protection, a cyclisation is achieved, the HMPB or Rink linker is cleaved, and the C-terminus is exposed for further manipulation on a support.

Finally, it is worth noting that sometimes a C-terminal conjugate is conveniently obtained by immobilising the conjugate group to a support via an orthogonal linker and assembling the peptide chain on an amino function of this conjugate group. 1,2,3,4-Tetrahydroisoquinoline, for example, has been attached to a vinylsulfonyl linker by Michael addition of the secondary amino group and the peptide is assembled on its 5-aminoalkoxy substituent.[229–231] After completion of the peptide synthesis, the isoquinoline nitrogen atom is quaternised by alkylation and cleaved from the resin by Hoffman elimination.

Peptide aldehydes are useful starting materials for C-terminal conjugation in solution and, their solid-supported synthesis in also of considerable interest. The most straightforward method of synthesis is either the Boc[232] or Fmoc[233] chemistry on a semicarbazide linker, to which the C-terminal aldehyde is immobilised. The linker withstands anhydrous TFA, but it may be cleaved by acid-catalysed hydrolysis. Another closely related approach involves the immobilisation of the C-terminal residue as an aldehyde to a support-bound serine or cysteine, giving a solid-supported oxazolidinyl[234,235] and thiazolidinyl[236] linker, respectively. The peptide chain is assembled by the Fmoc chemistry on the oxazolidinyl linker and by the Boc chemistry on the thiazolidinyl linker. Alternatively, the C-terminal aldehyde may be reacted with L-cysteine in solution and the resulting *N*-methyl-1,3-thiazolidine-4-carboxylic acid is then immobilized to an amino-support.[237] The oxazolidine ring can be hydrolysed with dilute acids and the thiazolidine ring with copper salts, which releases the peptide aldehyde. A 10,11-dihydroxyundecanoic acid linker has been used for the immobilisation of the C-terminal aldehyde as an acetal.[238]

Weinreb amide- (**68**)[239,240] and phenolic ester- (**69**)[239] based linkers have also been used to obtain peptide aldehydes. The C-terminal residue is immobilised as a carboxylic acid and the assembled peptide is cleaved reductively with $LiAlH_4$ (from **68**) or $LiAlH(OtBu)_3$ (from **69**). On using the phenolic ester linker (**69**), the peptide alcohol is formed as a by-product and, additionally, side-chain carboxy functions protected as esters may also be reduced upon cleavage from the resin and, hence, such protections must be removed before cleavage. Peptide

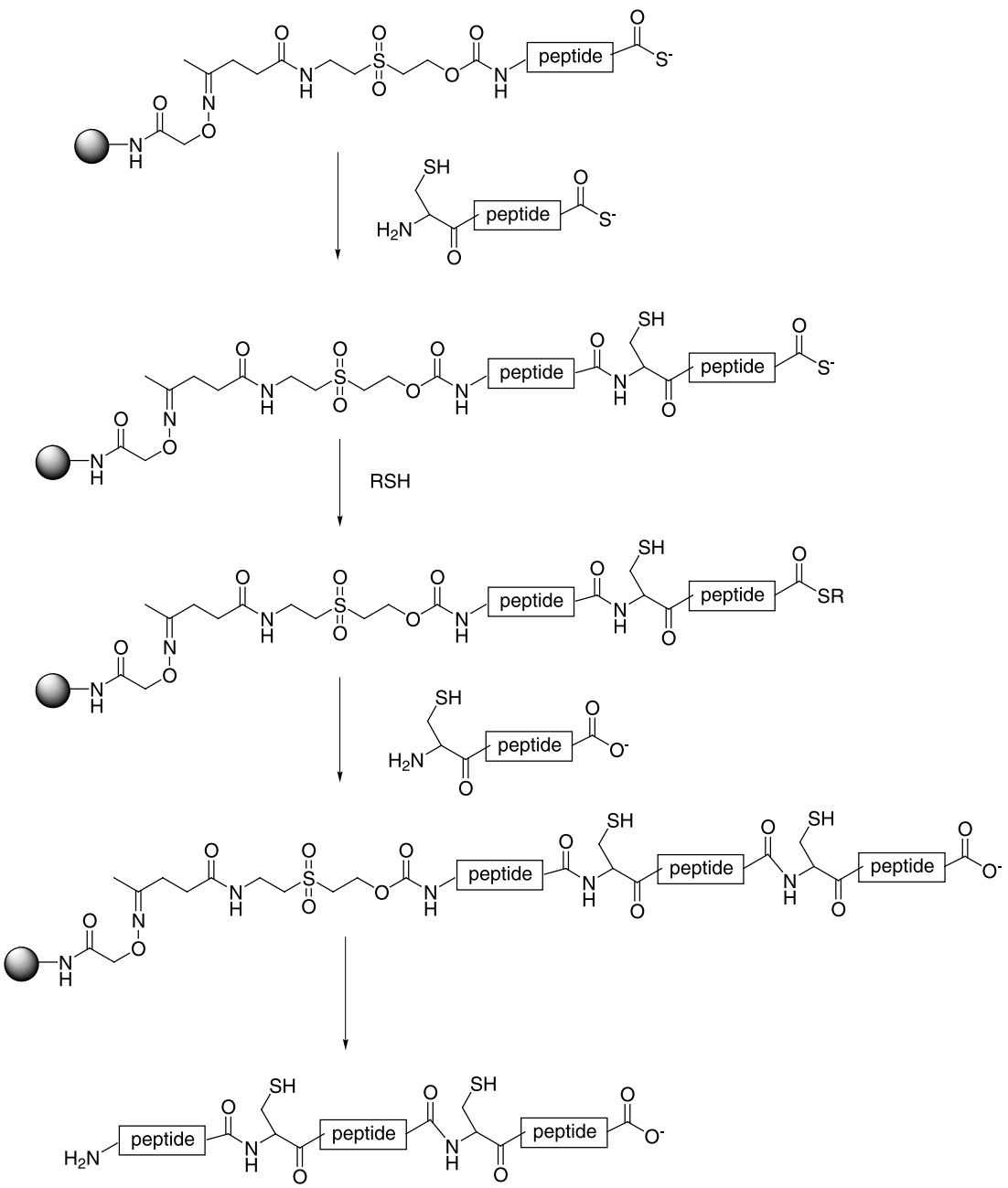
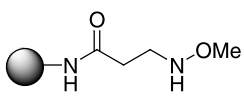


Figure 11. Native ligation on a solid support.[209]

aldehydes have also been prepared as the more stable semicarbazones on a dibenzosuberyl linker (**70**), which is cleaved with TFA.[241] Cleavage from a 4-hydroxymethylphenylacetic acid linker with aminoacetaldehyde–dimethylacetal gives, in turn, peptide aldehydes as acetals.[242] Solid-supported α,β-unsaturated δ-amino acids have also been used as a C-terminal linker.[243–245] The peptide chain is assembled on this linker, and the double



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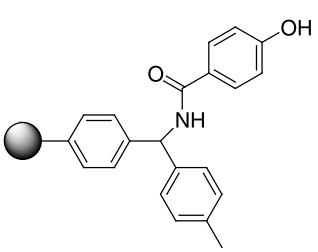

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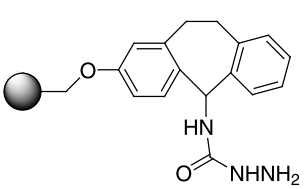

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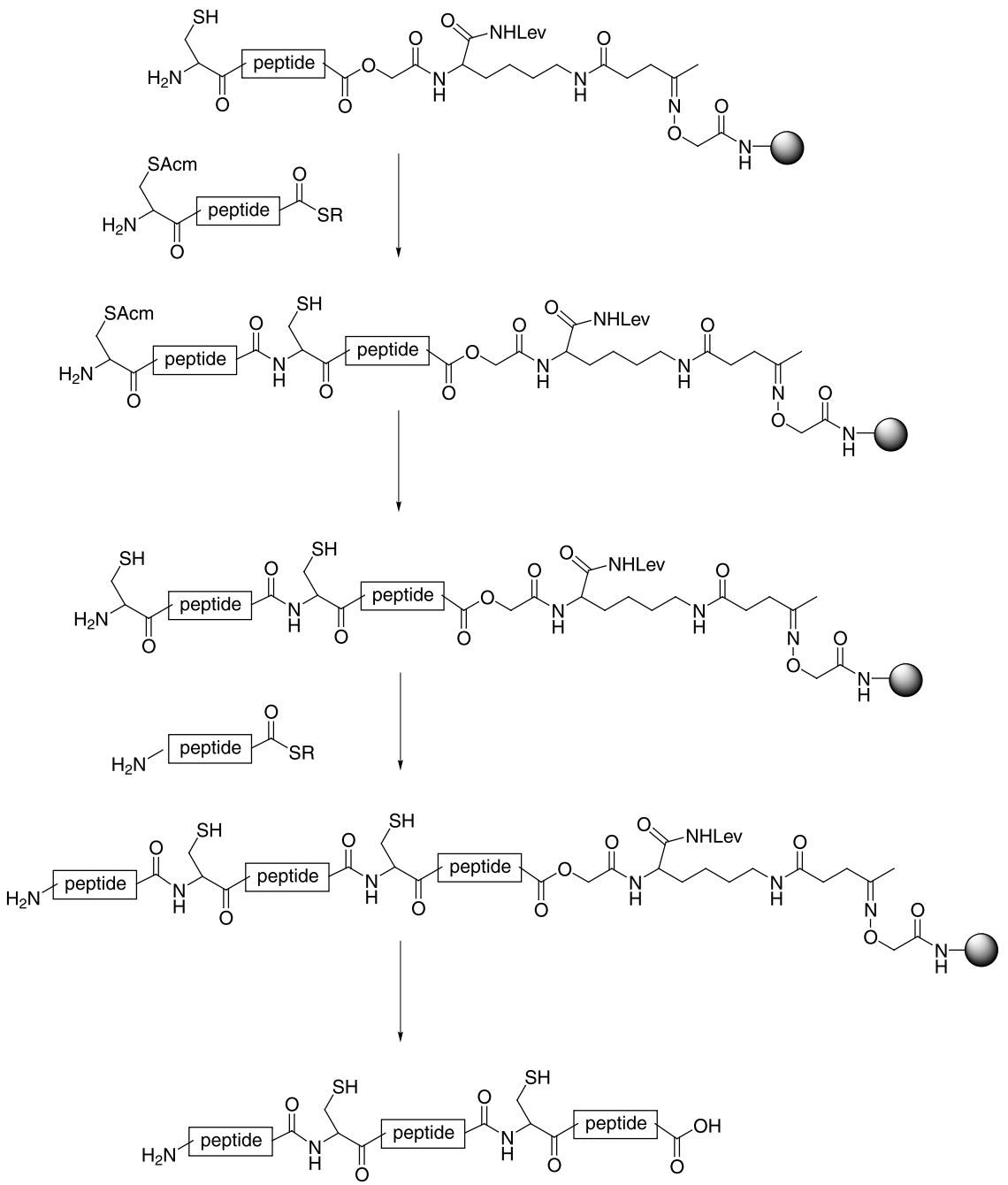


Figure 11 (*continued*)

bond is oxidised with ozone to an ozonide. The latter derivative is cleaved by consecutive treatments with thiourea and dimethyl sulfide to release the peptide aldehyde. 2,3-*O*-Isopropylidene-D-tartrate has been used as a linker for the solid-support synthesis of C-terminal peptide α-oxo aldehydes.[246] After completion of the peptide elongation, the linker is deprotected together with the peptide and the exposed 1,2-diol is cleaved by periodate ion oxidation.

3.4. Phosphopeptides

Phosphopeptides have been prepared both by using appropriately protected prephosphorylated serine, threonine or tyrosine building blocks or by utilising post-synthetic phosphorylation. As far as the building block approach is concerned, the introduction of tyrosine appears to be the least susceptible to complications. *O*,*O*-Dimethyl-,[247–250] *O*,*O*-dibenzyl-,[251–253] and *O*,*O*-di-*t*-butyl[252,254] protected phosphotyrosine monomers have all been successfully employed in a solid-support synthesis. While the benzyl and *t*-butyl protections are removed during normal cleavage from the support with TFA and scavengers, the methyl protections are more stable and a separate on-resin demethylation with Me_3SiI in MeCN has been recommended.[255] The fully protected phosphate group tends to undergo cleavage to the phosphodiester upon removal of the Fmoc protecting group with piperidine. This side reaction

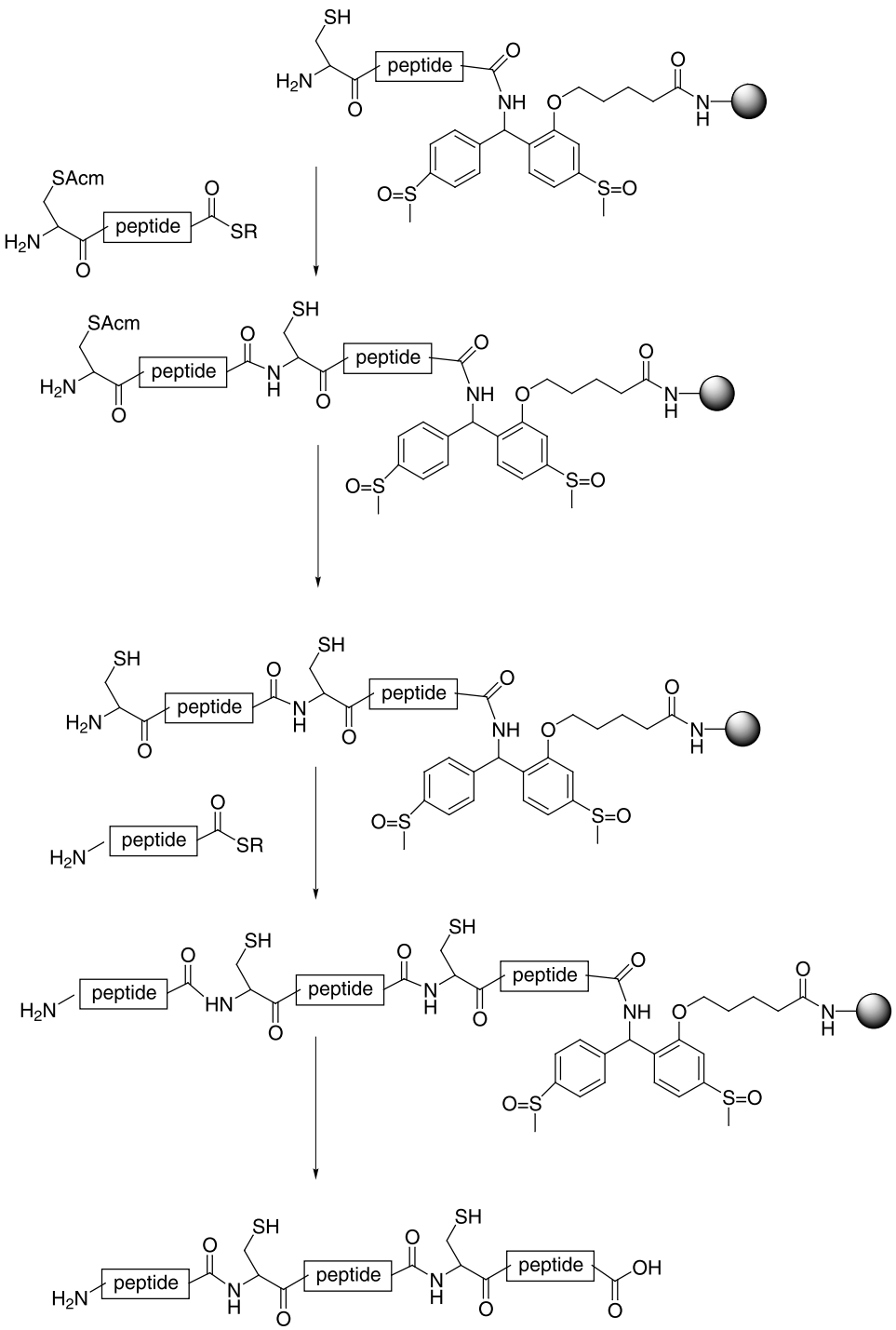


Figure 12. Native ligation on a solid support.[210]

may almost entirely be avoided by using DBU for the Fmoc deprotection.[256] More recently, *N*,*N*-dimethylamino[257] and *N*,*N*-diisopropylamino[258] groups have been introduced for phosphate protection. These bis(phosphoramidates) are stable against nucleophiles, such as piperidine, and can be hydrolysed with 95% TFA. 2-(Trialkylsilyl)ethyl triester protection has, in turn, been recommended for the preparation of tryptophan-containing sequences, since the high β-elimination propensity of these alkyl groups minimises the undesired alkylation of the indole residue and they may be cleaved with mild TFA treatment.[259] Finally, it is worth noting that phosphotyrosine may also be used in a solid-support synthesis without phosphate protection, as long as relatively short peptides are employed.[260–262] Some intermolecular pyrophosphate formation may take place, as well as transient O-acylation of the phosphate group.[263,264] Very recently, the use of the monobenzyl diester has been preferred over the triester protection.[265,266]

The synthesis of phosphoserine- and phosphothreonine-containing peptides by the prephosphorylated building block approach is complicated. The protected phosphotriesters are unstable, undergoing facile β-elimination under

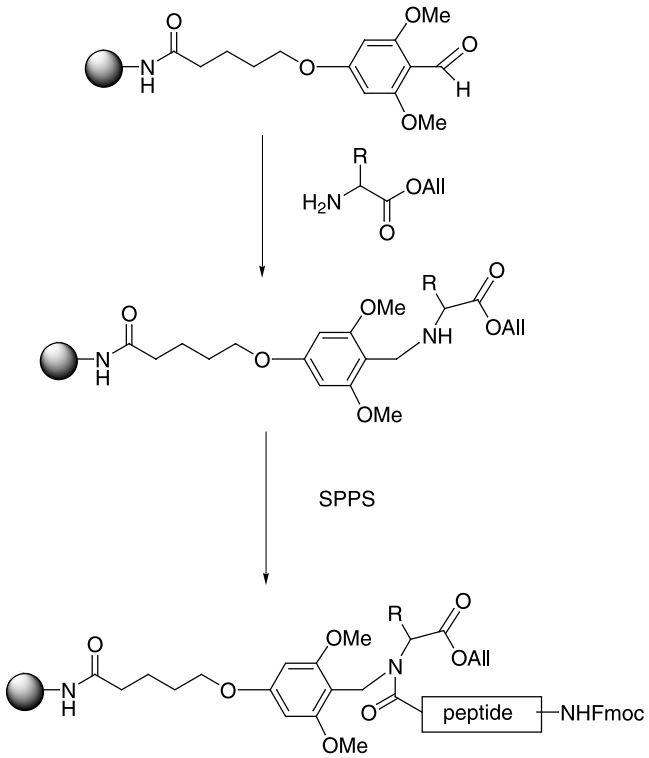


Figure 13. C-Terminal modifications on a BAL linker.[223]

basic conditions. In order to avoid this side reaction, the Boc chemistry was preferred in earlier work.[249,267] Alternatively, an Alloc group has been used for the N^{α}-protection.[268] This group is removed by hydrostannolysis with tributyltin hydride, $Pd^0(PPh_3)_4$ and acetic acid in CH_2Cl_2 and, hence, the basic conditions are avoided. Segment coupling after incorporation of an unprotected phosphoserine residue has also been utilised.[269,270] Nevertheless, as with phosphotyrosine, the monobenzyl phosphodiester is nowadays widely regarded as the building block of choice for phosphoserine and threonine residues.[271–276] Diesters undergo β-elimination much less readily than the triesters.

As mentioned above, an alternative method for the preparation of phosphopeptides involves the post-synthetic introduction of a protected phosphate group into a side-chain hydroxy function of a solid-supported peptide. The free hydroxyl group is obtained either by using a side-chain unprotected amino acid in the chain assembly or by utilising an orthogonally removable protecting group, such as a fluoride ion-sensitive TBDMS group.[277] For the introduction of the phosphate group, dialkyl-[278] and diaryl-phosphorochloridates,[279] alkyl H-phosphonates,[280,281] and dialkylphosphoramidites have been used. The latter reagents are by far the most frequently used and dimethyl,[282] dibenzyl,[283,284] bis(*p*-chlorobenzyl),[285] di-*tert*-butyl,[286–288] *tert*-butyl 2-cyanoethyl[289] and bis(2-trimethylsilylethyl)[290] groups have been introduced in this manner and subsequently oxidised to a phosphate ester. The H-phosphonates may be formed as side products by the monodealkylation of the phosphite triester intermediate. Oxidation with iodine converts the H-phosphonate diesters to the desired phosphate diesters,[291] but the methionine, cysteine and tryptophan residues may be simultaneously oxidised. It has been suggested that these oxidative side reactions may be minimised by using anhydrous *tert*-butyl hydroperoxide under argon as the oxidising agent.[292] *tert*-Butyl esters undergo cleavage less readily than the benzyl esters to the H-phosphonate byproducts. The asymmetrically protected phosphoramidite, tBuOP(OCH_2CH_2-CN)N^{i}Pr$_2$, can be converted to a monoprotected form after oxidation. The advantage of the asymmetrical *tert*-butyl 2-cyanoethyl triester is that both the 2-cyanoethyl and the Fmoc groups can be removed in a single step with 20% DBU in CH_2Cl_2. The resulting phosphodiester is not prone to β-elimination and, hence, the exposed amino terminus can be used for further peptide chain elongation. A recently reported method represents a combination of the building block strategy and post-synthetic phosphorylation. Serine or tyrosine is introduced without hydroxy group protection, but, immediately after incorporation, the hydroxy function is phosphitylated with *tert*-butyl H-phosphonate, oxidised with iodine, and chain elongation by the Fmoc chemistry is continued.[281]

Peptides bearing a thiophosphate group have also been obtained by a post-synthetic phosphitylation and subsequent sulfurisation. For the sulfurisation, phenylacetyl disulfide,[293] S_8/CS_2, tetraethylthiuram disulfide[294] and dibenzoyl tetrasulfide[295] have been employed. The building block approach has been attempted for thiophosphorylated tyrosine, but the method seems to be prone to several side reactions.[296]

4. Oligonucleotide peptide conjugates

4.1. Stepwise chain assembly

Peptide conjugates of ODNs have gained a special interest during the past decade, since it was hoped that the peptides which are known to be actively transported through a cellular membrane could be exploited as carriers of antisense oligonucleotides into the cell. These are structural analogues of natural ODNs which are stabilised towards enzymatic degradation and which are aimed at silencing gene expression by forming a double helix highly selectively with a given messenger RNA. Although the solid-phase synthesis of both oligonucleotides and peptides has been well established, it is still a considerable challenge to assemble oligonucleotide–peptide conjugates from monomeric units on a single support. A generally applicable method for the preparation of 3′-peptide conjugates of ODNs has recently been described.[297] An LCAA-CPG support is first acylated with glycollic acid and the solid-supported hydroxy function is then esterified with N^{α}-Fmoc-N^{ε}-Boc-lysine. The peptide moiety is assembled by the normal Fmoc chemistry on the α-amino group of the lysine handle. The acid-labile side-chain protections, including Tr on histidine, Boc on tryptophan and *t*Bu on serine, are removed, together with the Boc protection of the lysine handle, and the exposed ε-amino group is acylated with DMTr-protected glycollic acid. The unprotected side-chain functionalities of the peptide moiety are then acetylated and the oligonucleotide is assembled on the detritylated hydroxy function of the glycollic acid residue by the phosphoramidite strategy. The conjugate obtained is

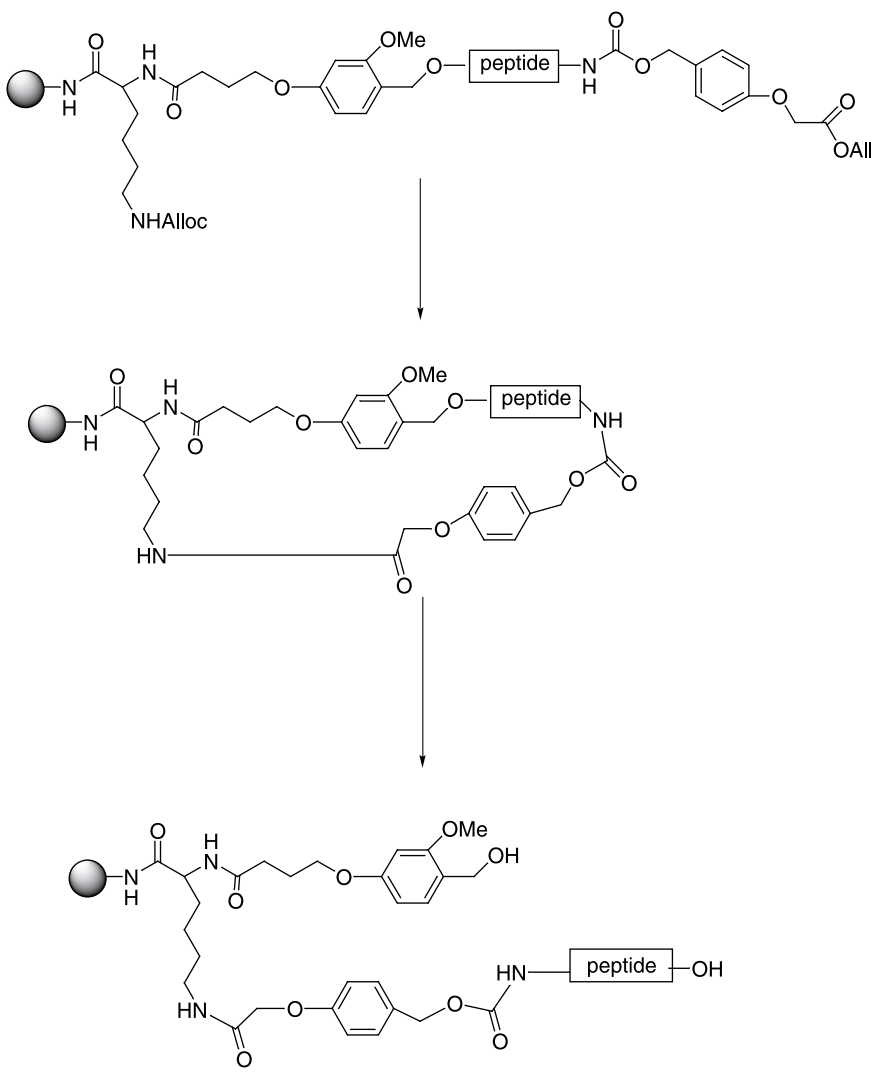


Figure 14. Method of internal resin capture.[227,228]

deprotected and cleaved from the support with aqueous sodium hydroxide.

Several more straightforward, but less generally applicable, methods have been developed. As far as peptide moieties containing no side-chain fuctionalities are concerned, the synthesis may be carried out on a branched linker bearing an Fmoc-protected amino group and a DMTr-protected hydroxy function. The peptide moiety is first assembled by the Fmoc chemistry and the ODN moiety then by the phosphoramidite chemistry. An early example of such an approach is provided by the synthesis on linker **71** in Figure 15, from which the conjugate is cleaved by successive treatments with en in ethanol and IO_4^- in aqueous acetonitrile.[298] More recently, the linkers **72**[299] and **73**[300,301] that both allow cleavage by normal ammonolysis have been employed. In fact, the amino acids incorporated may even contain side chain functionalities, but only those that can be protected with orthogonal or base-labile groups which still withstand the Fmoc removal. Some acid-labile groups have additionally been incorporated. On using the linker **74**, for example, the side chains of cysteine, lysine and serine have been protected with Tr, Boc and TBDMS groups, respectively, and these protections have been removed after the ODN assembly, but before the ammonolysis.[302] Conjugates containing a 13-mer peptide moiety have been prepared in this manner.

The Fmoc chemistry has been used to assemble the peptide moiety on an *N*-(10-hydroxydecanoyl) linker (**75**) using Dde protection for the lysine side-chain amino protection and another 10-hydroxydecanoyl unit to link the peptide and the ODN moieties.[303] A closely related approach involves the consecutive acylation of two molecules of 6-aminohexanoic acid to 3-aminopropyl-CPG (**76**), peptide synthesis by the Fmoc chemistry and acylation of the terminal amino group with 4-hydroxybutyric acid to allow the assembly of the ODN chain.[304] *N*-(4-Hydroxybutyryl)-3-aminopropyl-CPG (**77**) has also been employed as a support and the 4-hydroxybutanoyl or 4-[(6-hydroxyhexyl)amino]-4-oxobutanoyl group as a spacer.[305]

Another possibility is to assemble the peptide moiety first by the Boc chemistry, insert into the amino terminus a linker that affords a hydroxy function for the ODN synthesis and then assemble the ODN chain. A recent example of such an approach consists of the treatment of 3-aminopropyl-CPG with γ-butyrolactone, esterification of the resulting hydroxy

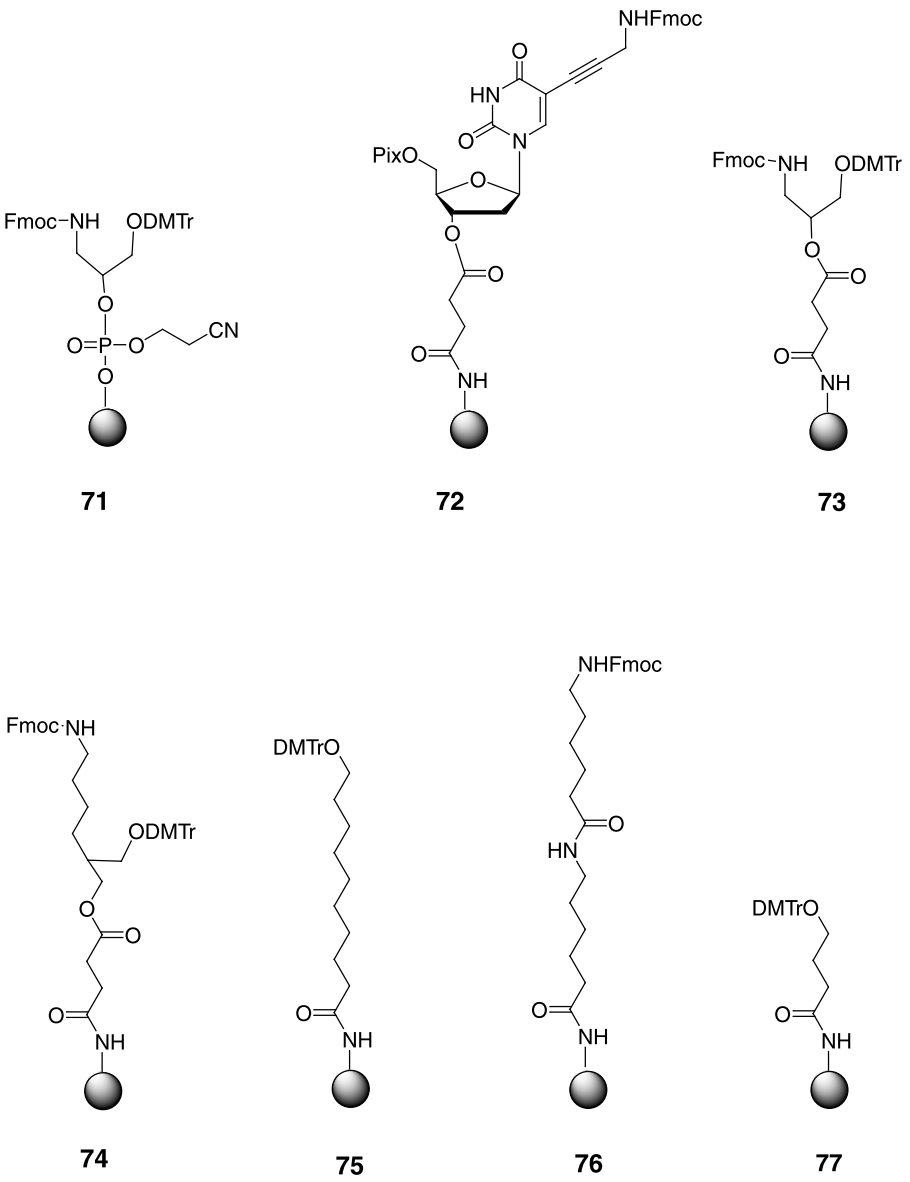


Figure 15. Linkers used for the preparation of oligonucleotide–peptide conjugates.[298–305]

function with N^{α}-Fmoc-N^{ε}-Boc-lysine and assembly of the peptide chain to the ε-amino group.[306] 4-Hydroxybenzoic acid is then acylated to the terminal amino group and the oligonucleotide chain is assembled on the phenolic hydroxy group. Unfortunately, acid-labile side-chain protections cannot be used. A PEG–PS support derivatised with an *o*-nitrophenylethanol handle has been employed similarly, using base-labile Fmoc and Fm groups for the side-chain protection and *N*-succinyl-6-aminohexanol as a spacer between the peptide and the oligonucleotide moiety.[307,308] The other examples include synthesis on *N*-(10-hydroxydecanoyl)-3-aminopropyl-CPG using Fmoc and Ts groups for the side-chain protection for lysine and histidine, respectively, and another 10-hydroxydecanoyl unit as a spacer between the peptide and the ODN moieties.[309] *N*-(4-Hydroxybutyryl)-3-aminopropyl-CPG has also been used as a support and 4-[(6-hydroxyhexyl)amino]-4-oxobutanoyl group as a spacer.[310] 3′-Conjugates of a minor groove-binding agent, dihydropyrroloindole tripeptide, have been synthesised on a 3-aminopropanol linker esterified to succinylated LCAA-CPG, using 4-hydroxybutyric acid to provide the starting point for the ODN chain assembly.[311]

A closely related stepwise method consists of peptide synthesis by the Boc chemistry on a 2-nitrophenylethanol or 9-fluorenylmethanol linker (**43**) and subsequent ODN chain assembly on a side-chain functionality of the N-terminal amino acid, viz. tyrosine, threonine or serine.[312–314] The following side-chain protecting groups have been employed: Fm for aspartic acid,[315,316] Fmoc or trifluoroacetyl for lysine, Fmoc for arginine, formyl for tryptophan and acetyl for serine, threonine and tryptophan.[317] Methionine containing conjugates are obtained by introducing methionine as a sulfoxide and reducing it back to methionine post-synthetically.[318]

The ODN chain may also be assembled in the inverse 5′→3′ direction and the peptide moiety containing a serine phosphoramidite is then coupled to the 3′-terminal hydroxy function of the support-bound chain.[319] In order to avoid

deprotection under drastically basic conditions, allyl protection has been utilised for the phosphate linkages and the C-terminus of the peptide and Alloc protection for the nucleoside bases and the N-terminus of the peptide. Another similar method involves immobilisation of 3′-*O*-TBDMS-5′-*O*-DMTr-2′-deoxycytidine through the N^4-atom to a succinylated support, assembly of the ODN moiety in the inverse direction on the 5′-hydroxy group, phosphitylation of the 3′-hydroxy group with 6-aminohexylphosphoramidite and synthesis of the peptide moiety on the amino group by the Fmoc chemistry.[320] It is also possible to insert 5′-amino-5′-deoxythymidine at the 5′-terminus of the support-anchored ODN and elongate the chain by peptide synthesis using either stepwise or fragment coupling.[321] By this approach, 5′-terminal PNA-conjugates have also been prepared.[322] 5′-Amino-5′-deoxythymidine phosphoramidite has additionally been exploited in the preparation of peptide–ODN hybrids consisting of two terminal ODN sequences and an intervening peptide joined to each other without any additional linker.[323] The 3′-ODN segment is first assembled on a sarcosine linker by the phosphoramidite chemistry, using 5′-*N*-MMTr-5′-amino-5′-deoxythymidine 3′-allyl-*N*,*N*-diisopropylphosphoramidite as the last building block. The peptide sequence is then assembled on the deprotected amino group by the Fmoc chemistry, and the 5′-terminal ODN sequence is assembled on the terminal amino group. The use of an allyl protecting group is essential, since it is compatible with the Fmoc peptide chemistry when DBU is used for the removal of the Fmoc group.

3′,5′-Dipeptidyl ODNs have been prepared on a CPG support bearing ω-hydroxylauric acid linkers.[324] The C-terminal amino acid of the 3′-peptide moiety is esterified to the support-bound hydroxy groups, and the peptide synthesis is terminated by N-acylation with 3-hydroxy-2,2-dimethylpropionic acid. The ODN chain is then assembled on the hydroxy function by the phosphoramidite strategy, using 5′-amino-5′-deoxythymidine as the 5′-terminal nucleoside. The latter unit allows the attachment of the 5′-terminal peptide through a direct amide link. This approach has also been applied to obtain 3′,5′-dipeptide conjugates where the 5′-bonded peptide has a branched trilysyl skeleton bearing quinoline derived intercalators.[325] This type of conjugates have also been cyclised by HBTU/HOBt-induced peptide bond formation between the 3′- and 5′-linked peptides.[326] A closely related approach has been applied to obtain ODN-3′-PNA[322,327] and 2′,5′-oligo(A)-3′-PNA[328] chimeras which exploits commercially unavailable PNA building blocks having base-labile protecting groups at the amino functions of the base moiety. More recently, a protocol using commercially available building blocks having Bhoc protection at the anino functions of the nucleic acid bases has been introduced.[329] The key point is that, after the assembly of the PNA sequence by the Fmoc chemistry, the Bhoc groups are removed with TFA and replaced with conventional benzoyl protections on the support. Finally, the terminal Fmoc group is removed, and the ODN chain is assembled on the exposed amino group.

4.2. Fragmental coupling

ODN–peptide conjugates have also been prepared by fragmental coupling on a solid support. The ODN has, for example, first been assembled on an LCAA-CPG, an α,ω-alkanediamine is tethered to the 5′-hydroxy function by 1,1′-carbonyldiimidazole activation and a prepared N^{α}-protected peptide is coupled to the amino group by PyBOP/HOBt-promoted peptide bond formation.[330] Another widely applicable conjugation method involves the coupling of presynthesised peptides to the *N*-(3-aminopropanoyl) side arm of a 2′-amino-2′-deoxy-arabino nucleotide within an otherwise protected oligonucleotide chain that is still attached to a solid support.[331]

5. Glycopeptides

5.1. General

Glycopeptides constitute another class of bioconjugates that consists of monomeric units of two different biopolymers. Such compounds have received increasing interest after identification of the carbohydrate moieties of glycoproteins as antigenic epitopes on viruses and cancer cells and the discovery that the pharmacological and pharmacokinetic properties of biologically active peptides may be tuned by glycosylation. Usually, glycopeptides are obtained by a solid-supported assembly of appropriately protected building blocks of glycosylated amino acids. The presence of an acid-labile glycosidic linkage and numerous hydroxy functions, however, necessitates some changes to the conventional protocols of solid-phase peptide synthesis.

5.2. Linkers and coupling

The linkers used for the synthesis of glycopeptides are those generally employed for peptide synthesis and are discussed in Section 3. The acid lability of the glycosidic bond excludes linkers which require hydrogen fluoride for cleavage, but several more acid-labile linkers may still be used. These include Wang (**35**), HMPA (**36**), MBHA (**39**), PAL (**41**) and Rink (**38** and **40**) linkers. With these linkers, cleavage from the support is achieved with 95% TFA in the presence of scavengers. The PAL (**41**) and Rink amide (**40**) linkers yield the peptides as the amides and the rest as the free acids. Glycosidic bonds tolerate such conditions for up to 2 h. In addition to these extensively employed acid-labile linkers, those which may be cleaved under orthogonal conditions and are, hence, compatible with both the Fmoc and Boc chemistry are of special interest for glycopeptide synthesis. Such linkers include allylic linkers, such as HYCRON (**46**), cleaved with Pd(0)-catalysts,[332,333] and silyl ether-based linkers cleaved with fluoride ion.[334] Since a sugar or serine side-chain hydroxy group is used for the immobilisation, both a stepwise elongation of the N-terminus and a fragmental coupling at the C-terminus is possible.

Usually, the conventional activators, including DCC, DIC, HBTU, TBTU, HATU, BOP and PyBOP, are used for coupling. In addition to these agents, a novel reagent, *N*,*N*-*N*′,*N*′-bis(tetramethylene)-*O*-pentafluorophenyluronium hexafluorophosphate (PfPyU), has recently been suggested to be particularly suitable for the synthesis of glycopeptides, exhibiting a reactivity comparable to that of HATU and 8-fold higher than that of TBTU.[335,336] Another interesting coupling method useful in glycopeptide synthesis is the use

dimethylphosphinothioic mixed anhydrides (Mpt-MA). On applying this method, no protection for the sugar hydroxy groups is needed.[337]

5.3. Protecting groups

Some early studies apart,[332,338,339] glycopeptides have been prepared by the Fmoc strategy. It has been shown that removal of the N^{α}-Fmoc group by 50% piperidine, or even by the more effective 2% DBU in DMF, does not result in β-elimination and the Fmoc chemistry may, hence, be safely used for assembling glycopeptides.[340]

Glycosyl amino acids are typically coupled as the active esters of carboxylic acids generated in situ from HOBt or HOAt with the aid of an appropriate activator. Another extensively used approach, originally introduced by Meldal and Jensen,[341] is the use of prefabricated Pfp esters. The Pfp ester serves the dual purpose of a protecting group during glycosylation of a monomeric amino acid and an activator for the peptide bond formation.

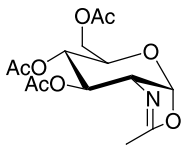
Base-labile acetyl and benzoyl groups are usually used for the protection of the sugar hydroxy groups of the glycosylated amino acids. Several examples of such syntheses are discussed in Section 5.5. The electron-withdrawing acyl groups stabilise the glycosidic bond towards the acidic conditions used for the cleavage of the glycopeptide from the resin and deprotection of the amino acid side chains. This is especially important when building blocks derived from acid-labile 2-deoxysugars or keto-sugars are employed in the solid-phase synthesis.[342] Acyl protections, however, also suffer from a serious drawback, namely that, during the coupling of a glycosylated amino acid, acyl migration from oxygen to nitrogen atom may occur. In addition, glycosylated serine and threonine residues may undergo β-elimination upon removal of the acyl protection under basic conditions. For these reasons, protection of the sugar moieties as benzyl ethers has recently been preferred.[343–346] Benzylated sugars readily undergo glycosidation and no *O*⟶*N* acyl migration may take place during the coupling reaction. The benzyl groups can be removed by catalytic hydrogenation under mild conditions. Unfortunately, acidic cleavage of the glycopeptide from the resin leads to several partially debenzylated products and catalytic hydrogenation is incompatible with sulfur-containing amino acids.

Some acid-labile protecting groups have additionally turned out to be suitable for the sugar moiety protection.[347–350] Amino acids bearing carbohydrate moieties protected with TBDMS, isopropylidene and 4-methoxybenzyl groups have been introduced by the Fmoc chemistry and removed upon acidic cleavage from the support. In some cases, glycosylated amino acids have been coupled unprotected.[351–358] In fact, such amino acids have been reported to couple faster[355,356] and to tolerate the TFA treatment without marked cleavage of the *O*-glycosidic bonds, at least, as long as the common monosaccharides are utilised.[356,357] On the other hand, large unprotected glycosyl amino acids are difficult to activate for peptide coupling, because of their low solubility in organic solvents.[357] An on-support segment-coupling of two glycopeptides bearing an unprotected oligosaccharide has been carried out.[359]

5.4. Glycosylations on a solid support

Only few examples of glycosylation of a solid-supported peptide are available. The serine hydroxy group of an otherwise fully protected resin-bound peptide has been glycosylated with 3,4,5-tri-*O*-acetyl-D-glucose oxazoline (**78**).[360] The reaction is, however, rather inefficient, since a repeated two-day treatment has been reported to give yields ranging from 20 to 35%. A comparable yield has been obtained on applying Koenigs–Knorr glycosidation with 2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl bromide (**79**).[361] The latter reaction has been shown to yield only the α-mannosylated conjugate. More recently, a sterically more-hindered threonine hydroxy group has been glycosidated with 1-*O*-(*N*-allylcarbamoyl)-2,3,4,6-tetra-*O*-acetyl-D-glucose (**80**) using *S*-methylbis(methylthio)sulfonium hexachloroantimonate as a promoter.[362] Even in this case, the efficiency of the glycosidation is similar to that observed in the previous studies. Very recently, more promising results have been obtained, the side-chain hydroxy functions of a support-bound peptide being glycosylated with glycosyl trichloroacetimidate donors in high yield.[363]

In striking contrast to the O-glycosidations discussed above, *N*-glycosyl bonds are easily formed on a support because the amide bond formation may be exploited. The aspartic acid side chains of support-bound peptides have been converted into Pfp esters and subjected to acyl substitution by various glycosylamines.[364] The yields are high on using simple mono- or disaccharides and range from 50 to 80% with



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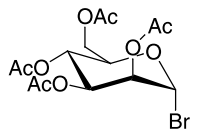

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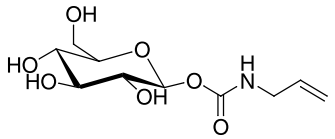

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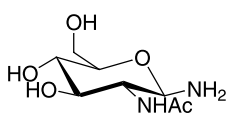

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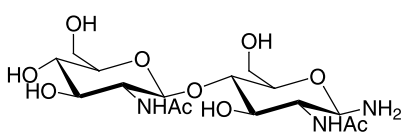

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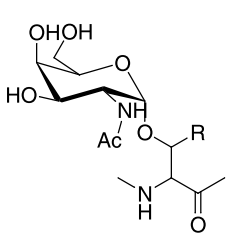
uncharged penta- and heptasaccharides. Charged glycosylamines, such as those derived from galactose sulfates or galacturonic acid, give lower yields. Aspartimide is formed as a side reaction. The side-chain carboxy group of aspartyl residues has also been reacted with *N*-acetyl-β-D-glucoamine (**81**) or *N*,*N*′-diacetylchitobioseamine (**82**) through in situ BOP/HOBt activation.[365] In this case, protection of the aspartyl backbone amide linkage with an AcHmb group has markedly reduced the formation of aspartimide as a byproduct.

5.5. Glycopeptides synthesised

GalNAcα-*O*-Ser/Thr (**83**) is the most common of the *O*-glycosidic bonds found in glycoproteins, and numerous this kind of *O*-glycopeptides containing mono-,[334,340,341,366–368] di-[343,369–373] or trisaccharide[374,375] sugar moieties have been prepared. The most generally applied method involves the assembly of the conjugate by the Fmoc chemistry on a PAL (**41**) or a Rink amide (**40**) linker attached to a kieselguhr-supported polydimethyl acrylamide resin.



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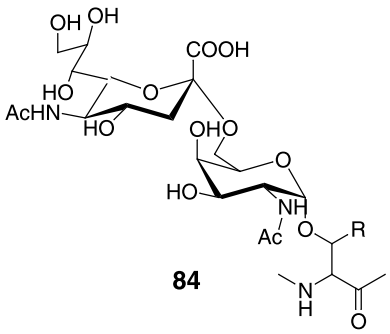

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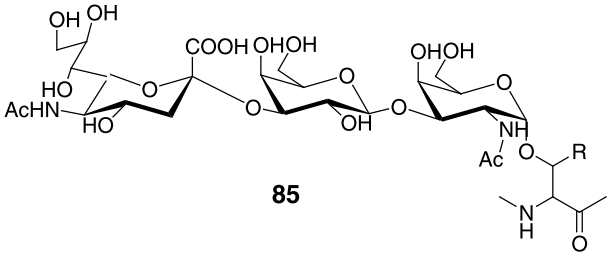

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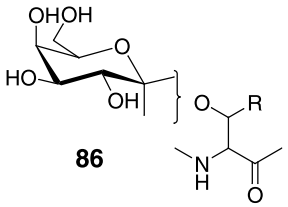

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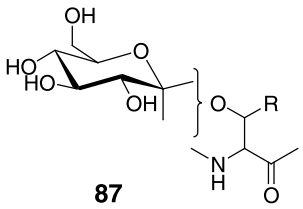

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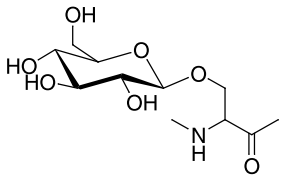

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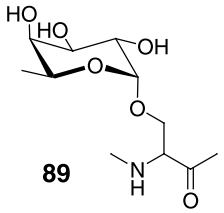

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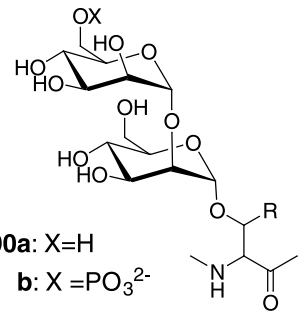
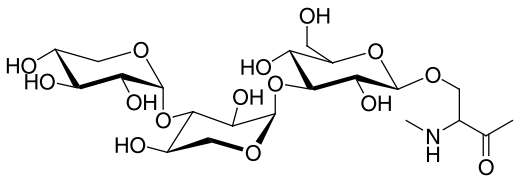


90a: X=H

b: X =PO_3^{2-}



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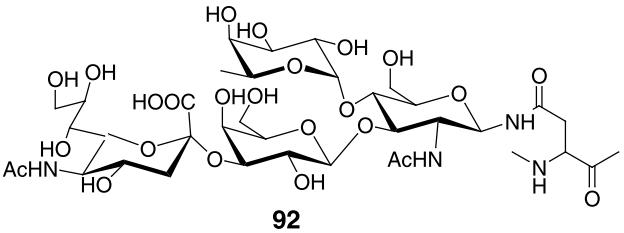

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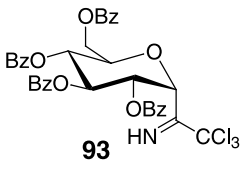

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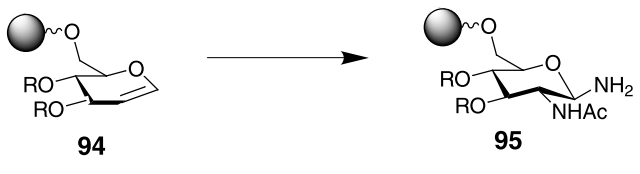
Non-glycosylated amino acids are coupled as Dhbt esters and the glycosylated amino acids as Pfp esters using Dhbt-OH as an auxiliary nucleophile. Amino sugars are introduced as the fully acetylated azido or *N*-Teoc amino derivatives and converted to *N*-acetyl sugars on the support, either with thioacetic acid or with $Zn/Ac_2O/AcOH$. The conjugate is cleaved with TFA and deacetylated with a catalytic amount of sodium methoxide in methanol. Several contiguous glycosylated amino acids have been inserted by this method.[376] More recently, the sialyated derivatives of GalNAcα-*O*-Ser/Thr-containing peptides, viz. NeuNAc(α2→6)GalNAcα-*O*-Ser/Thr (**84**)[377–379] and NeuNAcα(2→3)Galβ(1→3)GalNAcα-*O*-Ser/Thr (**85**),[380] have been synthesised. The latter structures are related to tumour-associated antigens present in glycoproteins on the surface of cancer cells. Ester and acetal protections have been used to mask the sugar moieties and the glycosylated amino acid has usually been coupled as an HOAt ester. HYCRON (**46**),[378,379] Wang (**35**)[380] and Rink amide (**40**)[377] linkers have been used.

The other naturally occurring *O*-glycosidic linkages inserted into peptides on a solid support include Galα,β-*O*-Ser/Thr (**86**),[341,352,381] Glcα,β-*O*-Ser/Thr (**87**),[382,383] GlcNAcβ-*O*-Ser (**88**),[339] and Fucα-*O*-Ser/Thr (**89**).[342,384] For addition, Manα(1→2)Manα-*O*-Ser/Thr (**90a**),[385] its 6-*O*-phosphate (**90b**)[386,387] and Xyl(α1→3)Xyl(α1→3)Glcβ-*O*-Ser (**91**)[357] containing peptides have been prepared by the Fmoc/Pfp chemistry on an HMPA (**36**) or a Rink amide (**40**) linker. Besides serine and threonine, tyrosine,[388] 4-hydroxyproline,[389] 5-hydroxylysine[350,390] and 5-hydroxy-L-norvaline[347,348] have been used as the carriers of glycosyl groups.

Several glycopeptides carrying simple mono- or disaccharide residues, such as Galβ,[354] Glcβ,[338,354] GalNAcβ,[354] GlcNAcβ,[355,391–393] Galβ(1→3)GalNAcα,[375] Galβ (1→3)GlcNAcβ,[333] GlcNAcβ(1→4)GlcNAcβ,[354] Glcα,β(1→4)Glcβ,[349,351,353,394] linked to the amido nitrogen of an asparagine residue have been prepared by methods closely resembling those discussed above. The solid-phase methodology described for the synthesis of peptides containing the GalNAcα-*O*-Ser/Thr linkage, i.e. coupling of the glycosylated N^{α}-Fmoc-protected amino acid as a Pfp ester in the presence of Dhbt-OH to a HMPA anchored peptide, has been successfully applied to the synthesis of glycopeptides containing a large *N*-asparaginyl-linked triantennary undecasaccharide moiety.[395] The sugar hydroxy functions are kept unprotected. A perbenzylated asparagine-linked branched pentasaccharide has been coupled to a solid-supported peptide in high yield using DCC/HOBt activation.[345] A partial sequence of the P-selectin glycoprotein ligand in which the *O*-glycosylated threonine is substituted by a sialyl-Lewis A asparagine moiety (**92**) has been prepared on a HYCRON (**46**) linker using TBTU/HOBt coupling.[396]



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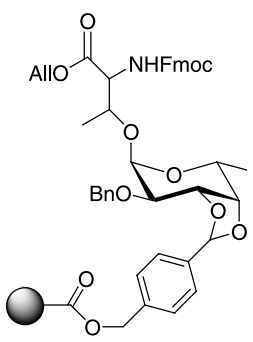

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5.6. Elongation of the oligosaccharide chain on a solid-support

An attractive alternative for the preparation of glycopeptides bearing a large oligosaccharide moiety is elongation of a glycosyl acceptor present in a resin-bound peptide. 8-Mer monoglycosylated peptides having only one of their sugar hydroxy groups unprotected have been successfully glycosylated with perbenzoylated glycosyl trichloroacetimidates (**93**) using Me_3SiOTf as an activator.[397] On using orthogonally removable hydroxy protections, consecutive glycosylations may be carried out.

Enzymatic elongation offers another even more useful approach. The evident advantages of the chemoenzymatic approach are the regio- and stereospecificity of the glycosylation and the avoidance of sugar protection. An early example is offered by the galactosylation of a 2-*N*-acetyl-2-deoxy-β-D-glucopyranosyl group of a support-bound peptide with UDP-galactose catalysed by β-1,4-galactosyltransferase[398] and subsequent sialylation with CMP-sialic acid using α-2,3-sialyltransferase as a catalyst. Finally, the sialylated glycopeptide is released from the support and fucosylated to a sialyl-Lewis X glycopeptide. Later, a more complex *O*-glycopeptide containing sialyl-Lewis X (Sle^X) tetrasaccharide has been prepared by a chemoenzymatic approach.[399] An *O*-glycopeptide containing an O-unprotected GlcNAcβ-*O*-Thr residue is first assembled on an orthogonally cleavable HYCRON (**46**) linker. Both the acid-labile amino acid side-chain protections and the base-labile acyl protections of the sugar moiety are removed and the starting monosaccharide is elongated by a galactosyltransferase-catalysed reaction with UDP-galactose and subsequently by a sialyltransferase-catalysed reaction with CMP-NeuAc. The final enzymatic fucosylation is performed in solution also in this case after a Pd(0)-catalysed cleavage from the support.

5.7. Elongation of the peptide chain on a support-bound oligosaccharide

In principle, glycopeptides may also be prepared by assembling the peptide chain on a functional group of a solid-supported oligosaccharide. Only few examples of this approach are, however, available. An oligosaccharide terminating in a glycal has been synthesised on a solid support utilising the glycal assembly method.[400]

The terminal glycal (**94**) is transformed into *N*-acetylglucosamine (**95**) by iodosulfonamidation with anthracenesulfonamide. The latter derivative can be coupled in a convergent manner to the γ-carboxyl of an aspartyl residue on a prefabricated peptide. A similar approach has been applied to obtain a small library of fucopeptides.[401] An allyl ester of a fucosyl threonine building block is immobilised as an acetal through its 3,4-*cis*-diol to an AMBA linker (**96**). Removal of the allyl group with Pd(0) and dimedone liberates the C-terminus, which may then be modified through esterification or amidation processes. Removal of the Fmoc group from the N-terminus of the glycosylated thereonine and subsequent stepwise amino acid coupling afford the protected fucosylpeptide.

5.8. Neoglycopeptides

In addition to the naturally occurring *O*- and *N*-glycopeptides, some neoglycopeptides have been prepared by solid-phase synthesis. Thioglycosides derived from 3-mercaptopropionic acid have been attached by normal peptide coupling to the N-terminus of support-bound peptides.[402] Similarly, *C*-glycosides functionalised with a carboxy group have been coupled to the N-terminus of solid-supported peptides.[403] Diglycosylated peptides have been obtained by first coupling a *C*-glycoside aldehyde by reductive amination and then a *C*-glycoside carboxylic acid to the terminal secondary amine.[404] Glycosylacetylene-phenylalanine building blocks have been introduced in peptides by the Fmoc chemistry.[405] A solid-supported method based on three orthogonal amino protections has been described for the construction of branched glycopeptides.[406]

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