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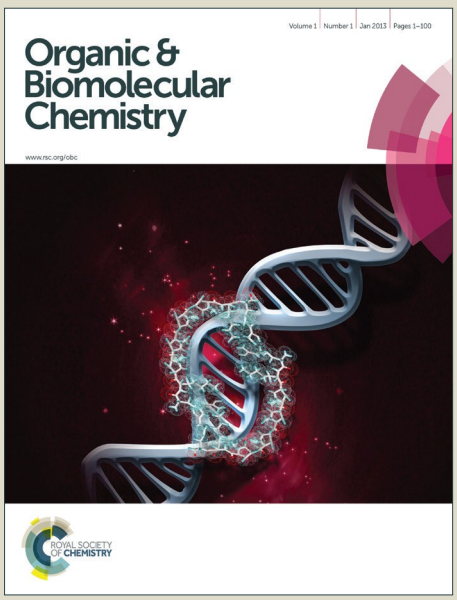

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An Update on New Methods to Synthesize Cyclotetrapeptides

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Cyclotetrapeptides are important bioactive lead drug molecules that display a wide spectrum of pharmacological activities. However, the synthesis of cyclotetrapeptides from their linear precursors is challenging due to the highly constrained conformation required for cyclisation, thus hampering their progress to a clinical setting. This review provides an account of the reported methods used for the synthesis of cyclotetrapeptides.

1. Introduction

Cyclotetrapeptides isolated from natural products are an interesting group of macrocycles which exhibit a variety of bioactivities. Cyclotetrapeptides are attractive pharmacological lead compounds, when compared to cyclopeptides of larger ring size, due to their close compliance to Lipinski's rules (less than 5 hydrogen bond donors, 10 hydrogen bond acceptors and a molecular mass less than 500 Da).[1] Five examples of cyclotetrapeptides with proven biological activity are shown in Figure 1, and these are briefly described herein. AS1387392 (**1**) is an immunosuppressant isolated from *Acremonium sp.* No. 27082, which has a strong inhibitory effect against mammalian histone deacetylase and T-cell proliferation, as well as good oral bioavailability.[2] A structurally similar peptide, chlamydocin (**2**), showed potent antimalarial activity (IC_{50} = 0.06 μM) and was cytotoxic against MCF-7, KB, and NCI-H187 cancer cell lines.[3] Some cyclopeptides containing *N*-methylated residues have been isolated from natural sources; such as hersutide (**3**) and other related analogues (**4**-**6**),[4, 5] which act as cardiac calcium channel blockers. A family of nobilamides which include the cyclodepsipeptide nobilamide D (**7**) were isolated from a mollusc-associated bacterium. Nobilamides are potent antagonists of the vanilloid receptor TRPV1 which responds to multiple pain stimuli.[6] Teixobactin (**8**) is an 11-mer peptide that contains a cyclotetradepsipeptide within its structure and shows potent activity against *M. Tuberculosis* (MIC = 0.1 μM). Teixobactin inhibits the synthesis of important bacterial cell wall components (peptidoglican and teichoic acid) by binding to their lipid precursors. This unique mechanism of action is believed to minimize the development of resistance.[7] Many other examples of cyclotetrapeptides are also found in the literature.[8]

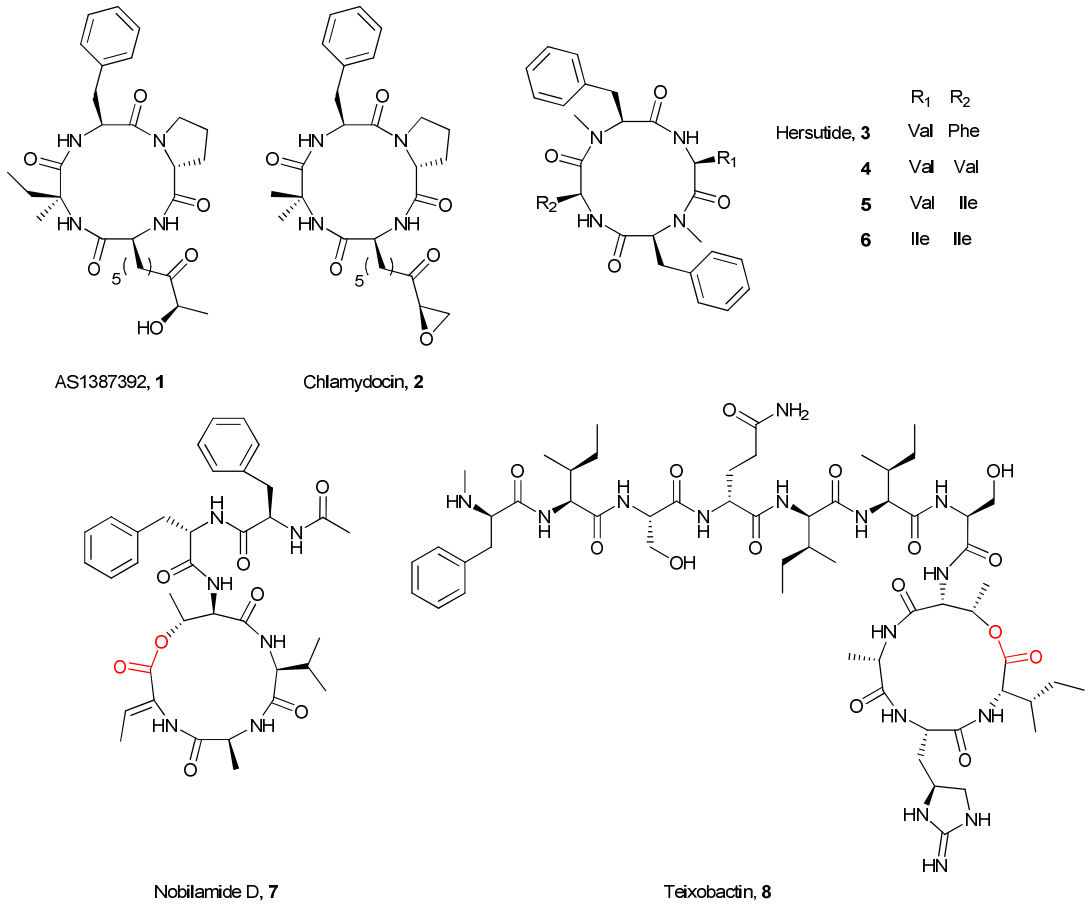


Figure 1. Biologically active cyclotetrapeptides.

Several impressive general reviews on peptide cyclisation strategies can be found in the literature.[9-11] However, this review focuses specifically on the synthesis of the more difficult cyclotetrapeptides, including both previously reported methods and novel approaches.

The cyclisation of tetrapeptides using natural amino acids can lead to the formation of a range of 12- to 18-membered ring homodetic or heterodetic cyclotetrapeptides depending on the mode of the cyclisation (Figure 2). Peptide cyclisation usually occurs via a macrolactamisation or macrolactonisation reaction. The synthesis of 12-membered ring cyclotetrapeptides is particularly challenging given the geometric constraints required for the cyclisation of their linear precursors.[12] Therefore, this review will focus on methods for their preparation however, a brief discussion on the synthesis of 13-membered cyclotetradepsipeptides is also included.

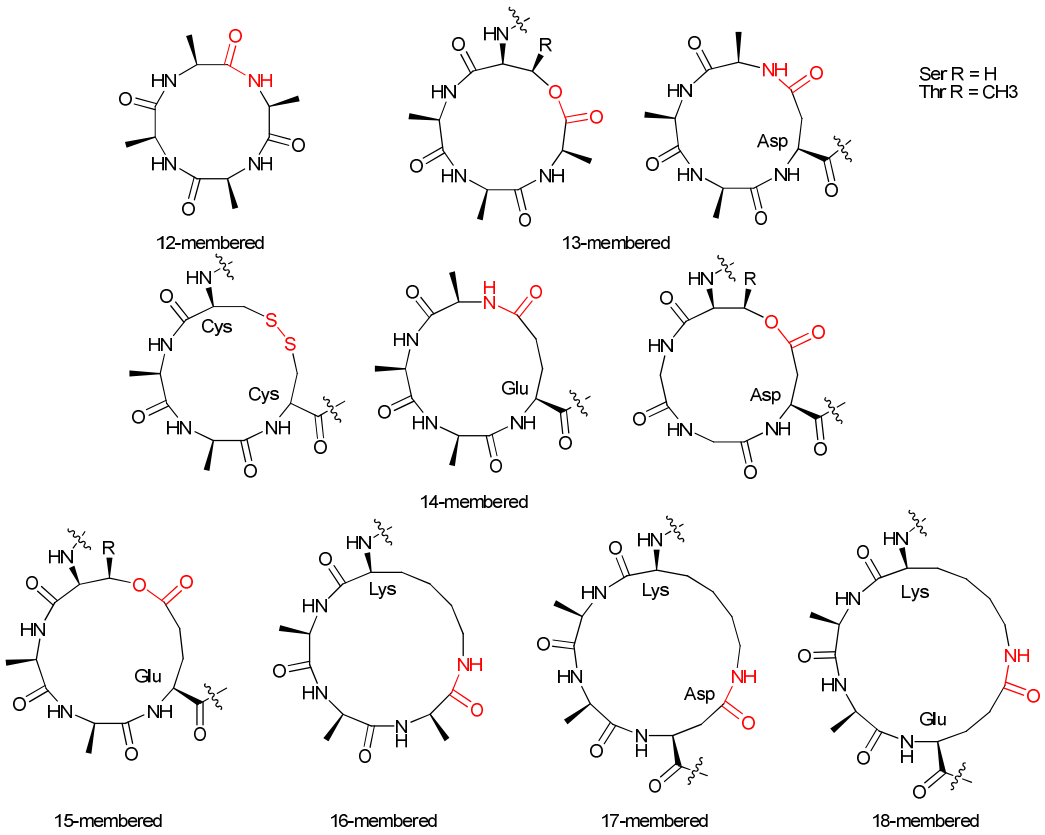


Figure 2. Different possible cyclisation products for a tetrapeptide containing natural amino acids.

An illustrative parameter that measures cyclopeptide strain in relation to the number of amino acids (**n**) within the cyclic structure has been determined by quantum chemical calculations and was subsequently defined by the mean plaque junction energies (MPJE).[12] MPJE is an averaged measure of the energetic cost or gain of joining **n** plaques (each plaque comprised of C^{α}-CO-NH-C^{α} backbone, Figure 3a) through the C^{α} carbon. The MPJE values determined for cyclopeptides of different sizes in an all *cis*-conformation are shown in Figure 3b (dashed line). For small linear peptides an all *cis*-conformation (Figure 3a) is close to ideal to achieve cyclisation,[13] however, adopting this conformation entails a considerable amount of strain (MPJE >+25 kcal/mol). This large MPJE value highlights the problem that synthetic chemists face when trying to prepare cyclic tetrapeptides. Moreover, this strain is less pronounced for smaller and larger cyclopeptides (n = 2 or > 4). In fact, cyclopeptides composed of at least six amino acids exhibit relatively stable all *cis*-conformations. It is important to note that the calculated most stable conformation of cyclotri- and cyclotetrapeptides is also not energetically favoured (MPJE >+1.2 and 0.6 kcal/mol respectively, Figure 3, solid line).

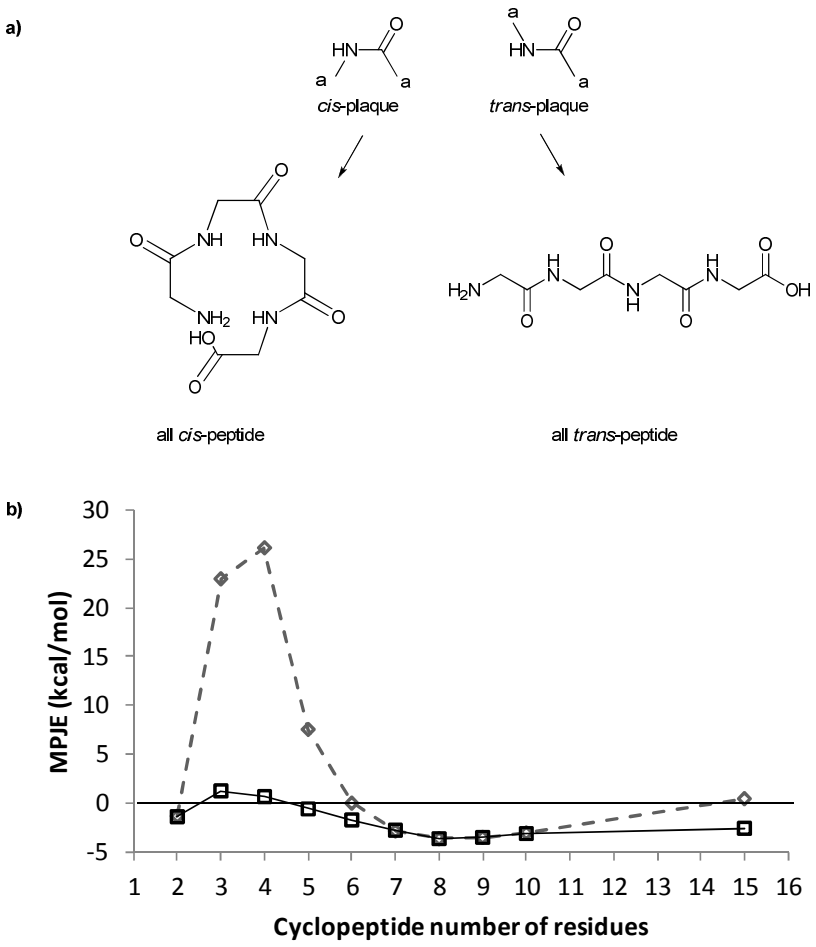


Figure 3. a) All *cis*-, *trans*-tetrapeptide conformations. b) Mean plaque junction energies for cyclopolyglycines; dashed line shown refers to MPJE values for an all *cis*-cyclopeptide constrained in a planar-symmetry while the solid line reflects MPJE values in the cyclopeptide most stable conformation.[12]

2. Synthesis of cyclotetrapeptides

2.1 Traditional head-to-tail cyclisation in solution phase.

Traditional peptide cyclisation is carried out in solution phase by activating the C-terminal carboxylic acid of the linear peptide, usually via the formation of a reactive ester. This ester is intramolecularly attacked by the nucleophilic *N*-terminal amino group to afford a macrolactam. This method is known as head-to-tail peptide cyclisation, and was the method of choice for the early synthesis of cyclotetrapeptides. A discussion of the synthesis of cyclotetrapeptides using this traditional head-to-tail cyclisation approach is provided.

Cyclotetrapeptides containing one or more turn-promoting amino acids such as proline, D- or *N*-methyl amino acids or the achiral glycine residue, should in principle be easier to form since these amino acids favour *cis*-amide bonds which in turn should reduce the potential energy of the cyclic structure (Figure 4).[12, 14, 15]

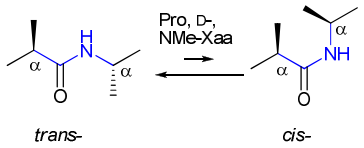


Figure 4. *Cis*- and *trans*-peptide bonds. Xaa represents any amino acid.

In contrast, the preparation of cyclotetrapeptides containing all L-amino acids is very challenging. For instance, attempts to synthesise the cyclo(L-Pro-L-Pro-L-Pro-L-Pro) peptide failed despite the presence of several Pro residues.[16] Moreover, when a pair of L-Pro residues were substituted for D-Pro, the corresponding cyclotetrapeptides cyclo(D-Pro-L-Pro-D-Pro-L-Pro) and cyclo(D-Pro-D-Pro-L-Pro-L-Pro) were readily synthesised.[17] Also the peptides cyclo(L-Xaa-L-Pro-L-Xaa-L-Pro) (where Xaa = Gly, Leu, Val or Phe) were difficult to prepare and rather the corresponding cyclooctapeptides were isolated.[18, 19] Interestingly, when pseudoprolines such as Ser(Thr) $\Psi^{MeMe'}$pro were introduced in the linear peptide sequence H-Xaa-Ser(Thr) $\Psi^{MeMe'}$pro-Xaa-Ser(Thr) $\Psi^{MeMe'}$pro-COOH (Xaa = Ile, Leu, Phe, Val), then the corresponding cyclic peptides were readily prepared in good to moderate yields (20-73%).[20] This observation is in agreement with the better turn inducing capabilities of pseudoprolines than proline.[21] Similarly, the synthesis of the tyrosine inhibitor cyclo(L-Pro-L-Val-L-Pro-L-Tyr) has been elusive.[22] Ring closure could only be achieved when starting from H- L-Pro-L-Val-L-Pro-L-Tyr-COOH , however, only the diastereomer cyclo(L-Pro-L-Val-L-Pro-D-Tyr) was obtained in 31% yield.[18] Some of the few examples that one can find in early reports of synthesis of cyclotetrapeptides containing all L-amino acids using the traditional head-to-tail cyclisation are cyclo(L-Pro-L-Leu-L-Ala-L-Ile) [23] and cyclo(L-Phe-L-Arg-Gly-L-Asp).[24]

Cyclotetrapeptides containing D-amino acids show less synthetic difficulties as evidenced from the successful synthesis of cyclo(D-Pro-L-Pro-D-Pro-L-Pro), cyclo(D-Pro-D-Pro-L-Pro-L-Pro), cyclo(L-Pro-L-Val-L-Pro-D-Tyr), [17] and cyclo(L-Pro-L-Val-L-Pro-D-Tyr).[18] This is in agreement with the fact that D-amino acids favour cisoid conformations. However, the selection of the cyclisation point is still important. For instance, the cyclo(D-Pro-D-Pro-L-Pro-L-Pro) peptide could not be obtained when starting from either H-D-Pro-D-Pro-L-Pro-L-Pro-COOH or H-L-Pro-L-Pro-D-Pro-D-Pro-COOH, but it was successfully prepared from H-D-Pro-L-Pro-L-Pro-D-Pro-COOH.[17] Some attempts to rationalize the preferred point of cyclisation for a given tetrapeptide sequence can be found in the literature, but the most important requirement is the capability of the linear peptide to reach the right geometry for cyclisation in the transition state.[25, 26] The preparation of cyclotetrapeptides containing at least one D-amino acid was reported to improve when the synthesis was carried out under microwave irradiation.[15]

N-Methylated amino acids are also known to induce *cis*-bond formation which in turn favours the cyclisation of linear peptides. [27] This effect is clearly illustrated when comparing the 5% yield obtained in the synthesis of cyclotetraglycine to the 58% yield of cyclotetrasarcosine.[28] Remarkably, the formation of cyclodipeptides during the cyclisation step of a series of linear tetrapeptides containing a combination of L- and *N*-methylated aminoacids was reported (Table 1).[29] The exact mechanism for dimer formation is unclear, but it could be attributed to an acid mediated peptide fragmentation (the cyclotetrapeptides were prepared by slowly adding the hydrochloride salts of the corresponding 2,4,5-trichlorophenyl (Tcp) ester linear peptides in DMF to a solution of pyridine in DMF (2.4 mM final peptide concentration) at 115 ˚C). Peptide fragmentation was observed during

removal of peptides containing *N*-methylated amino acids from a solid support under acidic conditions.[30] In a recent report, the synthesis of hersutide (compound **3** in Figure 1) from its linear free base precursor H-L-Phe-L-*N*Me-Phe-L-Val-L-*N*Me-Phe-OPfp (Pfp = pentafluorophenyl) (in CH_2Cl_2 with *N*-methylmorpholine at 0 ˚C and a 142 mM final peptide concentration) was successfully accomplished in 81% yield.[31] The synthesis of an *N*-benzylated glycine cyclotetrapeptide (peptoid) was also recently reported in excellent yield (80%).[32]

Table 1. Yields of cyclic di- and tetra-peptides from parent linear trichlorophenyl ester tetrapeptides.

Linear peptide	**Yield (%)**	
	Cyclodipeptide	**Cyclotetrapeptide**
H-Sar_4-OTcp[a]	21 cyclo(Sar_2)	58
H-L-Ala-Sar_3-OTcp	33 cyclo(Sar_2) 16 c(L-Ala-Sar)	25
H-Sar-L-Ala-Sar-L-Ala-OTcp	--	Traces 1 cyclo(Sar-L-Ala-Sar- D-Ala)
H- L-Ala-Sar-L-Ala-Sar-OTcp	64 cyclo(L-Ala-Sar)	-- Traces cyclo(D-Ala-Sar- L-Ala-Sar)
H- Sar-L-Ala_2-Sar-OTcp[a]	--	2.5
H- Sar_2-L-Ala_2-OTcp	30 cyclo(Sar_2) 13 cyclo(L-Ala_2)	--
H-L-Ala_3-Sar-OTcp	--	4.5
H-L-Ala_4-OTcp	--	5
H- Gly-Sar_3-OTcp[a]	6 cyclo(Gly-Sar) 4 cyclo(Sar_2)	23
H-Sar-Gly-Sar-Gly-OTcp	--	42
H-Sar-Gly_2-Sar-OTcp[b]	--	3
H-Sar_2-Gly_2-Sar-OTcp[a]	25 cyclo(Sar_2) 10 cyclo(Gly_2)	Traces
H-Gly-Sar_2-Gly-OTcp	57 cyclo(Gly-Sar)	--
H-Sar-Gly_3-OTcp	--	3
H-Gly_4-SCH_2COOH[a]	--	4.5

Sar = sarcosine. A 3% [a], and 13%[b] yield of cyclooctapeptide was obtained.

2.1.1 Effect of the carboxylic acid activation step

For linear tetrapeptides intramolecular cyclization is an inherently slow process, and side reactions, such as cyclodimerization, dimerisation and/or epimerization at the C-terminal residue may dominate. Thus, the coupling agent and conditions used in the cyclisation step should be carefully selected in order to minimise these undesired side reactions. Cyclodimerization and dimerization are usually minimised by performing the cyclisation step under high dilution conditions (< 1 mM). However, this slows down the reaction further, which is impractical for large scale production. For these slow cyclisations, the coupling agent used to activate the terminal carboxylic acid should be carefully selected to be devoid of side reactions, such as epimerisation at the C-terminal residue and degradation of the activated acyl species.

Some of the conventional coupling reagents used in solid phase peptide synthesis (SPPS) might not be suitable for tetrapeptide cyclisation, for instance, *N,N,N',N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (Figure 5) and analogues are problematic as they can cap the *N*-terminal amine by forming a guanidylated side-product, and are also prone to hydrolysis in

solution over extended periods of time.[33 34] Amino acid racemisation, which usually occurs through formation of an oxazolone intermediate, can take place with most of the known carboxylic acid activating reagents used for peptide synthesis, but racemisation can be minimized by careful selection of reagents and conditions (e.g. strength and amount of base, solvent, temperature). Acyl azides are the only activated amino acids for which the oxazolone intermediate has not been detected and therefore they can be considered as ideal intermediates for tetrapeptide cyclisation. However, the aminolysis of acyl azides is slow when compared to other activated acyl derivatives and also acyl azides can rearrange to alkyl isocyanates at higher temperatures.[33] Cyclisations with Pfp esters are also known to occur with minimal racemisation.[35]

Several successful macrolactamisations of linear tetrapeptides have been reported when using the corresponding Pfp esthers,[23, 31] diphenylphosphoryl azide (DPPA),[17] pentafluorophenyl diphenylphosphinate (FDPP),[20] 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM·BF_4), [20, 24] and 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU),[19, 36, 37] with lower yields obtained with the latter (Figure 5).

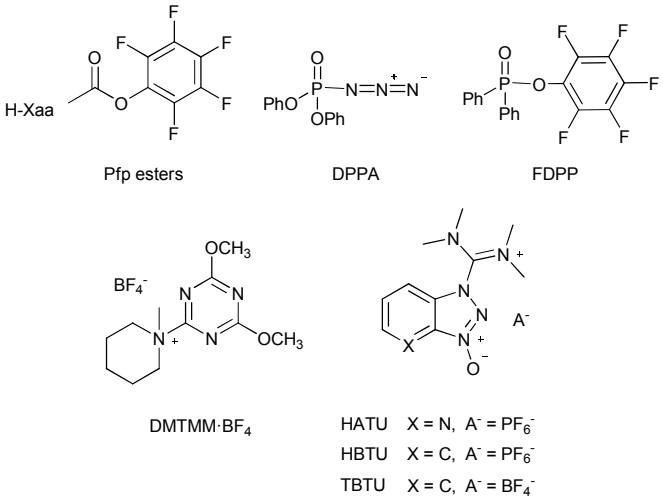
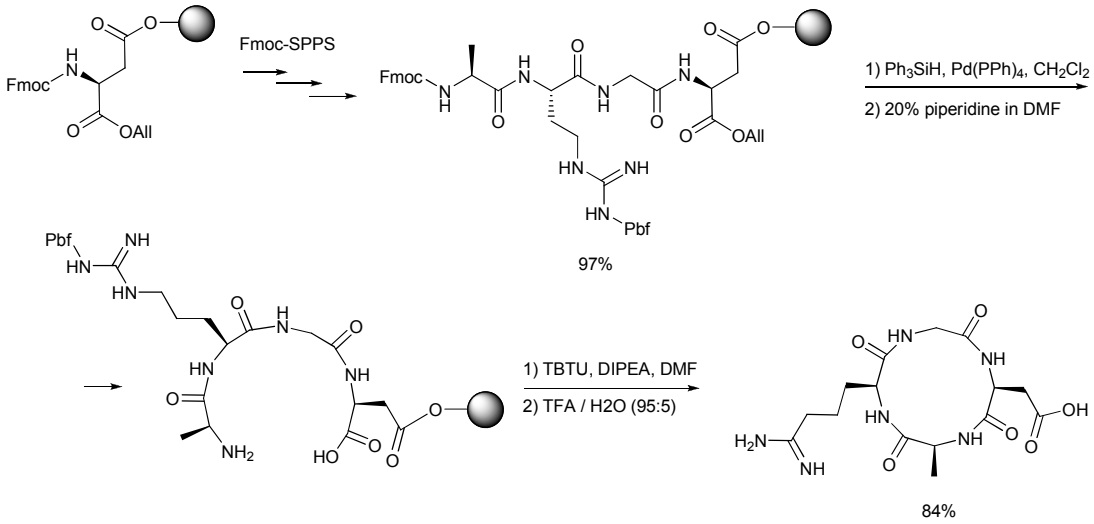


Figure 5. Structures of a peptide-OPfp ester and coupling reagents commonly used for the cyclisation of linear tetrapeptides.

2.2. Cyclisation on solid phase

The cyclisation of tetrapeptides on solid phase has been used to minimize the formation of cyclodimers by taking advantage of the pseudodilution phenomenon. Moreover, solid phase cyclisation requires an amino acid with a side chain functionality which can be selectively attached to the solid support (e.g. Asp, Glu, His, etc.). Thus, a three dimensional orthogonal protection approach (for instance 9-fluorenylmethylcarbonyl (Fmoc)/*tert*-butyl (tBu)/allyl (All)) is needed for completion of the head-to-tail cyclisation. A series of cyclotetrapeptides of sequence cyclo(Xaa-L-Arg-Gly-L-Asp) (where Xaa = L-Ala, L-Phe, L-Phenylglycine (Phg), D-Ala, D-Phe or D-Phg) were prepared by SPPS. Cyclisation was carried out between the Xaa and L-Asp, with the later attached to the solid support through its acetate side chain (Scheme 1). The highest yields (71-90%) were obtained when using a Wang resin and *N,N,N',N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium tetrafluoroborate /*N*,*N*-

diisopropylethylamine (TBTU/DIPEA) (Figure 5) for the cyclisation step. The lowest levels of racemisation were observed when Xaa = Ala < Phe < Phg, which correlates with the increased steric hindrance of the *N*-terminal amino acid.[38]



Scheme 1. An example of solid phase peptide cyclisation via a three dimensional orthogonal approach (Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl).

An overall analysis of solid phase cyclisation of a library of cyclotetrapeptides was recently reported.[39] A library that was composed of the linear peptide Xaa^1-Xaa^2-Xaa^3-Asp-OAll attached to a solid support was assembled, where Xaa was L-2-aminobutyric acid, L-norleucine or any of the 20 proteinogenic amino acids except Cys and Met. The results indicated that a high proportion of "hard to cyclise" sequences contained either Lys or Arg in residues Xaa^1 and Xaa^2, and Thr in Xaa^2 and Xaa^3. Significant amounts of cyclic dimers were obtained with the fast cyclising sequences. It was also shown that the cyclo(L-Pro-L-Asn-L-Pro-L-Glu) and cyclo(L-Thr-L-Thr-L-Lys-L-Glu) peptides were synthesised on solid phase in good to moderate yields (99 and 39% respectively) while reduced amounts of the same peptides were obtained when the synthesis was performed in solution.

At this point it should be obvious to recognize that the synthesis of all L-cyclotetrapeptides (12-membered ring) is problematic and might not be possible for many sequences using the conventional cyclisation techniques described above.

2.3 Ring contraction strategies

The ring contraction strategy (RCS) has emerged as an important technique to prepare difficult cyclotetrapeptides.[40] The principle behind the RCS is based on the cyclisation of a larger ring (>12 membered ring) which is less constrained than the original target, followed by intramolecular contraction to generate the desired peptide. Earlier examples of the RCS relied on the formation of a cyclodepsipeptide via a Ser residue followed by an intramolecular *O*-to-*N* acyl migration. However, for the highly constrained cyclic tetradepsipeptides the final *O*-to-*N* migration was not observed (13- to 12-membered ring contraction) (Figure 6).[41]

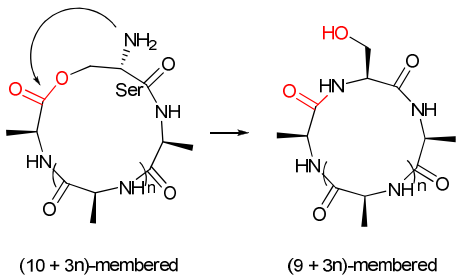
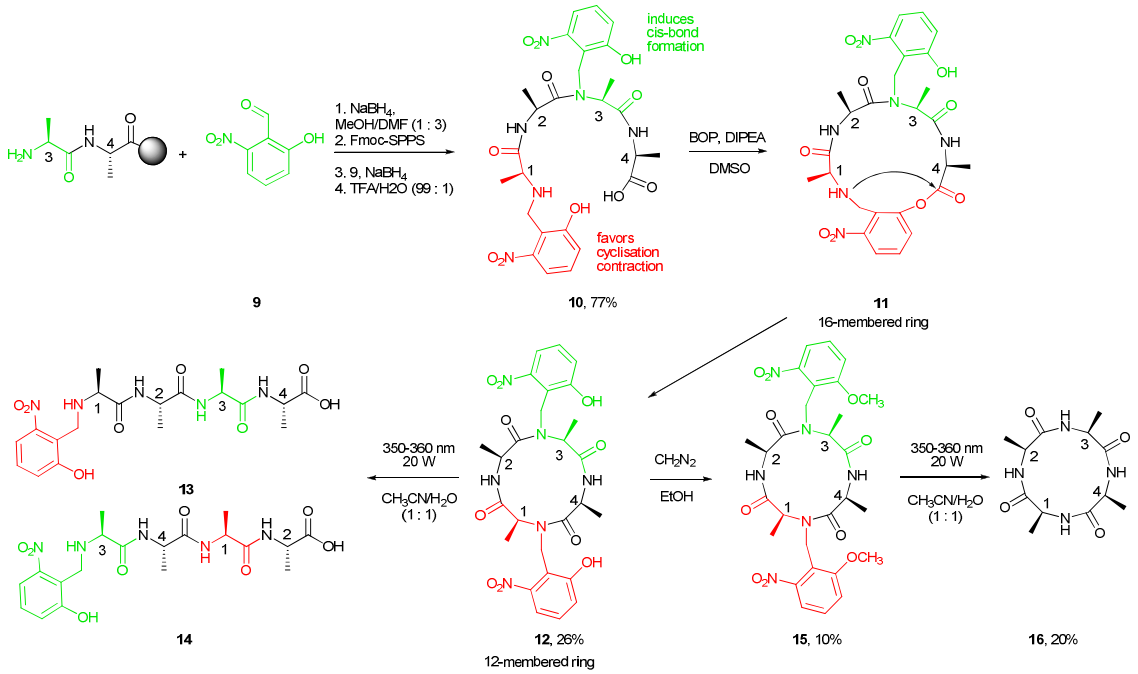


Figure 6. An illustration of the *O*-to-*N* acyl migration of cyclodepsipeptides to cyclopeptides (n = 1 for a cyclotetrapeptide).

The use of auxiliary molecules for the RCS synthesis of cyclopeptides has been reported.[42, 43] In this case the auxiliary molecule helps to form a less constrained ring and after the ring contraction step the auxiliary is removed. Examples of these auxiliary mediated approaches that have been used for the synthesis of cyclotetrapeptides are described below.

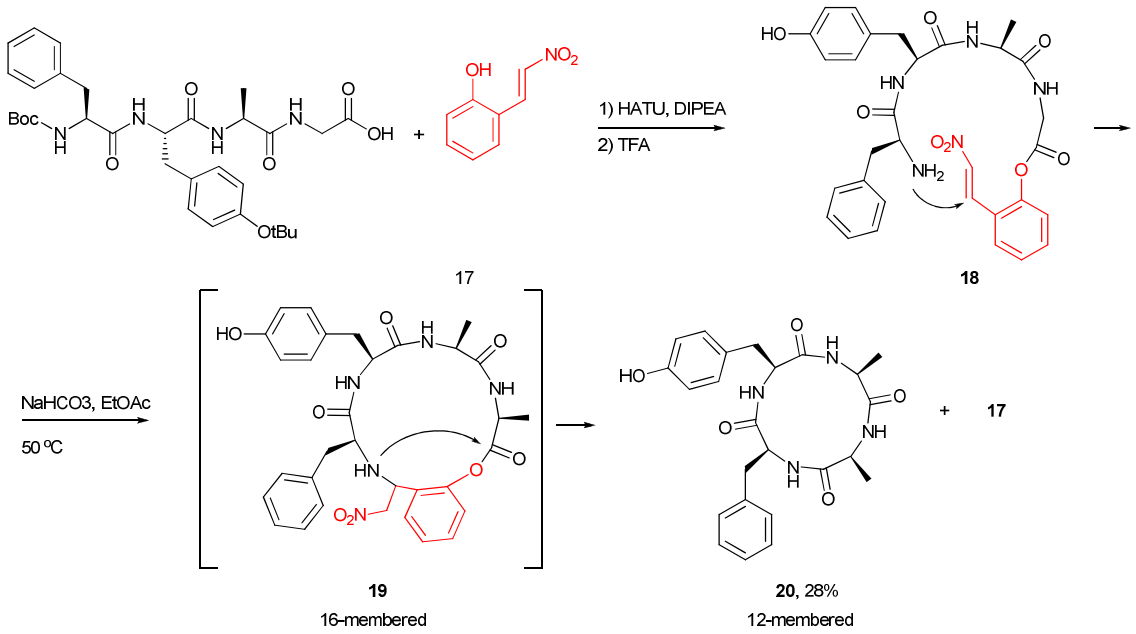
In one strategy an auxiliary molecule such as 2-hydroxy-6-nitrobenzaldehyde (**9**) (Scheme 2) is attached to the *N*-terminal amino acid of a peptide chain by reductive amination. An intramolecular cyclisation between the C-terminal carboxyl and the hydroxyl group of the auxiliary generates the corresponding depsipeptide **11** (16-membered ring), which yields the *N*-substituted cyclotetrapeptide **12** via an *O*-to-*N* acyl transfer. Finally, the 2-hydroxy-6-nitrobenzyl (HnB) group is removed by photolysis. A library of cyclotetrapeptides with the sequence H-L-Xaa-L-Xaa-L-Xaa-Gly was prepared using this method in modest yields (4-29%) (cyclisation and ring contraction was accomplished in a 1 mM peptide solution in DMSO by overnight heating at 70°C with 1 eq. of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 10 eq. of DIPEA).[44]

However, the real utility of this technique cannot be assessed from the successful synthesis of sequences containing a C-terminal glycine, since glycine is achiral and therefore not prone to racemisation. Later, it was demonstrated that an all L- cyclotetrapeptide could be synthesised by introducing two HnB groups (**10**) (Scheme 2),[45] which is equivalent to having two *N*-methylated amino acids. Initial attempts to prepare cyclo(L-Tyr-L-Arg-L-Phe-L-Ala) from ([HnB]-L-Tyr-L-Arg-L-Phe-L-Ala-COOH failed, however cyclo([HnB]-L-Tyr-L-Arg-[HnB]-L-Phe-L-Ala) and cyclo([HnB]-L-Tyr-L-Arg-[HnB]-L-Phe-D-Ala) were obtained in 26% and 33% yield respectively from [[HnB]-L-Tyr-L-Arg-[HnB]-L-Phe-L-Ala-COOH. Surprisingly, attempts to remove the [HnB] auxiliary by photolysis resulted in the formation of only mono [HnB]-tetrapeptides **13** and **14**, presumably by reverse *N*-to-*O* acyl transfer and hydrolysis. HnB removal was complete when the hydroxyl groups of HnB were methylated (**15**) prior to photolysis, thus, due to these post-cyclisation modifications the all L-cyclotetrapeptide **16** was only prepared in low yield.



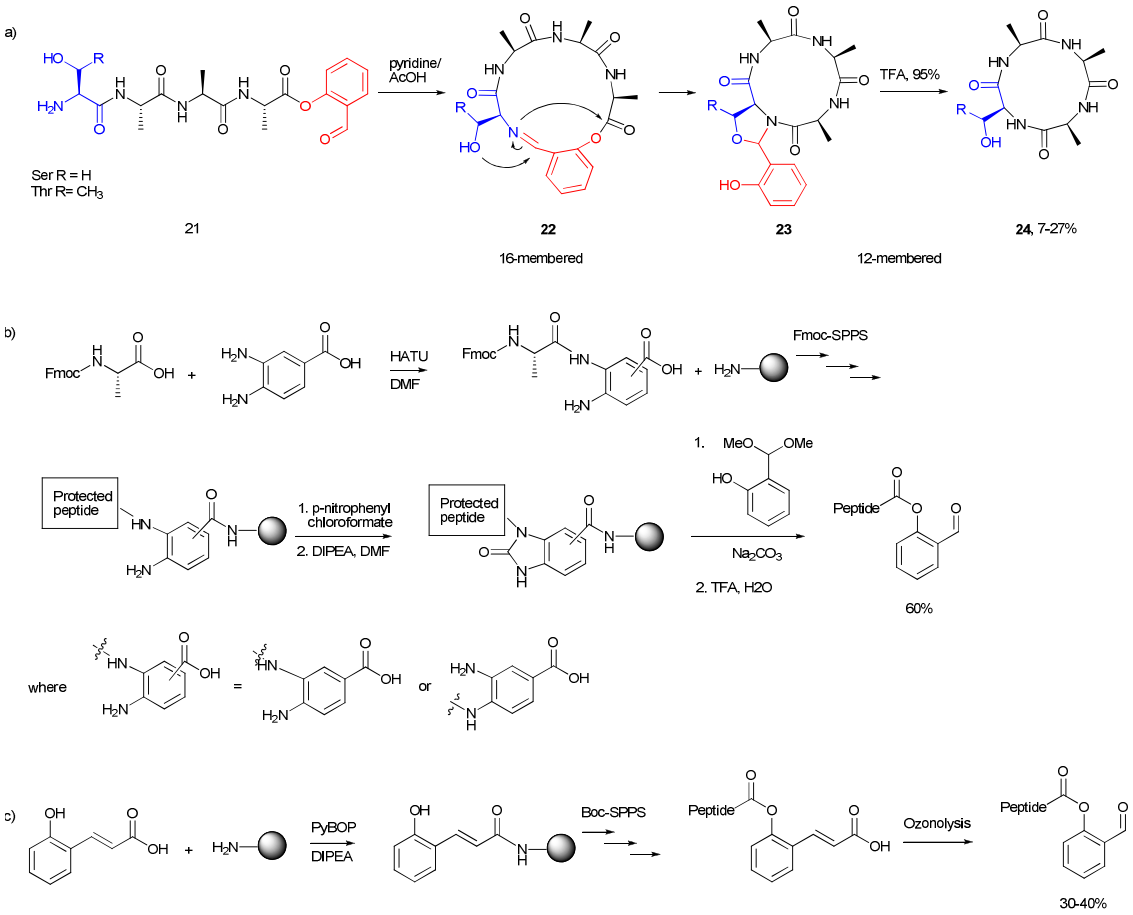
Scheme 2. An example of the synthesis of an all L- cyclotetrapeptide by introduction of two HnB groups (1 = L-Tyr, 2 = L-Arg, 3 = L-Phe and 4 = L-Ala).

(*E*)-2-(2-Nitrovinyl)phenol (**17**) (Scheme 3) is a C-terminal auxiliary that has been used for the synthesis of cyclotetrapeptides by RCS.[46] With this auxiliary an *N*-terminal Boc protected peptide is first used to form an ester with the phenol group (**18**). Next, the Boc protecting group is removed to release the *N*-terminal amino group, which upon basification attacks the alkene moiety of the auxiliary via an intramolecular Michael addition reaction giving **19**. *O*-to-*N* acyl transfer followed by simultaneous release of the auxiliary yields the desired cyclic peptide **20**. With this method, the peptide cyclo[L-Phe-L-Tyr-L-Ala-Gly] was prepared in 28% yield, this yield is higher than the 11% yield reported using the HnB auxiliary.[44] The improved yield obtained when using **17** can be attributed to the simplified synthesis compared to the HnB route.



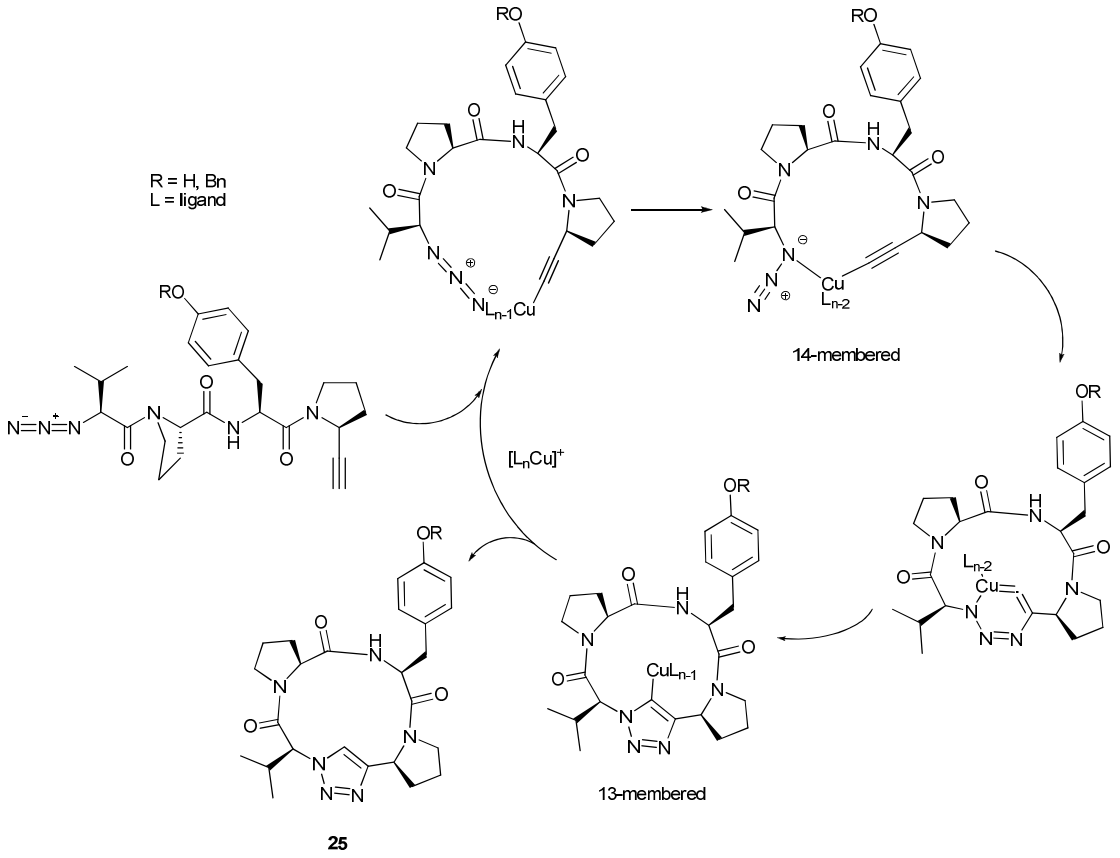
Scheme 3. Synthesis of a cyclotetrapeptide via a ring contraction strategy using the (*E*)-2-(2-nitrovinyl)phenol (**17**) auxiliary.

Intramolecular Ser/Thr ligation is another type of RCS which requires a Ser or Thr residue at the *N*-terminus and a 2-phenylcarboxaldehyde ester at the C-terminus (**21**) (Scheme 4). The peptide ester can be prepared without racemization by both *tert*-butoxycarbonyl (Boc)-SPPS (Scheme 4a)[47, 48] and Fmoc-SPPS (Scheme 4b),[49] in moderate yields. The cyclisation proceeds through intramolecular head-to-tail imine formation to give a 16-membered macrocycle (**22**). Subsequent intramolecular ring contraction by an *O*-to-*N* acyl transfer gives an *N*,*O*-benzylidene acetal (**23**) which upon acidic treatment affords the cyclic peptide (**24**). Several cyclotetrapeptides containing all L-amino acids were recently prepared using the Ser/Thr ligation via Boc-SPPS. In most cases the cyclotetrapeptide was the main product but as the dimer cyclopeptide was also formed in a high proportion, the overall reaction yields were low.[47] Interestingly, in the original report by Wong *et al.*[47] the cyclisation step was carried out in pyridine/acetic acid (1:2 mol/mol) at 1 mM peptide concentration at room temperature for 4 h. Moreover, in other work reported by the same authors the cyclisation was carried out in a mixture of trifluoroethanol (TFE)/ pyridine/acetic acid (2:1:1 mol/mol) at a peptide concentration of 0.1 mM to minimize dimerisation.[48] Unfortunately, the effect of TFE on peptide cyclisation was not further discussed, but it has been recently reported that polyfluorinated alcohols stabilize peptide folding thus enhancing peptide cyclisation.[50] Furthermore, intramolecular Ser/Thr ligation is limited to peptides containing Ser/Thr at the *N*-terminus, although it could potentially be extended to amino acids containing the thiol functionality.[51]



Scheme 4. a) Intramolecular Ser/Thr ligation. The indicated yield range is the one reported for a series of tetrapeptides. Synthesis of the 2-phenylcarboxaldehyde ester intermediate via: b) Boc-SPPS, where the indicated yield corresponds to the Ser-Ile-Pro-Leu-Phe-Pro-Ile peptide; and c) Fmoc-SPPS, where the indicated yield range is the one reported for a series of tetrapeptides (PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate).[47]

Another example of RCS is provided by the synthesis of a triazole-containing analogue **25** (Scheme 5) of the potent tyrosinase inhibitor cyclo(L-Pro-L-Val-L-Pro-L-Tyr),[22] for which the synthesis has been elusive up to date. The triazole-peptide was efficiently prepared via Cu(I) catalyzed click cyclisation (70% yield) introducing a 1,4-disubstituted 1,2,3-triazole as a surrogate of a *trans*-amide bond [52] In this case cyclisation is facilitated by the increased ring size of triazole analogues and the apparent "ring contraction" mechanism of Cu(I)-catalyzed alkyne-azide cycloaddition (Scheme 5).[53] The triazole-peptide and two other analogues were more than or as active as the parent natural product,[54] which further validates the utility of the triazole linkage as an isostere of the amide bond.



Scheme 5. Cu(I)-catalyzed alkyne-azide cycloaddition mechanism for the synthesis of the tyrosinase inhibitor analogue (**25**) of cyclo(L-Pro-L-Val-L-Pro-L-Tyr).

Other peptide bond surrogates that could be used for the synthesis of cyclopeptide mimetics are 1,2,3-oxadiazoles and *E*-alkenes as isosteres of the *trans*-peptide bond or 1,5 substituted 1,2,3-triazoles, o-substituted benzenes, tetrazoles, 1,2-pyrroles and *Z*-alkenes for the *cis*-peptide bond (Figure 7).[55] However, none of these have been used for the synthesis of cyclotetrapeptides to date.

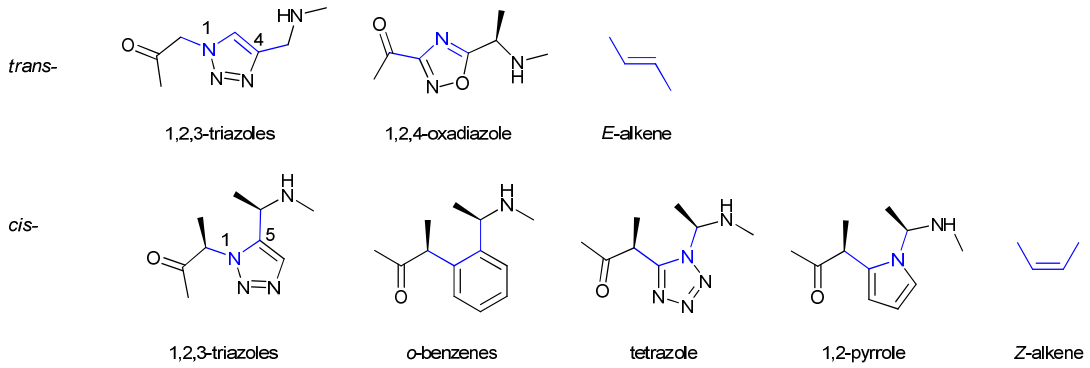
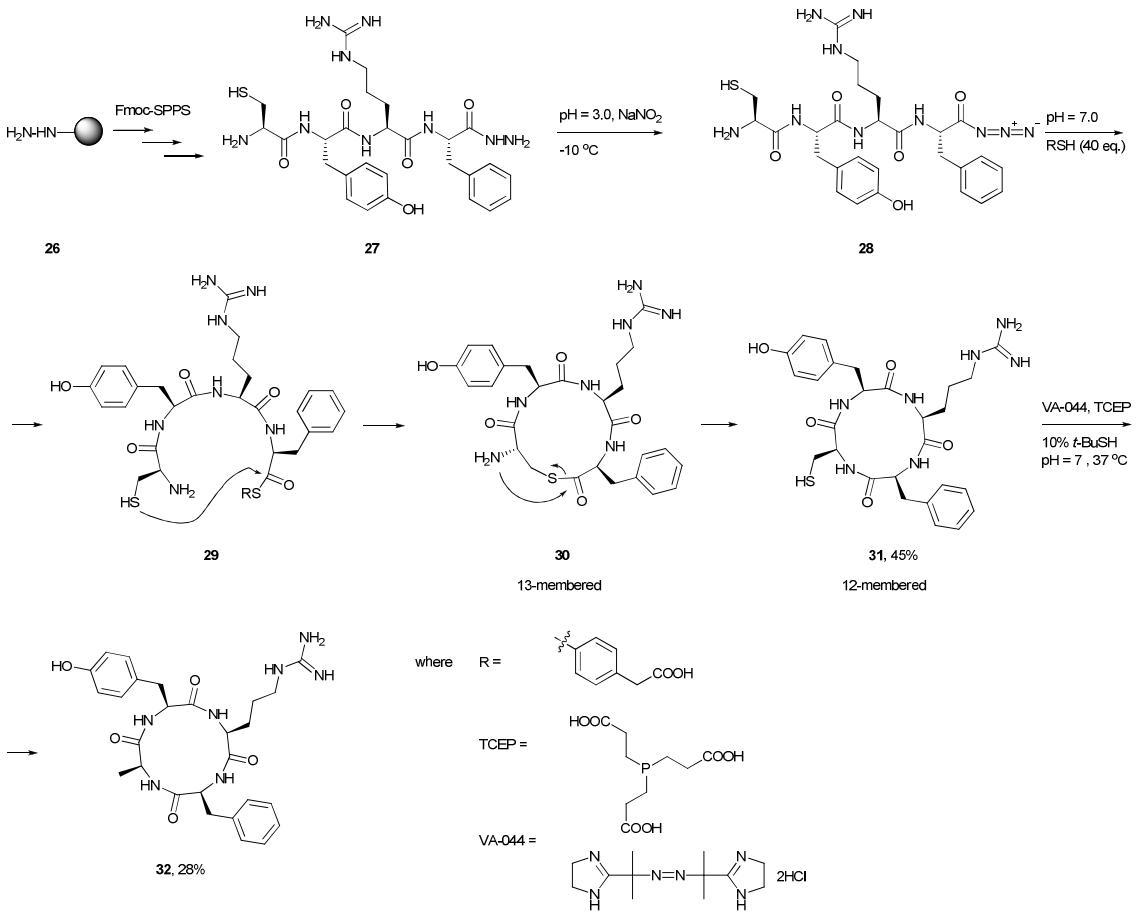


Figure 7. Peptide bond surrogates.

An RCS sulfur-mediated cyclisation of tetrapeptides was recently reported. Thus, four cyclic tetrapeptides containing all L-amino acids cyclo(L-Cys-L-Leu-L-Ala-L-Leu), cyclo(L-Cys-L-His-L-Gly-L-Trp), cyclo(L-Cys-L-Val-L-Gly-L-Ile) and cyclo(L-Cys-L-His-L-Trp-L-Gly) have been successfully prepared in moderate yields (~40%) through intramolecular native chemical ligation (NCL) (Scheme 6).[56 57] Fmoc-SPPS on a hydrazine 2-chlorotrityl resin (**26**) was used to prepare the peptide hydrazide (**27**) and its conversion to acyl azide (**28**) in solution followed. The peptide hydrazide was used as a precursor to the corresponding peptide thioester (**29**), which undergo NCL (**30**) to afford the corresponding cyclotetrapeptide (**31**). Peptide thioesters are usually prepared via Boc-SPPS, however this technique is problematic for acid labile peptides , such as those containing *N*-methylated amino acids.[30] Methods to prepare peptide thioesters by Fmoc-SPPS are also known[58] or alternatively they can be prepared in solution from the corresponding carboxylic acid precursors by conventional coupling protocols, but epimerization of the C-terminal amino acid can occur.[26] Thus, advantages of the hydrazide protocol are the ease of access to peptide thioesters through Fmoc-SPPS and little or non-racemisation of the C-terminal amino acid, given their formation via the acyl azide precursor. The strategy is limited to peptides containing the thiol functional group at the *N*-terminus; however, it can be easily extended to introduce other amino acids such as Ala(**32**) after desulfurisation of (**31**) or Phe, Val, Asp or elimination of the thiol functionality to dehydro-amino acids.[59-61]

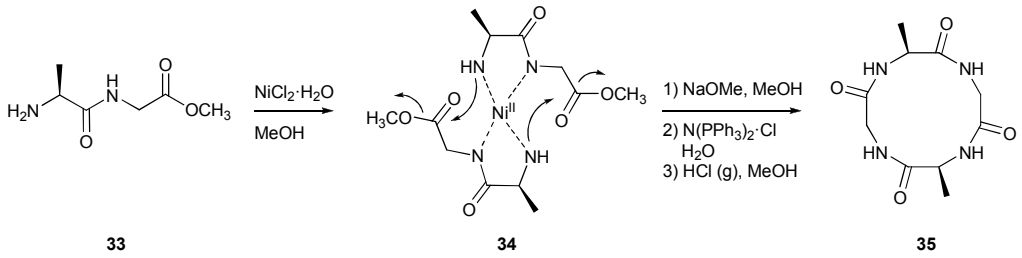


Scheme 6. An example of a ring/contraction strategy sulfur-mediated cyclisation of tetrapeptides.

2.4 Other synthetic strategies

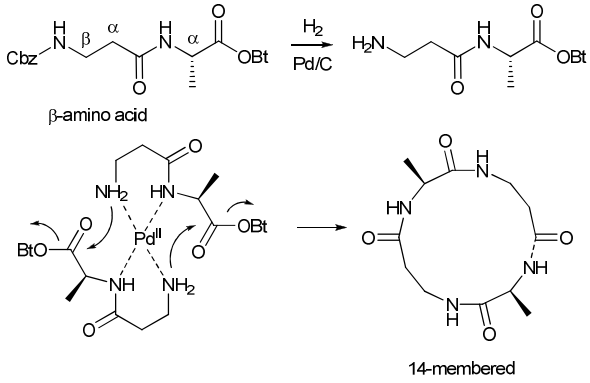
There have been other reports on methods used for the synthesis of cyclopeptides but their specific application to the synthesis of highly constrained cyclotetrapeptides has not been documented. We describe these alternate methods in this section.

The concept of template metal cyclisation was introduced in 1998.[62] This method relies on the formation of a metal complex with two units of a dipeptide ester (**33**) (Scheme 7). Metal complexation of **33** positions both the reactive *N*- and C-termini in close spatial proximity (**34**) thereby facilitating ring closure. It was found that cyclotetrapeptides (12-membered rings) could be formed only with Ni(II) since it is believed to have the ideal ionic radius for the cyclisation of tetrapeptides (i.e. Ni(II) is smaller than Pd(II)). In the original report only cyclo(Gly-L-Ala-Gly-L-Ala) (**35**) was synthesised in 21% yield. However, this method has not been cited more generally for the synthesis of cyclotetrapeptides.



Scheme 7. Nickel(II) template cyclisation of tetrapeptides.

Reports on the successful preparation of cyclotetrapeptides containing a combination of α- and β-amino acids using the metal template strategy can be found in the literature, which further substantiates the difficulty experience when synthesising 12-membered cycles compared to 13- or 14-membered ring analogues.[63, 64] For example, a Pd-promoted tandem deprotection/cyclisation dimerization method was used to prepare C_2 symmetrical cyclotetrapeptides from *N*-Cbz-dipeptidoyl benzotriazole (Bt) sequences (Cbz = carboxybenzyloxy) containing either β- or α- and β-amino acids (Scheme 8).[50]



Scheme 8. An example of palladium-promoted tandem deprotection/cyclisation dimerization method for the synthesis of cyclotetrapeptides containing β-amino acids.

Tetrapeptides, linear or cyclic, were used to create molecularly imprinted cavities in polymer-cellulose composites. These cavities served as templates for the synthesis of cyclotetrapeptides. The process consisted of three stages. In the first stage, cellulose was derivatized with 3-methacryloxypropyltrimethoxysilane to create a hydrophobic base. The second stage involved the polymerisation using 2,2'-azobisisobutyronitrile in presence of acrylamide, *N*-acryltyramine , *N*,*N'*-ethylene bisacrylamide and the tetrapeptide at high temperatures to increase the population of linear peptides in a turned conformation. For the third stage, the imprinted polymer was used as a scaffold to constrain the incoming linear tetrapeptides in an appropriate folded geometry for cyclisation (Figure 8).[16]

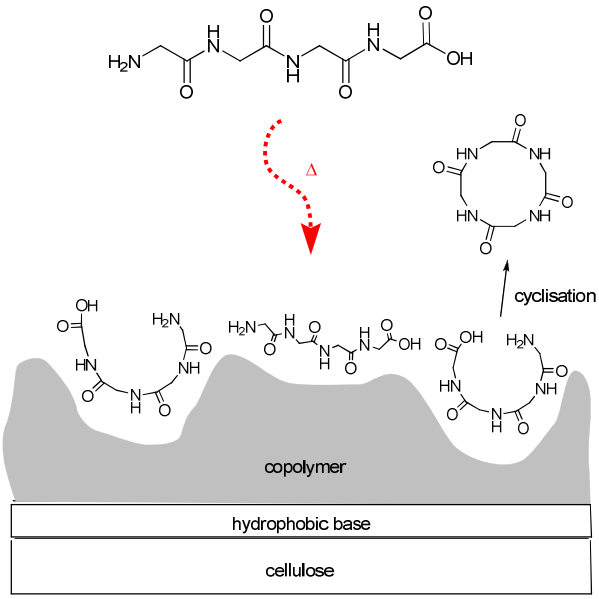


Figure 8. An illustration of the synthesis of cyclotetrapeptides using molecularly imprinted polymers.

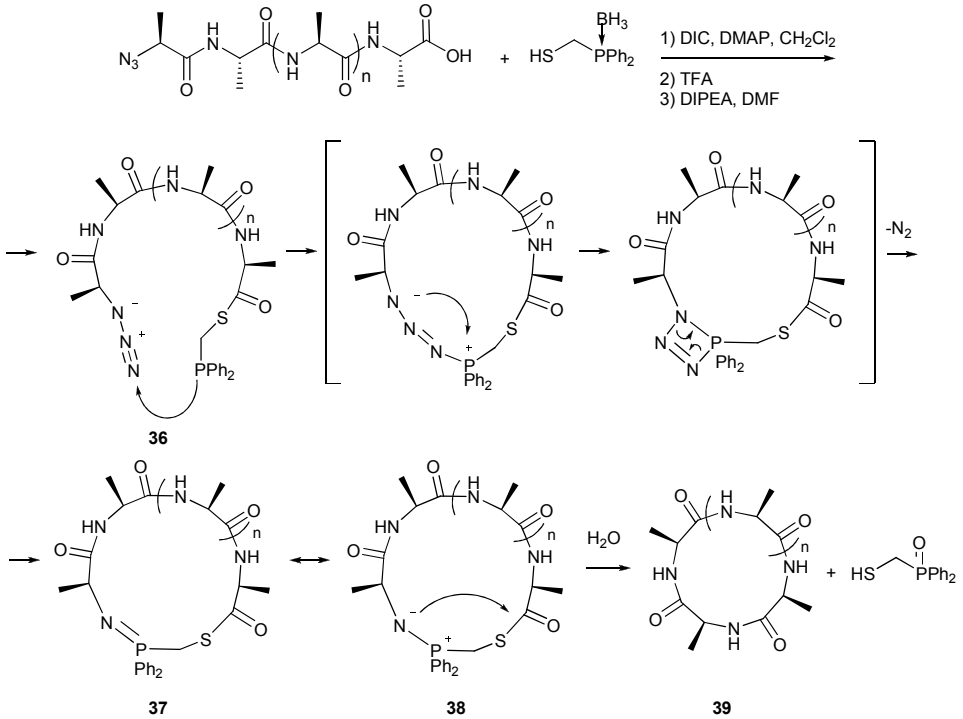
The best results were obtained when preparing the imprinted polymer using the cyclic peptides as templates. Thus cyclo(Gly-Gly-Gyl-Gly) and cyclo(L-Phe-L-Phe-L-Phe-L-Phe) were prepared in moderate yields (42.8 and 54.4%, respectively). However, the elusive cyclo(L-Pro-L-Pro-L-Pro-L-Pro) peptide, for which the cyclic template was not available, could not be prepared using the linear tetrapeptide as template.

2.5 Other potential methods for the synthesis of cyclotetrapeptides

One method that could be applied to the synthesis of cyclotetrapeptides consists of using polyfluorinated alcohols (PFAs) as co-solvents during the cyclisation step.[50] Mixtures of PFAs and halogenated solvents were originally used to solubilise insoluble protected peptide fragments and to couple them without racemisation during the synthesis of large peptides.[65, 66] It is also known that PFAs either stabilize or induce peptide folding by coating the surface of the peptide, thus favouring the formation of intramolecular hydrogen bonds which promotes the formation of secondary structure.[67, 68] This results in an increased population of peptides in a folded conformation in solution. Thus, it is attractive to consider that such peptide folding enhancement and no racemisation in PFAs would increase the success of peptide cyclisation. This principle was recently

demonstrated with the successful synthesis of the cyclohexapeptide cyclo(L-Thr-L-Ala-L-Ala-L-Thr-L-Ala-L-Ala) from three of its possible linear precursors.[50] The best yield for the monocyclic peptide (82%) was obtained from the linear precursor H-L-Thr-L-Ala-L-Ala-L-Thr-L-Ala-L-Ala-COOH by performing the reaction for 1 hour using diisopropylcarbodiimide and 7-aza-1-hydroxybenzotriazole (DIC/HOAt) (1/1) in TFE/CH_2Cl_2 (1/1; v/v) at a 10 mM linear peptide concentration. Under these conditions the cyclic dimer and the TFE ester were observed as side products (11.2 and 2.5% respectively). The percent of cyclic dimer was lowered when hexafluoroisopropanol (HFIP)/CH_2Cl_2 (1/4) was used as the solvent mixture (5.8%). This effect was attributed to the larger steric hindrance or stronger hydrogen bonding capacity of HFIP when compared to TFE. However, the proportion of HFIP ester (11.3 %) was higher than the corresponding TFE ester.

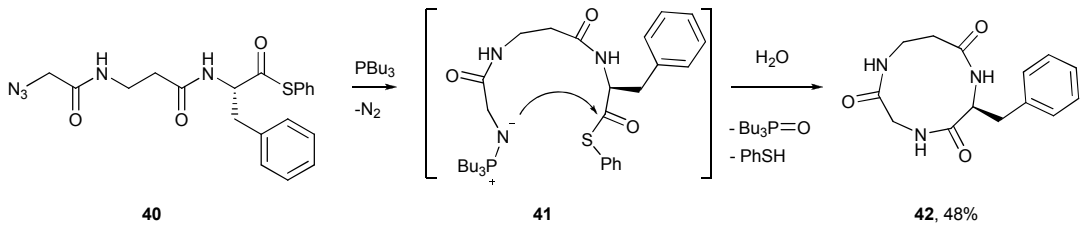
Another method that could be applied to the synthesis of cyclotetrapeptides is the traceless Staudinger ring contraction strategy (SRCS). This strategy has been used for the synthesis of three cycloundecapeptides, which were obtained in moderate yields (20-36%) (Scheme 9).[69] The SRCS involves the intramolecular reaction of an *N*-terminal azide with a C-terminal phosphine (**36**) yielding an iminophosphorane (**37**), which reacts intramolecularly as the aza ylide (**38**) with the thioester yielding a native amide bond (**39**). The SRCS is another type of RCS, which if applied to the synthesis of a cyclotetrapeptide a 15-membered ring iminophosphorane would be formed prior to the ring contraction step, thus suggesting the potential utility of this technique for the synthesis of constrained cyclotetrapeptides. However, application of SRCS to the synthesis of cyclotetrapeptides has not been demonstrated to date.



Scheme 9. An illustrative example of a Staudinger ring closure ligation with a hypothetical all L-alanine cyclopeptide (n = 1 for a cyclotetrapeptide and DMAP = 4-dimethylaminopyridine).

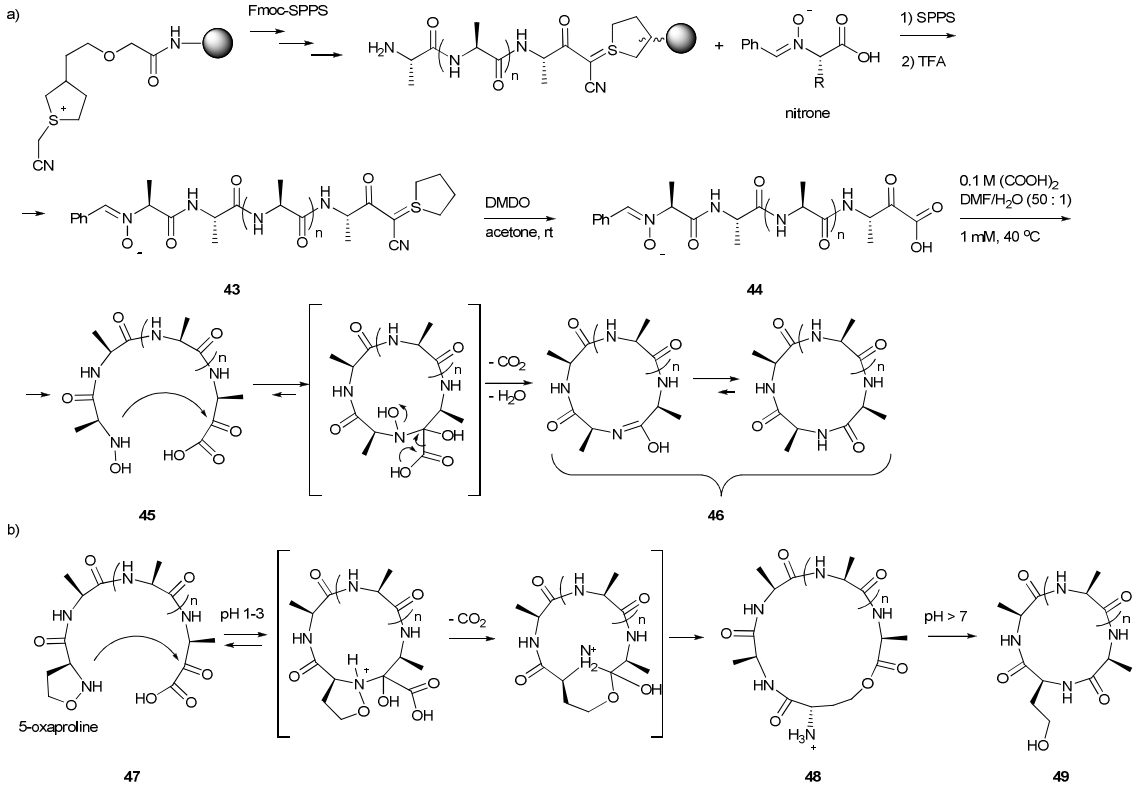
Another variant of the Staudinger mediated cyclisation has been reported to be useful for the synthesis of small cyclo di- and tri-peptides (**42**).[70] This method uses an azide as the *N*-terminal

residue and a thioester at the C-terminus (**40**) (Scheme 10). The cyclisation of **40** is promoted in the presence of a phosphine via an aza ylide (**41**). However, application of this method to the synthesis of cyclotetrapeptides has also not been demonstrated to date.



Scheme 10. Tributylphosphine activated *N*-azide peptide thioester Staudinger ligation.

The ligation of a C-terminal peptide α-ketoacid and an *N*-terminal hydroxylamine (KAHA) has recently been used for the synthesis of cyclopeptides.[71] Similar to NCL, the KAHA intramolecular ligation offers the advantage of not requiring protecting groups for the reactive side chains of the amino acids during the cyclisation step. In addition, it also does not require a cysteine or thiol containing amino acid in the *N*-terminal residue. The synthesis of α-ketoacids using Fmoc-SPPS can be performed through a sulphur ylide intermediate (**43**) (Scheme 11a).[72] Initially, the *N*-terminal hydroxylamine (**45**) can be prepared either in solid[73] or in solution phase, after which it is introduced into SPPS as the *N*-benzylidene nitrone protected *N*-hydroxyamino acid.[74] The sulphur ylide **43** is oxidised with dimethoxyldioxirane (DMDO) yielding the corresponding α-ketoacid **44**. Deprotection of the *N*-hydroxylamine group of **44** gives **45** which undergoes a traceless cyclisation process to give the cyclopeptide **46**.[75, 76] Cyclopeptides of different sizes (15- to 30-membered rings) were prepared with KAHA ligation and were obtained in poor to moderate yields (9-36%). The smallest cyclopeptide reported was a cyclopentapeptide derived from Gramicidin S; cyclo(D-Phe-L-Pro-L-Val-L-Orn-L-Leu) which was obtained in 15% yield. The low overall yield is independent of the cycle size and is related to the stability of the nitrone protecting group.[71] However, the KAHA ligation is not an RCS and thus might not offer any advantage for the synthesis of cyclotetrapeptides. An alternative KAHA ligation employs an α-ketoacid and an *N*-terminal 5-oxaproline (**47**) (Scheme 11b) which forms depsipeptide (**48**), which is then converted under basic conditions via an *O*-to-*N* acyl shift to a peptide (**49**).[77] This reaction offers an interesting alternative for the synthesis of 12-membered ring cyclopeptides given the formation of an expanded ring (**48**). However, an unnatural amino acid is introduced in the cycle when starting from 5-oxaproline.



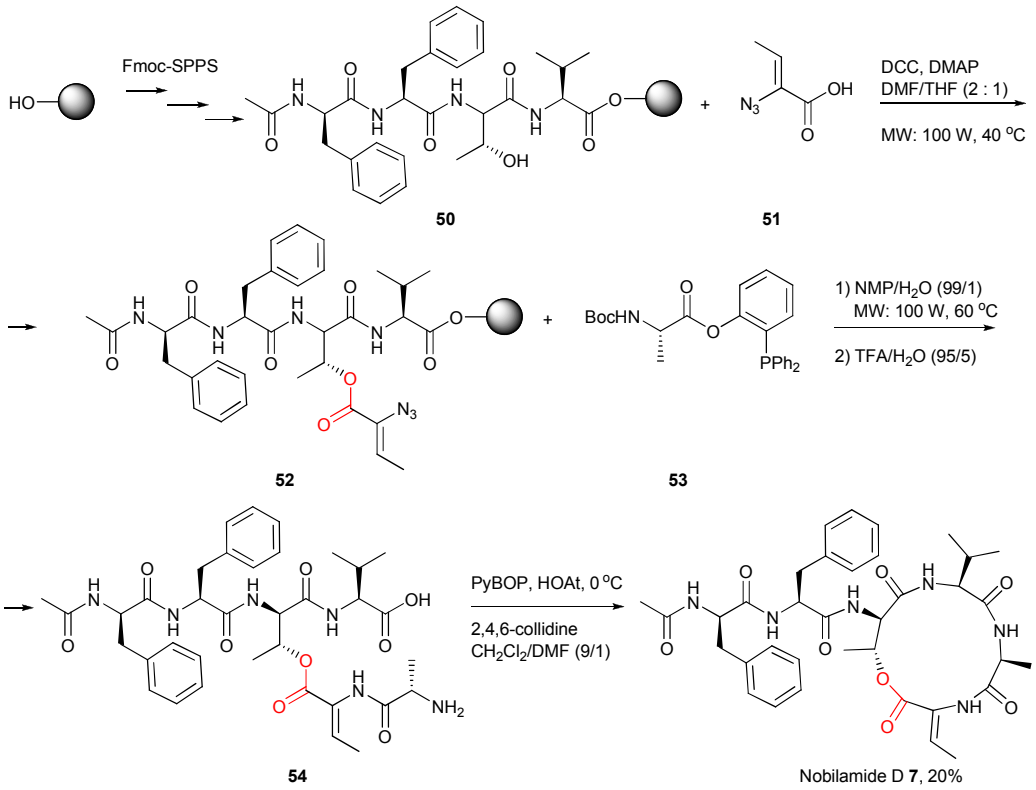
Scheme 11. Illustrative example of the synthesis of a hypothetical all L-alanine cyclopeptide via the intramolecular ligation of a C-terminal α-ketoacid and an *N*-terminal hydroxylamine (a) or 5-oxaproline (b) (n = 1 for a cyclotetrapeptide).

3. Synthesis of cyclotetradepsipeptides

Cyclodepsipeptides with important biological activities have been recently reviewed in the literature.[78] Both solution [79] and solid phase[80, 81] methods for the synthesis of cyclodepsipeptides have been reported. A traditional method for either inter- or intra-molecular esterification of peptides in solution and solid phase employs carbodiimide reagents in the presence of catalytic amounts DMAP, which functions as an acyl transfer catalyst. Other methods, although used less frequently include esterification with succinimide esters and Mitsunobu or Yamaguchi esterifications.[82]

In general, due to the limited stability of the ester bond, the possibility of racemization under basic conditions and its limited compatibility with deprotection and cleavage conditions used in SPPS, macrolactamizations have been the method of choice for depsipeptide ring closure. This was demonstrated by the recent synthesis of nobilamide D (Scheme 12).[83] The cyclic portion of the peptide was prepared via macrolactamization rather than macrolactonization, due to the known higher nucleophilicity of the nitrogen atom as compared to the oxygen atom. Thus, an *N*-acetylated fragment of the peptide (**50**) was synthesised on a solid support by Fmoc-SPPS. The secondary alcohol of Thr was then esterified with *Z*-didehydroazidobutanoic acid (**51**) giving **52**. Notice that an Fmoc-derivative of *Z*-didehydroaminobutanoic acid was not used in the esterification step given the poor stability of the ester to the basic conditions used for removal of the Fmoc protecting group and

the possibility of a competing Michael addition reaction taking place. The *N*-Boc-L-Ala-diphenylphosphinophenol ester (**53**) was attached via a Staudinger ligation to **52** followed by protecting group cleavage and release from the solid support to yield peptide **54**. The final macrolactamisation was performed in solution to afford cyclotetradepsipeptide **7** in 20% overall yield.



Scheme 12. Synthesis of nobilamide D.

4. Conclusion

Cyclotetrapeptides are an important class of peptide derivatives that could potentially lead to the development of new pharmaceuticals. However, the efficient synthesis of these highly constrained macrocycles remains an important chemical challenge. Ring contraction strategies offer an alternative method for the preparation of particularly difficult sequences (e.g. all L-cyclotetrapeptides), and amongst these the use of peptide bond mimetics provides a promising avenue to access this important cyclic structures.

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