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MINIREVIEW ARTICLE

Solid-phase peptide synthesis (SPPS), C-terminal vs. side-chain anchoring: a reality or a myth

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Abstract Here we review the strategies for the solid-phase synthesis of peptides starting from the side chain of the C-terminal amino acid. Furthermore, we provide experimental data to support that C-terminal and side-chain syntheses give similar results in terms of purity. However, the stability of the two bonds that anchor the peptide to the polymer may determine the overall yield and this should be considered for the large-scale production of peptides. In addition, resins/linkers which do not subject to side reactions can be preferred for some peptides.

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The importance of peptides in modern science has grown exponentially in recent years. In addition to that, peptides are considered a firm alternative to small molecules for the treatment of a large number of human and animal diseases (Albericio and Kruger 2012; Kaspar and Reichert 2013; Scognamiglio et al. 2013; Gongora-Benitez et al. 2014). The cosmetic and nutraceutical industries are also introducing peptides into their products (Mentel et al. 2012; Udenigwe and Howard 2013). Furthermore, peptides are also used in the development of drug delivery systems and diagnostic kits (Li et al. 2012; Vasconcelos et al. 2013). Finally, new biomaterials for a broad range of applications are currently prepared from peptides (Nune et al. 2013; Menzel 2013). The introduction of these biomolecules into our everyday lives (Zompra et al. 2009; Chandrudu et al. 2013) has unquestionably been fuelled by the development and optimization of the solid-phase synthetic strategy, first described by Merrifield (Merrifield 1963).

In brief, the solid-phase peptide synthesis (SPPS) is based on the concourse of a supported protecting group—a polymeric support—that facilitates the stepwise elongation of this biopolymer through sequential steps of coupling and deprotection of protected amino acids, thus allowing the use of large excess of reagents. At the end of the synthetic process, a chemical treatment is usually applied to remove the protecting groups and detach the peptide from the resin (Albericio et al. 2011; Gongora-Benitez et al. 2013).

All peptides have the following four types of functional groups which can in principle be used for attachment to the polymeric support: (1) C-terminal function (C to N strategy); (2) N-terminal function (N to C strategy); (3) backbone; and (4) side chain (if a trifunctional amino acid is present) (Fig. 1).

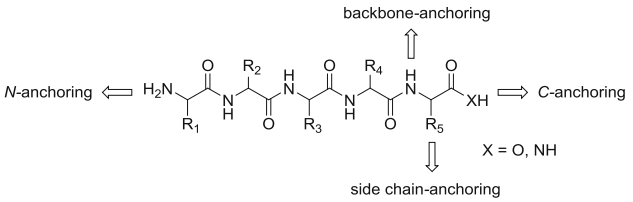


Fig. 1 Schematic representation of a peptide showing the anchoring sites to the polymeric support

SPPS through the C-terminal function is the most predominant strategy for the preparation of peptides because the formation of the peptide bond usually requires the activation of the carboxylic acid component. This is the key component of the reaction, which is the one added in solution and can be used in large excess driving the reaction to completion (Lloyd-Williams et al. 1997; Kates and Albericio 2000). Furthermore, the amino function of the activated amino acid component is commonly protected as a carbamate, whose slight electron-withdrawing effect does not facilitate oxazolone formation—the cause of racemization and poor yields—and does not overly enhance the acidity of the α-proton, thus minimizing the racemization through the enol mechanism (El-Faham and Albericio 2011).

Several attempts of SPPS through N-anchoring have been made (Thieriet et al. 2000). However, the need to activate acid terminal in this strategy jeopardizes its broad use, because potential racemization through oxazolone formation can occur at each step [route (a) in Fig. 2]. Furthermore, diketopiperazine can also form in each step [route (b) in Fig. 2].

Backbone anchoring is normally used when manipulation of the C-terminal function is required and the C-terminal amino acid is not trifunctional (Jensen et al. 1998, 1999). For trifunctional amino acids, except for Arg, side-chain

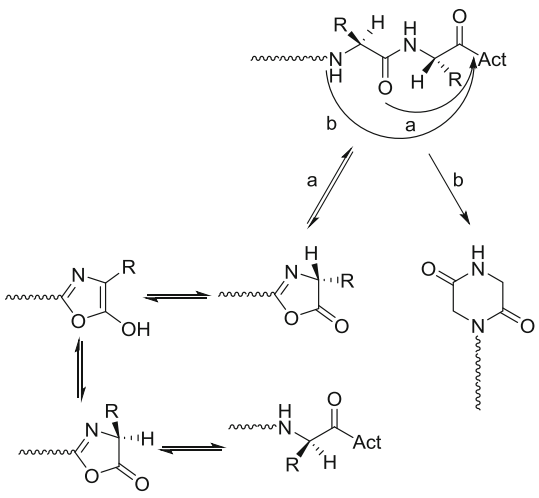


Fig. 2 Main side reactions during the N to C elongation

anchoring is preferred due the simplicity of the strategy. The backbone amide linker (BAL) is very useful for the synthesis of C-terminal-modified peptides, such as peptide aldehyde (Kappel and Barany 2005; Boas et al. 2009).

SPPS through side-chain anchoring is used for the following cases:

1. *Synthetic comfort* It is now widely accepted that the synthesis of peptide amides is more convenient than for their counterpart acids. The formation of the initial amide bond (side chain of Gln and Asn) is generally achieved with better yields and less racemization when compared with ester formation in Wang-type resins. Furthermore, the amide bond is on the whole more stable than the ester bond and, therefore, peptide amides are synthesized with better yields (Albericio et al. 1990; Breipohl et al. 1990).
2. *Minimization of side reaction formation* The synthesis of C-terminal Cys-containing peptide acids is accompanied by high level of Cys racemization and *N*-piperidyl-Ala through the formation of didehydro-Ala residue followed by a piperidine Michael addition. In this regard, Barany and co-workers demonstrated that the side anchoring of Fmoc-Cys-O*t*Bu to the solid support through a xanthenyl handle allows minimization of the above-mentioned side reactions in peptide synthesis (Barany et al. 2003; Han and Barany 1997). Alkoxybenzyl resins and linkers, such as Wang-type, PAL, Rink, BAL, are cleaved by different points during the TFA treatment, thus leading to several carbocations, which can be reattached to the peptide with the consequent formation of side products (Yraola et al. 2004; Cironi et al. 2004). These resins are used for the preparation of both acid and amide peptides. In this regard, the use of chloro-trityl chloride (CTC), developed by Barlos and co-workers, does not show this abnormal cleavage and therefore the synthesis, for instance, of Ser/Thr-NH_2 C-terminal peptides through the side-chain anchoring of Fmoc-Ser/Thr-NH_2 to CTC resins is advantageous for this kind of peptide (Ziovas et al. 2012; Barlos 2013a, b).
3. *On-resin cyclization* Cyclic peptides can be prepared on solid-phase by the side- chain anchoring while the C-terminal acid bears an orthogonal protecting group. The protecting group is then removed after the elongation of the sequence to be further activated for rendering the macrolactamization with a free amino function (N-terminal or side-chain amino function) (Kates et al. 1994; Rovero 2000).
4. *Manipulation of the C-terminal function* A similar strategy to that used for on- resin cyclization can be used to prepare modified peptides such as the C-terminal thioesters required for chemical ligation, after

Table 1 Resins and linkers used for side-chain anchoring of protected amino acids

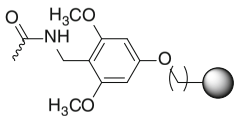
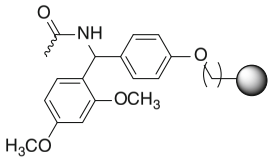
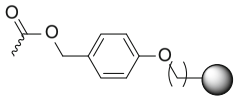
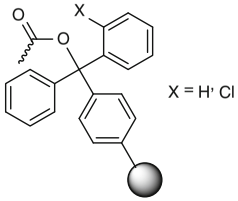
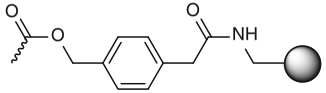
Amino acid(s)	Resin/linker (indicating the anchoring site)[b]	Name	Strategy	Cleavage conditions[c]	References
Asn/Gln		PAL linker	Fmoc/*t*Bu	TFA/thioanisole/β-mercaptoethanol/anisole (90:5:3:2)	(Albericio et al. 1990; Kates et al. 1993)
		Rink resin/linker	Fmoc/*t*Bu	TFA/H_2O (9:1)	(Breipohl et al. 1990; Trzeciak and Bannwarth 1992)
Asp/Glu[a]		Wang resin/linker	Fmoc/*t*Bu	TFA/phenol (95:5)	(McMurray 1991)
		Chloro-trityl chloride (CTC) resin	Fmoc/*t*Bu	TFA/DCM (2:98)	(Teixido et al. 2003)
		PAM (phenylacetamidomethyl) resin	Boc/Bzl	HF/anisole/dimethylsulfide (10:1:0.5)	(Rovero et al. 1991)

Table 1 continued

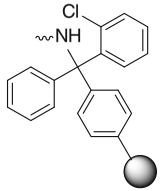
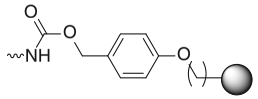
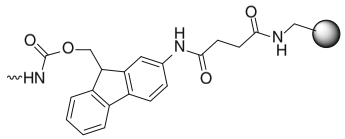
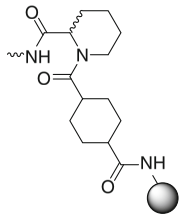
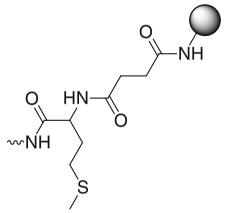
Amino acid(s)	Resin/linker (indicating the anchoring site)[b]	Name	Strategy	Cleavage conditions[c]	References
Lys		CTC resin	Fmoc/*t*Bu	TFA/DCM (3–5:97–95)	(Giraud et al. 2008; Barlos 2013a, b)
		Carbonate Wang resin	Fmoc/*t*Bu	TFA/thioanisole/ethanedithiol/anisole (90:5:3:2)	(Alsina et al. 1994)
		Carbonate Fluorenylmethyl resin	Boc/Bzl	Piperidine or morpholine/DMF (20:80)	(Alsina et al. 1994)
		Pipecolic linker	Fmoc/*t*Bu	TFA/TIS/H_2O (95:2.5:2.5)	(Masurier et al. 2012)
		Methionine linker	Fmoc/*t*Bu Boc/Bzl	CNBr (60 eq.) in CH_3CN/HOAc/H_2O (5:4:1)	(Kappel and Barany 2004)

Table 1 continued

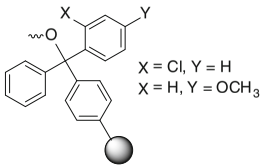
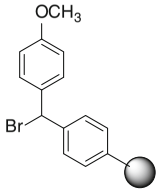
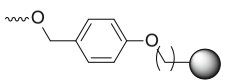
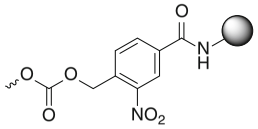
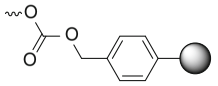
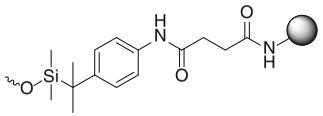
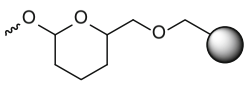
Amino acid(s)	Resin/linker (indicating the anchoring site)[b]	Name	Strategy	Cleavage conditions[c]	References
Ser/Thr		CTC and chloro-trityl methoxy resins	Fmoc/*t*Bu	TFA/DCM (3–5:97–95)	(Barlos 2013a; Rizzi et al. 2011)
		Bromo-(4-methoxyphenyl)methyl resin	Fmoc/*t*Bu	TFA/TIS/H_2O (95:2.5:2.5)	(Barlos 2013a)
		Wang resin/linker	Fmoc/*t*Bu	TFA/TIS/H_2O/thioanisole (92.5:2.5:2.5:2.5)	(Yan et al. 2004)
		Carbonate Nbb linker	Boc/Bzl	Photolysis at 350 nm in TFE/DCM (2:8)	(Alsina et al. 1997, 1998)
		Carbonate resin	Boc/Bzl	HF/anisole (9:1) or TFA/TFMSA/thioanisole/ethanedithiol/anisole (85:5:5:3:2)	(Alsina et al. 1997, 1998)
		Silyl linker	Fmoc/*t*Bu	TBAF/AcOH (1:1)	(Nakamura et al. 1999)
		Ellman resin	Fmoc/*t*Bu	TFA/DCM/EtOH (2:2:1)	(Graham et al. 2002; Villorbina et al. 2007)

Table 1 continued

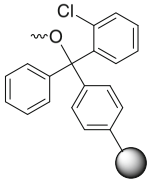
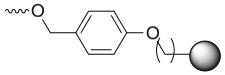
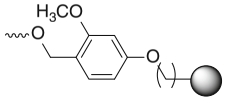
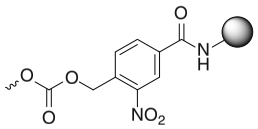
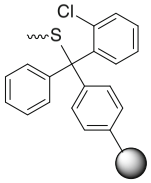
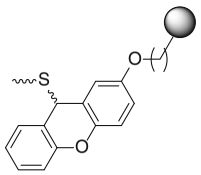
Amino acid(s)	Resin/linker (indicating the anchoring site)[b]	Name	Strategy	Cleavage conditions[c]	References
Tyr		CTC resin	Fmoc/*t*Bu	TFA/DCM (3–5:97–95)	(Bernhardt et al. 1997)
		Wang resin/linker	Fmoc/*t*Bu	TFA/TIS/H_2O/thioanisole (92.5:2.5:2.5:2.5)	(Cabrele et al. 1999)
		Sasrin resin	Fmoc/*t*Bu	TFA/TIS/H_2O/thioanisole (92.5:2.5:2.5:2.5)	(Graham et al. 2002)
		Carbonate Nbb linker	Boc/Bzl	Photolysis at 350 nm in TFE/DCM (2:8)	(Alsina et al. 1998)
Cys		CTC resin	Fmoc/*t*Bu	TFA/DCM (3–5:97–95)	(Bernhardt et al. 1997)
		*S*-Xanthenyl linker	Fmoc/*t*Bu	TFA/DCM/*i*Pr_3SiH (0.2:99.3:0.5)	(Han and Barany 1997)

Table 1 continued

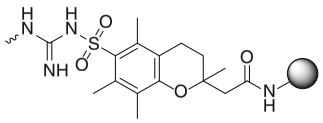
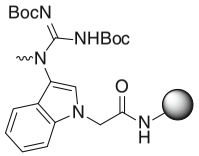
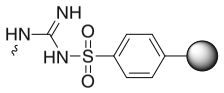
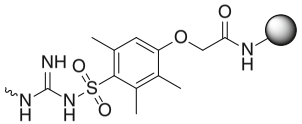
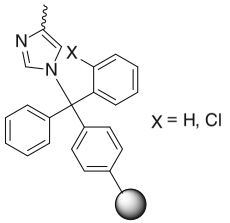
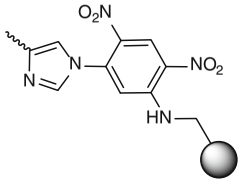
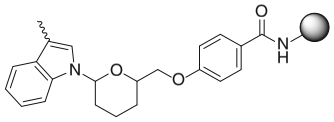
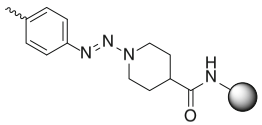
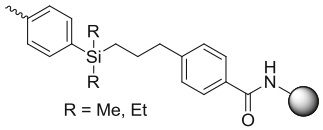
Amino acid(s)	Resin/linker (indicating the anchoring site)[b]	Name	Strategy	Cleavage conditions[c]	References
Arg		Pmc-based linker	Fmoc/*t*Bu	TFA/H_2O (10:1)	(Garcia et al. 2003)
		Indolylacetyl linker	Fmoc/*t*Bu	TFA/DCM (1:1)	(Beythien et al. 2006)
		Sulphonyl resin	Boc/Bzl	HF/anisole (4:1)	(Zhong et al. 1997)
		Polysubstituted Sulphonyl linker	Boc/Bzl	1 M TMSBr-thioanisole/TFA (10 mL)	(Edwards et al. 2000)
His		CTC and Trt resins	Fmoc/*t*Bu	TFA/DCM (3–5:97–95)	(Alcaro et al. 2003)
		2,4-Dinitrobenzene linker	Boc/Bzl	Deoxygenated thiophenol or dithiothreitol in DMF	(Isied et al. 1982)
Trp		Dihydropyranyl linker	Fmoc/*t*Bu	TFA/mDMB/DCM (0.5:0.5:9)	(Torres-Garcia et al. 2012a; Preciado et al. 2013)

Table 1 continued

Amino acid(s)	Resin/linker (indicating the anchoring site)[b]	Name	Strategy	Cleavage conditions[c]	References
Phe		Triazene linker	Fmoc/*t*Bu	(i) TFA/DCM (5:95) (ii) $Fe_2SO_4{\cdot}7H_2O$	(Torres-Garcia et al. 2012b)
		Ellman traceless linker	Fmoc/*t*Bu	TFA/thioanisole (25:1)	(Lee and Silverman 1999; Liu et al. 2005)

Most of the resins are commercially available except carbonate fluorenylmethyl resin, pipecolic linker, methionine linker, bromo-(4-methoxyphenyl)methyl resin, silyl linker, *S*-Xanthenyl linker, Pmc-based linker, indolylacetyl linker, sulphonyl resins, dihydropyranyl linker and triazene linker, which can be prepared using the methods described in the corresponding references

Schutkowski and co-workers have described the introduction of several Fmoc-AA-*p*-nitroanilides, including Arg, Gln, Citrulline, and Trp, among others, on CTC resin, with mixed results (Kaspari et al. 1996; Bernhardt et al. 1997)

Representative references are given in each case

[a] Although in some of the resins, just model small peptides have been synthesized, the use of side anchoring through a CTC resin has allowed the synthesis of peptides larger than 30 residues (Barlos 2013a, b)

[b] Parenthesis in the chemical structure indicates that both resin and linker have been used. Resin is when part of the solid support core forms part or all of the linker; Linker is when the proper linker is attached to the solid support core (adapted from Guillier et al. 2000)

[c] In the TFA linkers/resins other scavengers can be used but the proportion of TFA is advisable to be kept

reaction of the activated C-terminal carboxylic acid with the corresponding thiol (Diaz-Rodriguez et al. 2012; Ajish et al. 2009; Ficht et al. 2008; Tulla-Puche et al. 2004; Alsin et al. 2000). In the case of other modified peptides, such as the C-terminal *p*-nitroanilide, where the precursor is a poor nucleophile, the synthesis starts at the residue n-1 and at the end the C-terminal amino acid is incorporated in form of the *p*-nitroanilide, which is prepared in solution after cleavage of the peptide from the resin.

The following table describes the resins and/or linkers used for side-chain anchoring, with representative references (Table 1).

In addition to the four cases described above regarding the use of side-chain vs. C-terminal SPPS, there is the question as to whether side-chain anchoring has some additional (dis)advantages over the C-terminal strategy with respect to the peptide skeleton arrangement in relation to the polymer. Thus, in the 90 s, Larsen and Holm described a new concept, sequence-assisted peptide synthesis (SAPS) (Larsen and Holm 1996). This approach is based on the introduction of certain C-terminus-positioned short sequences $(Lys)_n$, which induce a structure in a subsequent peptide chain that can lead to improved synthesis of difficult sequences. This concept was demonstrated for the synthesis of several $(Ala)_n$ and $(Thr\text{-}Val)_n$ peptides, as well as other natural sequences corresponding to acyl carrier protein and insulin (Larsen and Holm 1998a). This concept was further reinforced when they showed that the use of 4-methoxymandelic acid as a Wang-type handle, which binds the polymer to the peptide through a three-atom moiety, renders better results than when a typical Wang linker, such as the 4-hydroxymethylphenoxypropionic acid, is used (Larsen and Holm 1998b). If the SAPS concept is correct, side-chain anchoring could impair bonding between the polymer and the peptide chain.

Although we demonstrated that the synthesis of Thymosin α1 via side-chain anchoring of the C-terminal Asn/Asp through the β-carboxylic acid of Asp to a Rink resin performs better than through the α-carboxylic acid of Asn to a CTC resin, this result does not validate the hypothesis that side-chain anchoring is more favorable than that of C-terminal synthesis, because more than one parameter was changed (linker and resin) (Garcia-Ramos et al. 2009).

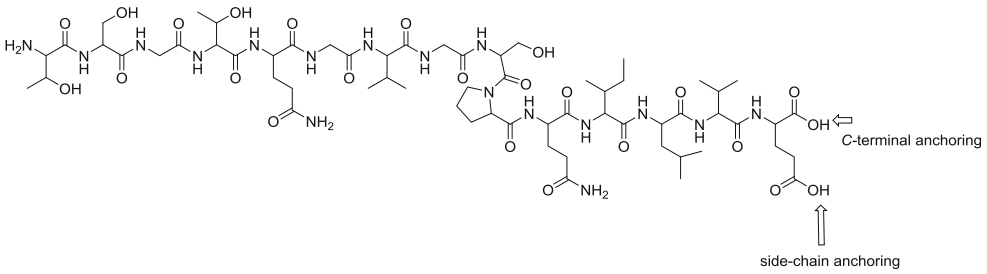


Fig. 3 Sequence of HIV-1 Rev (91–105)

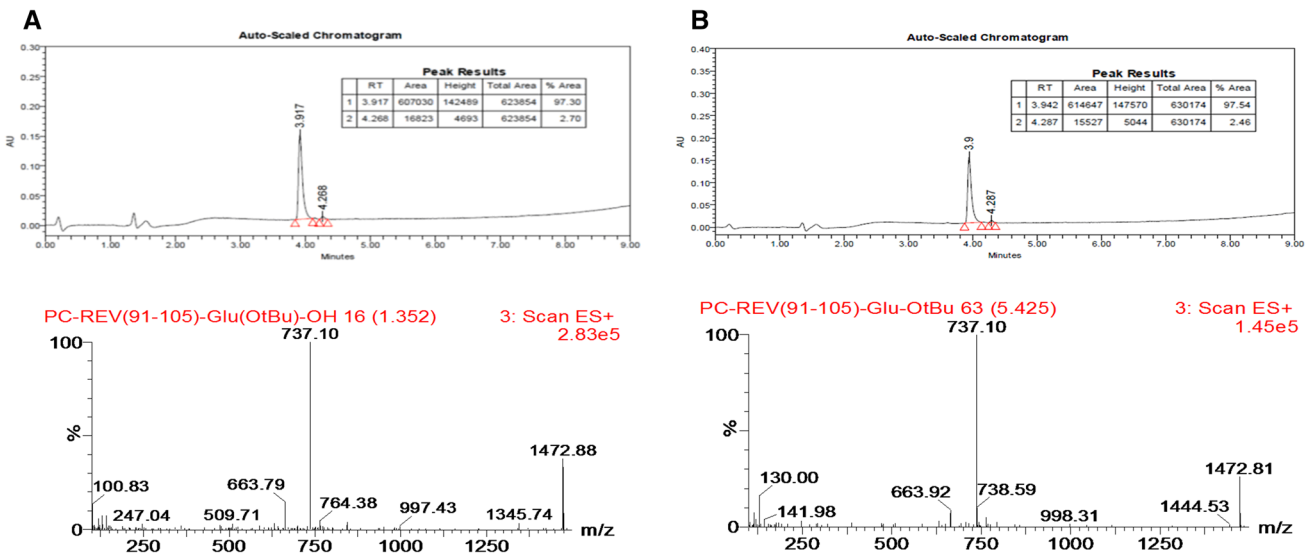


Fig. 4 HPLC and HPLC–MS of HIV-1 Rev (91–105); **a** from C-terminal anchoring; **b** from side-chain anchoring

Bearing all these factors in mind, we designed a simple experiment to determine whether there are any differences between C-terminal and side-chain anchoring. For this purpose, we synthesized a Glu C-terminal peptide. Glu or Asp is the most neutral amino acids for this experiment, because the same resin and the same protection for the remaining carboxylic acid can be used for both syntheses. As a model, the 15 amino acid peptide HIV-1 Rev (91–105) was chosen (Fig. 3).

Syntheses were performed by applying methods extensively used in our laboratories (Pelay-Gimeno et al. 2013). For this purpose, we anchored Fmoc-Glu(O*t*Bu)-OH and Fmoc-Glu-O*t*Bu (0.6 equiv.) to CTC resin (1.6 mmol/g) to render the starting resins [Fmoc-Glu(O*t*Bu)-O-CTC-resin, 202 mg, 0.77 mmol/g and Fmoc-Glu(O-CTC-resin)-O*t*Bu, 204 mg, 0.79 mmol/g]. Elongation of the peptide chain was carried out consecutively with Fmoc-Val-OH (3 equiv.), Fmoc-Leu-OH (3 equiv.), Fmoc-Ile-OH (3 equiv.), Fmoc-Gln(Trt)-OH (3 equiv.), Fmoc-Pro-OH (3 equiv.), Fmoc-Ser(*t*Bu)-OH (3 equiv.), Fmoc-Gly-OH (3 equiv.), Fmoc-Val-OH (3 equiv.), Fmoc-Gly-OH (3 equiv.), Fmoc-Gln(Trt)-OH (3 equiv.), Fmoc-Thr(*t*Bu)-OH (3 equiv.), Fmoc-Gly-OH (3 equiv.), Fmoc-Ser(*t*Bu)-OH (3 equiv.), Fmoc-Thr(*t*Bu)-OH (3 equiv.), using COMU (3 equiv.), OxymaPure (3 equiv.) and DIEA (6 equiv.) for 1 h in DMF. No recouplings were done. At the end of the syntheses, the global deprotection and cleavage of the peptide were carried out with TFA–H_2O–TIS (95:2.5:25) for 1 h. After precipitation of the peptide in cold ether and further washing with ether, it was dissolved in AcOH–H_2O (9:1) and lyophilized to give 180 mg (76 % overall yield) for C-terminal anchoring and 206 mg (87 % yield) for side-chain anchoring.

HPLC revealed that the crude peptides showed (Fig. 4) virtually identical purity (97.3 % for C-terminal anchoring and 97.5 % for side-chain anchoring), the main impurity being the same for both syntheses. Furthermore, the EI-MS showed a similar profile for both peptides.

We conclude that, in terms of purity, there are no major advantages of performing peptide synthesis through C-terminal vs./and side-chain anchoring. This conclusion is supported by the purity of the final products achieved. However, the better yield obtained (by approx. 10 %), which can be attributed to the better stability of the ester bond anchoring through the β-carboxylic (less acid and therefore worse leaving group) vs. the α-acid group, makes side-chain anchoring highly suitable for large-scale peptide synthesis. This improved stability can be translated into the preparation of longer peptides. In addition, this strategy is very convenient for the synthesis of cyclic and C-terminal-modified peptides. Finally, side-chain anchoring could allow the use of resins/linkers that do not subject to side reactions, which will make this strategy preferred in front of the C-terminal anchoring.

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Conflict of interest Authors declare that they do not have any financial conflict of interest.

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