Tetrahedron 67 (2011) 8595–8606

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Tetrahedron report number 952

# Aspartimide formation in peptide chemistry: occurrence, prevention strategies and the role of *N*-hydroxylamines

Ramon Subirós-Funosas [a,b], Ayman El-Faham [c,*], Fernando Albericio [a,b,d,*]

[a] *Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain*
[b] *CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain*
[c] *Alexandria University, Faculty of Science, Department of Chemistry, PO Box 246, Ibrahimia 21321 Alexandria, Egypt*
[d] *Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1-11, 08028 Barcelona, Spain*

ARTICLE INFO

*Article history:*
Received 8 August 2011
Available online 23 August 2011

**Contents**

1. Introduction ..... 8596
2. Appearance of aspartimide and derived byproducts ..... 8596
3. Factors influencing aspartimide formation ..... 8597
    3.1. Base ..... 8597
    3.2. Acid ..... 8598
    3.3. β-carboxyl protecting group ..... 8598
    3.4. Solid support ..... 8598
    3.5. Temperature ..... 8598
    3.6. Solvent ..... 8599
    3.7. Sequence (Asp-X) ..... 8599
    3.8. Conformation ..... 8599
4. Current approaches to minimization/suppression of aspartimide formation ..... 8599
    4.1. Sterically hindered protecting groups, bases and microwave irradiation ..... 8599
    4.2. Mild-cleavable linkers, backbone amide anchorage and $N^{\alpha}$-protecting groups ..... 8600
    4.3. Pseudo-Pro and Asp-X backbone amide-protecting groups ..... 8601
    4.4. Asp(OMe) conversion ..... 8602
5. *N*-Hydroxylamines as aspartimide suppressants ..... 8602
    5.1. Minimization during coupling in solution ..... 8602
    5.2. Minimization during β-carboxyl activation ..... 8603
    5.3. Minimization during Fmoc basic removal ..... 8603
6. Conclusions ..... 8603
    Acknowledgements ..... 8604
    References and notes ..... 8604
    Biographical sketch ..... 8606

*Abbreviations:* Acm, acetamidomethyl; API, active pharmaceutical ingredient; Boc, *tert*-butoxycarbonyl; CRH, corticotropin-releasing hormone; DBU, 1,8-diazabicyclo [5.4.0]undec-7-ene; DCM, dichloromethane; DEPBT, 3-(diethoxy-phosphoryloxy)-3*H*-benzo[*d*][1,2,3] triazin-4-one; DIEA, *N*,*N*-diisopropylethylamine; DIC, diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI-MS, electro spray ionization mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; HMPT, hexamethylphosphoramide; HOAt, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; Mtt, 4-methyltrityl; NMP, *N*-methyl-2-pyrrolidone; NMR, nuclear magnetic resonance; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran; PEGA, polyethylene glycol polyamide copolymer; Pmc, 2,2,5,7,8-pentamethylchroman; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TBAF, tetrabutylammonium fluoride; TBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene] *N*-methylmethanaminium tetrafluoroborate *N*-oxide; TFA, trifluoroacetic acid; THF, tetrahydrofuran; UV, ultraviolet.

* Corresponding authors. Fax: +34 93 403 71 26; e-mail addresses: aymanel_faham@hotmail.com (A. El-Faham), albericio@irbbarcelona.org (F. Albericio).

0040-4020/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2011.08.046

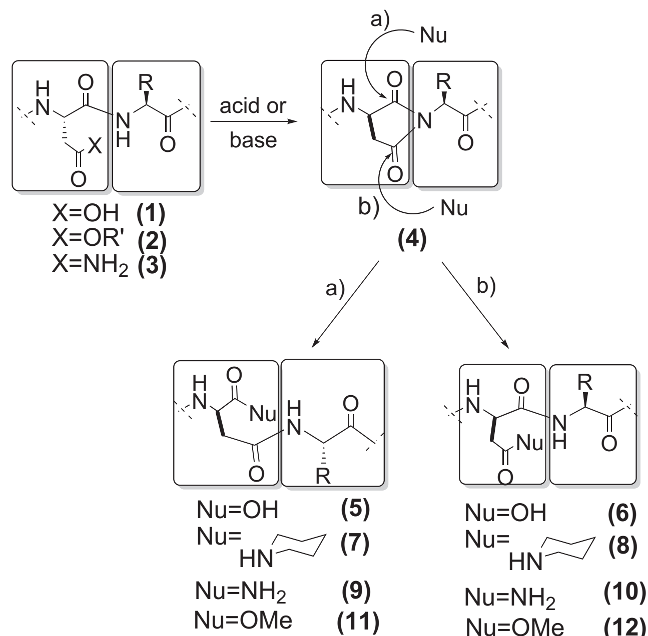
## 1. Introduction

Many side reactions may occur at various stages of solid-phase or solution peptide synthesis.[1] Some of these take place during activation of the C-terminus and coupling with the N-terminus, such as epimerization of the $\alpha$-carbon of the residue being activated, which is especially severe in the assembly of His and peptide fragments, or N-terminal guanidylation of the amino group when uronium/amminium salts are used, thereby blocking any further elongation of the peptide chain.[2–6] In these steps, certain amino acids might also undergo specific undesired reactions, like conversion to Orn, $\delta$-lactam formation (Arg), dehydration to nitrile, or succinimide and glutarimide formation (Asn, Gln).[7–11] Strong acidic conditions (HBr, TFA, HF) used for the removal of Boc, Bzl and $^t$Bu protecting groups or final cleavage of the peptide from the resin often cause alkylation on residues with nucleophilic side chains (Met, Cys) or those activated towards electrophilic aromatic substitution (Trp, Tyr), unless suitable cocktails of scavengers are added.[12–18] Acidolysis occasionally gives rise to undesired cyclizations (Glu, Gln, Met) or, under treatment with HF, provokes fragmentation of the peptide chain in Met, Ser, Thr or Asp-Pro-containing sequences.[19–25] Depending on the resin and sequence involved, basic media required for Fmoc removal can lead to diketopiperazine formation at the dipeptide stage and also to elimination of the thiol group of Cys to give dehydroalanine and piperidinylalanine derivatives.[26–29] The oxidation of Trp, Met and Cys has also been observed.[30]

*N*-Hydroxylamine-based additives greatly contribute to the success of peptide synthesis.[31] In addition to assisting in the reduction or suppression of several side reactions that occur during peptide bond formation, such as *N*-acylisourea formation, amino acid epimerization and guanidylation of the peptide chain, the unique acidic character of *N*-hydroxylamine-based additives also minimizes the impact of other non-coupling-related undesired reactions. Here we focus our attention on what could be considered the most documented and studied side reaction in peptide chemistry, namely aspartimide formation. Like most of the abovementioned detrimental impurities, aspartimide and its derivatives remain attached to the peptidic core, thereby hindering their removal, especially during the assembly of long peptides. This event is of concern in the case of APIs even when such impurities are encountered in very low amounts.

## 2. Appearance of aspartimide and derived byproducts

Aspartimides include amino-succinimide structures, formed or built as part of a peptide backbone (**4**, Scheme 1). Aspartimide units are often abbreviated as ‘Asu’ (*A*mino-*su*ccinyl).[32,33] However, it is more appropriate to use the term ‘Asi’ (*As*part*i*mide) or ‘Asc’ (*A*mino-*s*u*c*cinyl) when referring to this structure, since ‘Asu’ has also been used to denote $\alpha$-aminosuberic acid (herein we use ‘Asi’).[34,35] Asi-containing structures show properties and applications of interest in many fields. Given their biodegradability, polyaspartimides are promising solid supports for anchoring sialic acid linkers as inhibitors of viruses, like influenza.[36] Material science also takes advantage of Asi-based compounds, like bis(*N*-silylalkyl)aspartimides, which have recently been used to prepare surfactants, viscosity modifiers, primers and adhesives.[37] The presence of Asi moieties has been recently observed to support cell adhesion of peptides in vivo and in vitro.[38] Alternatively, Asi structures have proved useful as peptidomimetics during the synthesis of 2,5-dioxopiperazines as peptidomimetics, upon nucleophilic ring opening.[39] Studies based on X-rays, temperature dependence and circular dichroism spectroscopy on Boc-L-Pro-L-Asi-Gly-L-Ala-OMe concluded that the presence of Asi units induces type II′ β-turn in the peptide backbone conformation, as a result of stabilization by intramolecular hydrogen bonding.[40,41]




**Scheme 1.** Formation of aspartimide and byproducts derived from nucleophilic ring opening.

However, Asi (**4**) can be spontaneously generated during the elongation of Asp-containing peptides as result of nucleophilic attack from the amide nitrogen of the preceding residue to the β-carboxyl moiety of Asp (Scheme 1). Given the need for orthogonal protection schemes, the β-functional group in the side chain of Asp is normally masked as an ester (**2**), thereby acting as a leaving group in this side reaction. Nevertheless, under certain strongly acidic conditions, this unwanted cyclization is also reported to occur on the unprotected β-carboxyl group (**1**).[42–45] The early syntheses of Asp-containing peptides gave low yields and purities, although much effort was required to identify the source of the problem. It was only after ESI-MS detection of an $[M-18]^+$ ion in the crude mixture that Asi formation was envisaged, an event that was also supported by NMR.[46–48] Further proof of the Asp-based origin of the side reaction was obtained when the peptide fragment at the N-terminus of Asp was separately synthesized in a clean and efficient way.[48] The detection of Asi (**4**) formation can be troublesome in cyclic peptides, since the mass of this byproduct matches that of the desired cyclic material.[49]

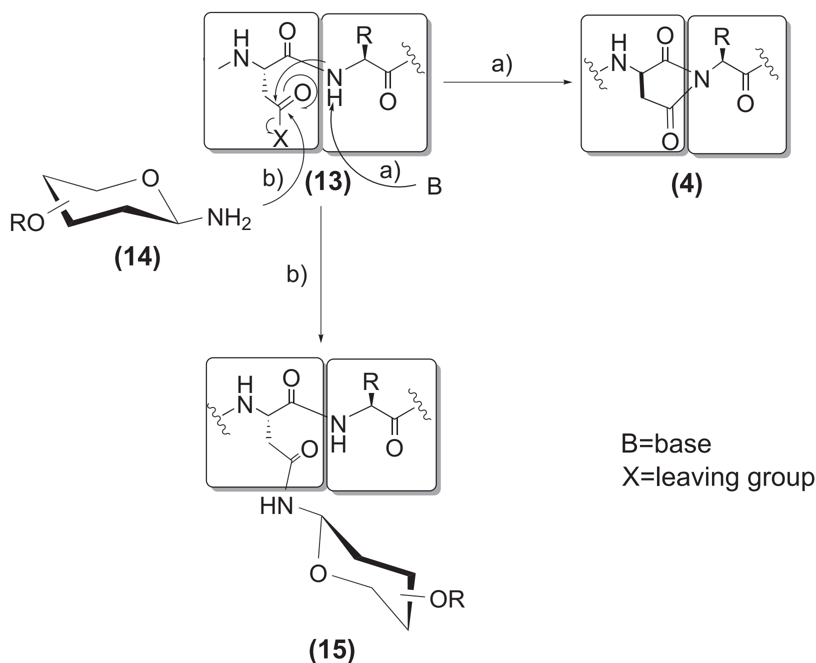
This byproduct (**4**) has been encountered in neutral, strongly acidic and basic media, either in solution or solid-phase synthesis.[33,43,45,48,50–52] It was originally found in the Boc/Bzl protection strategy, in the cleavage step of the peptide chain from the solid support using HF or MeSOOH.[42] In solution, this side reaction may take place during the removal of the Boc temporary protecting group and in the subsequent basic treatment to obtain the free amine, or during amide-bond formation using tertiary amine catalysis.[32,43] Later on, with the increasing presence of Fmoc/$^t$Bu protection schemes, this side reaction was found during standard basic Fmoc removal conditions.[42] Asi units can also occur when using a base-labile linker attached to the resin.[53] The wide variety of reaction conditions in which Asi are formed is not the major concern regarding this side reaction, but rather the additional byproducts derived from nucleophilic opening of the aminosuccinyl moiety (**4**).[52] In aqueous media, either in the synthesis or purification step, hydrolysis of Asi (**4**), which derives from attack to the β-carboxyl of Asp (path B, Scheme 1), leads back to the unmodified $\alpha$-peptide (**6**).[34] However, attack to the $\alpha$-carboxyl group (path A, Scheme 1) generates a product with unnatural backbone, the isoaspartyl-β-peptide (**5**).[54] Unprotected hydrolyzed peptides **5** and **6** may go unnoticed after amino acid analysis, since both

release Asp. Moreover their mass is identical.[42,44] The presence of the isoaspartyl-β-peptide (**5**) implies major difficulties in its separation from the target peptide.[34,55–57]

The impact of this detrimental side reaction is especially relevant in the Fmoc/$^t$Bu approach, the currently predominant choice of protecting strategy, not only because the basic treatment required for Fmoc removal takes place after each coupling/deprotection cycle, but also because this side reaction is faster in basic than in acidic media.[33,34,42,45,51,58] Moreover, in long sequences, stronger bases or extended reaction times are required for quantitative deprotection of the temporary group, thereby increasing the extent of Asi formation.[47] In addition, secondary nucleophilic amines, such as piperidine, are used to induce basic pH. Once an Asi ring (**4**) is present in the peptide backbone, piperidine can also open the cyclic structure in any of the carboxyl groups, thus resulting in a mixture of the piperidide of the α-peptide (**8**) and the piperidide of the β-peptide (**7**), in addition to the abovementioned hydrolyzed products (**5** and **6**).[45,47,48,51,52,54,58] Direct piperidine attachment to the β-carboxyl-protected group was considered by Yang and colleagues, although other authors rejected this mechanism.[45,55] These piperidide byproducts (**7** and **8**), which depend on the residue following Asp, can be easily identified by ESI-MS and show 47-mass units less than the target peptide.[45,47,48,59]

Thus, only 1 out of the 4 byproducts derived from the opening of the amino-succinyl ring leads to the desired α-peptide (**6**). This percentage of target peptide is even lower, bearing in mind that the content of piperidides **7** and **8** increases as does the number of deprotection steps performed. Moreover, the Asi ring (**4**) shows a remarkable activation towards epimerization of the α-carbon, which may produce additional aspartyl and piperidinyl derivatives.[47,52,55,57] Further studies also revealed that, as a result of electronic effects, the β-peptide byproducts (**5** and **7**) are more favoured than α ones (**6** and **8**).[32,55,60,61] Steric and conformational contributions might also be involved.[55] Finally, Asn (**11** and **12**) and Asp-methyl ester (**9** and **10**) derivatives have also been reported to occur after bubbling with ammonia at 0 °C or may arise accidentally as a result of washings with methanol in the presence of traces of DIEA in solid-phase.[34,51,55,62]

Synthetic methods to afford *N*-glycopeptides (**15**) as an alternative to enzymatic strategies are also hampered by this undesired cyclization (Scheme 2).[54] The two most representative approaches to bind an *N*-glycosylamine (**14**) to a peptide chain (**13**) involve the activation of the β-carboxyl group of Asp (**1**, convergent approach) or Asn (**3**, building block approach).[56,63,64] However, this enhanced reactivity of the side chain often results in nucleophilic attack of the preceding backbone amide nitrogen, which is catalyzed by the presence of base, such as DIEA to form the cyclic **4**, which occasionally represents the major product (Scheme 2). The competition between *N*-glycosylamine attachment to the β-carboxyl (**15**) and Asi formation (**4**) has been proposed by many authors.[54,63,64] Use of the sugar moiety as hydrogen-abstracting agent is one of the most efficient strategies to minimize this side reaction.[54]




**Scheme 2.** Base-mediated aspartimide formation during attachment of *N*-glycosylamines to Asp/Asn.

The generation of aspartimides (**4**) and derived byproducts during peptide synthesis causes a substantial decrease in yield and purity in addition to time-consuming purifications. Nevertheless, the effects of the appearance of these compounds in the peptide backbone are even more severe in vivo.[54] Although some exceptions are reported on the enhanced biological activity of Asi-containing sequences, the formation of the Asi (**4**) and, more important, the rearrangement to the β-peptide (**5**), which occurs in Asp (**1**) and Asn (**3**) residues, lead to dramatic events, such as the induction of flexible areas in proteins with secondary and tertiary structure and even their degradation.[54,60,65] The deamidation of Asn (**3**) to Asp (**1**), as a result of hydrolysis of the aspartimide on the β-carboxyl group, and racemization of Asp (**1**), are associated with protein ageing and degradation and with Alzheimer's disease, since many proteins associated with fibril aggregation show a high percentage of these modified backbones.[66,67] Recent studies revealed a link between Asi (**4**) formation and protein dimerization.[68]

## 3. Factors influencing aspartimide formation

### 3.1. Base

The effect of alkalis on base-catalyzed aspartimide formation was first tested in solution, after reports of the appearance of this

side reaction during coupling in this approach.[43] The stability of the Asp residue has been compared in the presence of various tertiary amines. The use of diisopropylethylamine (DIEA) has been shown to be safer than others, such as triethylamine ($Et_3N$). This result is attributed to its higher steric hindrance.[42,61] However, even when DIEA is chosen as the basic pH inducer, a small percentage of Asi or its derivatives might be formed (Tam and co-workers calculated that 0.002% of Asi is generated in each coupling step). This formation is higher in the synthesis of *N*-glycopeptides (**15**).[54,61,63] The extent of this unwanted cyclization is dependent not only on the type, but also on the total amount of base used in the coupling cocktail.[54,63]

Piperidine, the standard secondary amine applied in the removal of Fmoc in solid-phase, gives rise to a considerably higher percentage of Asi (**4**) than the previously mentioned tertiary amines, plus the additional presence of piperidides of the α- and β-peptide (**7** and **8**).[42] Even after long exposure to Asp-containing sequences, DIEA and $Et_3N$ do not cause this side reaction.[42,69] The concentration of the secondary amine also affects the amount and ratio of byproducts.[45,47] Stronger bases, such as DBU, TBAF, aqueous NaOH and $NH_3$, further accelerate the rate of this cyclization, compared to piperidine.[46,47,52,60,62,70,71] For instance, 50% piperidine in DMF produces a similar amount of byproducts to 2% DBU-2% piperidine in DMF.[47]

### 3.2. Acid

Cleavage of the peptidic material from the solid support by means of the Boc/Bzl protection scheme requires strong acid. Treatment of the resin with hydrofluoric acid (HF) causes contamination with Asi (**4**) and β-peptide (**5**), an occurrence, that is, particularly severe when using the 'low-high HF' cleavage strategy.[42] Other strong acids used to release the peptide chain, like trifluoromethanesulfonic acid (TFMSA) and concentrated TFA, also lead to these byproducts.[45,51,62,72] The latter organic acid has also been described to be problematic when simultaneously performing cleavage from the resin and removal of the Boc temporary group in the cyclic peptide argifin.[73] Diluted 1 N hydrochloric acid (HCl) might solve this problem, although higher concentrations (6 N) also give rise to Asi (**4**).[44,73]

Regarding the use of milder or less concentrated acids to remove the Boc temporary group in solution, acetic acid, hydrobromic acid (HBr) in acetic acid and 1:4 phenol/*p*-cresol are not recommended, since they may give rise to the undesired cyclization that produces Asi residues (**4**).[43] Alternatively, HBr-TFA mixtures or TFA alone render only traces of these byproducts.[43]

### 3.3. β-carboxyl protecting group

The nature of the β-carboxyl ester, acting as protecting group, is markedly influential on the impact of the aspartimide side reaction (Fig. 1). In the acidolytic-catalyzed cyclization, Asp(OBzl) (**16**) gives rise to high percentages of Asi (**4**) peptide and thus offers the poorest protection against this unwanted process.[32,43] The β-protection of Asp as cyclohexyl ester (OChx, **17**) results in increased prevention of Asi (**4**), possibly because of its high bulkiness.[61,74,75] Although Asp(OChx) (**17**) does not completely suppress the appearance of aspartimide and β-peptide during final cleavage with HCl, its content is much lower than that of Asp(OBzl) (**16**).[51,61,72] Low efficiency of the β-benzyloxy protection (**16**) in preventing this side reaction has prompted its selection when the formation of Asi units (**4**) is desired, after Nicolas and colleagues found that conversion from Asp(OBzl) (**16**) into Asi (**4**) could be quantitative, after only 10 min of acidic treatment.[34,42]

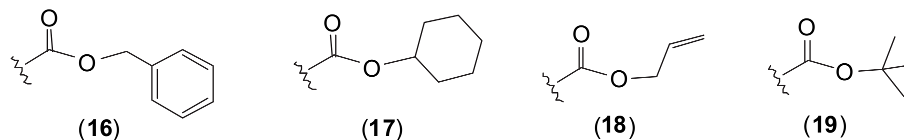



**Fig. 1.** Structure of some classical Asp β-carboxy protecting groups for Boc and Fmoc strategies.

β-cyclohexyloxy protection (**17**) is also more effective than β-benzyloxy one in the prevention of base-catalyzed aminosuccinyl formation (**4**).[32,72] In the presence of tertiary amines, Asp(OBzl) (**16**) induces up to 50% undesired cyclization, whereas Asp(OChx) (**17**) only renders <1%.[59] Moreover, the presence of the benzyl ester is associated with additional side reactions, such as 1,4-diazepine-2,5-dione ring formation, which can be the major product.[59] Nevertheless, Asp(OChx) (**17**) might give rise to Asi (**4**) when basic cleavage of the resin is performed.[58] Protection with the β-allyloxy group (**18**) does not result in improved prevention of the side reaction and is comparable to that achieved by β-benzyl ester protection (**16**).[49,52] Protection with the bulky *tert*-butyl ester (**19**) has shown greater efficacy than the abovementioned strategies in minimizing this side reaction in basic media.[49,54,70,73,76,77] In Fmoc removal conditions (treatment with piperidine), Asp(O$^t$Bu) (**19**) gives rise to considerably lower conversion into Asi residues than Asp(OChx) (**17**) and even Asp(OBzl) (**16**).[42]

### 3.4. Solid support

The resin used as solid-phase support may sometimes contribute to decreasing the formation of Asi (**4**). Thus, in certain syntheses, use of 2-chlorotrityl chloride resin, which offers the possibility of mild acidolysis of the peptide chain, prevents this undesired cyclization to a greater extent than Tenta Gel-derived solid supports.[62,73] Further improvements are achieved when polystyrene-type resins are replaced by less hydrophobic supports, such as PEGA and CLEAR (cross-linked ethoxylate acrylate-type resin).[60,64] By using the latter resin and 20% piperidine in NMP, only 8% aspartimide-related byproducts are detected in multi-Asp sequences.[78] Cebrian and colleagues reported that, when using the latter resin, the purity of the peptide increased from 10–30% to 80%, because the disruption of peptide chain aggregation translates into more efficient Fmoc removal steps, and thus basic treatments can be shortened, leading to minimization of Asi (**4**) and the formation of derived byproducts (**5**–**8**).[60]

### 3.5. Temperature

The temperature at which acidic or basic treatments are carried out contributes to a lower extent than previously mentioned factors in circumventing the formation of aminosuccinyl-containing peptide chains. However, high temperature alone (in the absence of solvent) also gives rise to Asi (**4**) formation, although at a slow rate.[68] However, a slight decrease in temperature might result in enhanced purity of target peptidic material.[54] Thus, Tam and co-workers observed that yield increased by 10% when the reaction temperature was lowered from 0 to −20 °C.[61] In contrast, when the temperature of the treatment of Fmoc-protected peptides with piperidine is increased to 45 °C, a greater content of Asi (**4**) and piperidides (**7** and **8**) is detected.[52]

### 3.6. Solvent

The properties of the solvent in which acidic or basic steps are performed have a great impact on the rate of undesired cyclization of Asp (**1**–**3**) to Asi (**4**). Extremely influential is the solvent polarity, which increases the percentage of Asi (**4**) formation in the order HMPT>DMSO>DMF>>THF>DCM.[47] Addition of water to DMSO solutions results in additional instability of the Asp residue.[64] In the synthesis of *N*-glycopeptides, through the activation of β-carboxyl group of Asp/Asn (**1**/**3**), DCM gives rise to lower percentage of aspartimide (**4**).[35] Moreover, Dölling and colleagues reported a substantial decrease in piperidide (**7** and **8**) formation when DCM or THF is used instead of DMF in the Fmoc removal step (0.5 vs 32%).[69] Among low-polarity solvents, the use of DCM is more efficient in the prevention of this side reaction than THF.[56]

Protic solvents, like MeOH, EtOH or BuOH, show a faster rate of Asi (**4**) formation than non-protic ones like DMF [complete conversion of Asp (**1**) into Asi (**4**) takes place in 15 days in DMF and 1–2 days in the previously mentioned protic solvents].[55] DMF is also involved in the ratio of byproducts observed, since formation of β-peptide (**5**) and epimerized byproducts are favoured in this solvent.[47,48,55] Finally, an efficient strategy for preventing Asi formation (**4**) consists of treating the Fmoc-peptide-resin with 30% piperidine in NMP (only 4 min per cycle), in conjunction with a fluoride-labile linker (2% aspartimides).[53]

### 3.7. Sequence (Asp-X)

Undoubtedly, the nature of the neighbouring amino acid located at the C-terminus of the aspartic acid (Asp-X) determines the degree of aspartimide formation, since the cyclization of Asp (**1**) to Asi (**4**) is initiated by attack of the amide backbone nitrogen of the preceding residue.[47] Thus, Gly (the least sterically hindered amino acid) shows the highest tendency towards formation of this unwanted cyclic structure (**4**) both under acid or base catalysis.[33,45,79–81] The syntheses of many peptidic compounds fail due to this marked instability, such as partial sequences of coat protein phage MS2, CRH hormone or thrombospondin.[60] Thus, it is not surprising that Asp-Gly-containing sequences, such as the 1–6 fragment of toxin II of scorpion *Androctonus australis Hector* (H-Val-Lys-Asp-Gly-Tyr-Leu-$NH_2$) and H-Glu-Asp-Gly-Thr-OH, have been widely used as models for testing aspartimide formation.[32,33,42,44,45,50,52,54,57,71,79,82–84]

Many other residues have great influence on base-catalyzed Asi (**4**) formation. One of the most prone amino acids is Asn, either protected as Asn(Trt) or Asn(Mtt). Most sequences containing Asn(Trt), such as partial fragments of MS2 or CRH, might fail in an initial attempt, although some exceptions are reported.[45,47,48,69,70] Asn(Mtt) is also very sensitive to Asi (**4**) formation.[71,79] Gln(Trt) and Asp(O$^t$Bu) have been shown to favour the unwanted cyclization.[45,47,64] The tendency towards this cyclization in other residues depends on the protecting group. Thus, Cys(Acm) and Arg(Pbf) induce this side reaction to a higher extent than Cys(Trt) and Arg(Pmc).[52,69–71] Ala [even when Asp is protected as Asp(OChx)], Phe and Ile also favour undesired Asi (**4**) formation.[45,55,58,62,64,71,76] In contrast, His(Trt), Ser($^t$Bu), Thr($^t$Bu), Tyr($^t$Bu), Leu and Val are relatively stable to this cyclization.[34,35,45,48,49,69–71,74] During glycosamine attachment on the Asp/Asn (**1**/**3**) side chain, Glu(OChx) or Ser($^t$Bu) gave higher percentages of aminosuccinyl-peptide chain than other residues.[35,56]

In acid-catalyzed Asi (**4**) formation, some residues that induce stability under basic treatment become markedly prone to this side reaction, thus proving that acid and basic catalysis go through distinct pathways. This is the case with Ser, Thr and His.[42,45,50] Other residues, like Gly and Asn, are as sensitive as in basic media.[42,45] Val is also stable under acidic catalysis.[32]

Revealing studies have been conducted on the tendency of unprotected amino acids preceding Asp residues (**1**) to give this undesired cyclization under basic conditions. On the one hand, Ser and Thr accelerate the rate of Asi (**4**) formation, compared to the average tendency of the overall residues.[32,70,71] It has been proposed that the favourable tendency towards this cyclization is due to the presence of a neighbouring-group effect from the free β-hydroxyl group.[32] On the other hand, amino acids bearing acidic β-functional groups, such as Asp, Glu and Tyr, show a lower tendency towards this process because the presence of the negatively charged side-chain functional group precludes the formation of a second negative charge, which is necessary to initiate the cyclization.[32] Asn and Gln are not as sensitive as their protected analogues.[54,69] Surprisingly, Met does not favour the aspartimide (**4**).[32] Hypotheses have been made about the formation of a six-membered cyclic structure with the Met side chain.[32]

### 3.8. Conformation

The conversion of Asp (**1**) into Asi (**4**) units is not only sequence-, but also conformation-dependent.[47] It has been observed that replacement of L-aa by D-aa results in enhanced byproduct generation, depending on the residue that has been changed.[69] Thus, introduction of D-Asp(O$^t$Bu)-D-Gln(Trt) in positions 25 and 26 of the CRH hormone increases the percentage of Asi (**4**) and piperidides (**7** and **8**) after repetitive Fmoc removal, compared to L-Asp(O$^t$Bu)-L-Gln(Trt). This observation would indicate the formation of a more favoured conformation.[47,48] The presence of D-amino acids in positions n+2 and n+3 induces a similar effect (n=Asp), whereas in more distant positions no increased tendency is detected.[48]

## 4. Current approaches to minimization/suppression of aspartimide formation

β-*tert*-butyloxy (**19**) and β-cyclohexyloxy (**17**) protection of Asp results in enhanced prevention of base- and acid-catalyzed Asi (**4**) formation, respectively, compared to the more prone β-benzyloxy (**16**) and β-allyloxy (**18**) groups. Nevertheless, contrary to initial impressions after evaluating this kind of protection, it was observed that, even when this choice of protecting group is supported by the presence of a relatively weak base, like piperidine, considerable amounts of Asi (**4**) and derived byproducts can arise.[35,45,47,48,52,55,57,69] In particular, using Asp(O$^t$Bu) (**19**) in the Fmoc/$^t$Bu scheme, substantial formation of aspartimides (**4**) and piperidides (**7** and **8**) is detected. As a result of these observations, and with the aim to minimize or suppress this detrimental side reaction, efforts have been made to improve the tools available in peptide synthesis.

### 4.1. Sterically hindered protecting groups, bases and microwave irradiation

Initially, the so-called 'temporary group' strategy was thought to be efficient in preventing the appearance of Asi (**4**) when following the Boc/Bzl scheme.[85] This approach consists of β-protection of Asp with a group showing orthogonality to the cleavage conditions, and the introduction of Fmoc-amino acids in the residues following Asp. Thus, these temporary β-protecting groups can be removed prior to HF treatment, which is envisaged as a safe scenario.[43,85] However, it was later discovered that acid-catalyzed Asi (**4**) formation also takes place in the free Asp (**1**).[42,44] Moreover, one of the first β-protecting groups proposed, the phenacyl ester, was found to be unstable to basic coupling conditions.[32,42] The introduction of aspartic acid as Asp(O$^t$Bu) (**19**) in this strategy results in similar or poorer performance than Asp(OBzl) (**16**) and Asp(OChx) (**17**).[42]

Alternatively, the use of a β-4-chloro-benzyloxy group in the standard Boc/Bzl strategy was not suitable, since this side reaction was enhanced in comparison with β-benzyloxy (**16**).[43]

In order to minimize the nucleophilic attack of the preceding amide backbone nitrogen atom to Asp, highly sterically hindered β-protecting groups for the Fmoc/$^t$Bu scheme have been designed (Fig. 2). On the one hand, increase of the bulkiness does not result in lower percentage of Asi (**4**), when the rigidity of the protecting group is simultaneously enhanced.[52,57] Thus, β-protection as Asp[Oada=β-(1-adamantyl)] (**20**), Asp[OPyBzh=β-(4-pyridyl-diphenylmethyl)] (**21**), Asp[OPhFl=β-(9-phenyl-fluoren-9-yl)] (**22**), Asp[OBO=β-(4-methyl-2,6,7-trioxabicyclo[2.2.2]oct-1-yl)] (**23**) or Asp[OPp=β-(2-phenylisopropyl)] (**24**) is less efficient than Asp(O$^t$Bu) (**19**) in preventing this undesired cyclization.[47,52,70,86] However, recent studies report that the latter approach, also referred to as Asp(OPhiPr) (**24**), dramatically reduces Asi (**4**) formation, in comparison with β-allyloxy protection (**18**).[64] The aromaticity of β-(4-pyridyl-diphenylmethyl) (**21**), β-(9-phenyl-fluoren-9-yl) (**22**) and β-(2-phenylisopropyl) (**24**) results in an excellent leaving group, which might favour the cyclization.[52] In contrast, the more bulky, although flexible, Asp[OMpe=β-(3-methylpent-3-yl)] (**25**) and Asp[ODie=β-(2,3,4-trimethyl-pent-3-yl)] (**26**) are considerably less sensitive than Asp(O$^t$Bu) (**19**) to Asi (**4**) formation.[52,57,71,79,87] Asp(ODie) is more efficient than Asp(OMpe) with prolonged exposure to piperidine treatment.[79] A further increase in bulkiness, as in the case of Asp[OTcm=β-(tricyclohexylmethanol-yl)] (**27**) and Asp[OTim=β-(triisopropylmethyl)] (**28**), results in extremely difficult syntheses. Moreover, this increased hindrance does not diminish the impact of this side reaction.[79] Regarding *N*-glycopeptide (**15**) chemistry, Asp [Bni=(β-5-bromo-7-nitroindoline)] (**29**) is the best choice for protection, because it efficiently combines the prevention of Asi (**4**) formation with possible activation towards glycosamine attachment under UV light.[54,56,88] Finally, β-protection with the 2% hydrazine hydrate-labile Dmab [4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl] (**30**) increases the tendency to cyclization. Therefore, this protecting group is used when the formation of Asi (**4**) units is desired.[62]

The effect of using highly sterically hindered bases has also been studied. The addition of bulky tertiary amines in the coupling cocktail, such as proton sponge, methyldibenzylamine and tribenzylamine, decreases the cyclization kinetics and also the rate of amide-bond formation, thereby resulting in inefficient couplings.[43] Pyridine analogues, like collidine, 1-methyl-2-pyrrolidone and 4-pyrrolidino-pyridine, behaved similarly.[34] The use of the guanidine-based TMG (1,1,3,3-tetramethylguanidine) is not recommended, since conversion of Asp (**1**) into Asi (**4**) increases.[52] The prevention of aspartimide (**4**) formation can be enhanced by using bases structurally resembling piperidine.[69] Thus, 4-hydroxypiperidine, diethanolamine, piperazine and morpholine substantially decrease the byproduct content, compared to piperidine (0.5 vs 32%).[35,58,60,89,90] In the synthesis of a CRH hormone analogue, piperazine does not give rise to Asi (**4**) or piperidides (**7** and **8**).[69,77] In addition, piperazine is less odorous than piperidine.[77,91]

By disrupting chain aggregation, microwave irradiation is a powerful tool for the synthesis of small organic molecules and peptides, especially in the assembly of hydrophobic sequences.[91,92] However, there is concern about the suitability of this technique in Asp-containing sequences, because the accomplishment of rapid coupling and Fmoc-removal steps has been associated with accelerated Asi (**4**) formation.[91] However, in fact microwave irradiation enhances the efficiency of weaker bases than piperidine, such as piperazine, which induce slow-rate deprotections in hydrophobic peptides.[77,91] In contrast, when Fmoc removal with piperazine was assisted by microwaves, deprotection was complete in only 3 min.[77,91]

### 4.2. Mild-cleavable linkers, backbone amide anchorage and $N^{\alpha}$-protecting groups

Asi (**4**) formation can also be suppressed by using synthetic strategies that prevent strong acidic or basic conditions (Fig. 3). Among these, linkers have been developed that allow cleavage from the resin under neutral or slightly basic conditions, which are particularly useful in the Boc/Bzl strategy, in which HF cleavage is the main source of Asi (**4**) formation.[53,58] For instance, Wagner and Kunz proposed the use of PTMSEL (2-phenyl-2-trimethylsilylethyl) linker (**31**), which bears a weak benzylic C–Si bond and is labile to

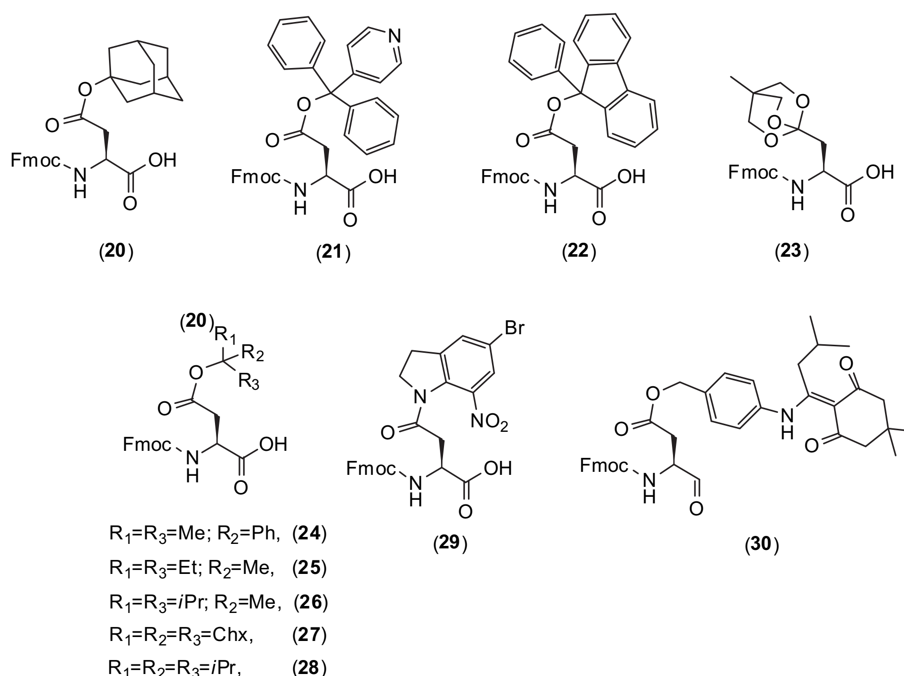



**Fig. 2.** Structure of Fmoc-Asp-OH residues featuring sterically hindered β-carboxy protection.

TBAF·$3H_2O$ in DCM, giving rise to only 2% of aspartimides (**4**).[53] Alternatively, the peptide chain can be anchored to the HMFS [*N*-[(9-hydroxymethyl)-2-fluorenyl] succinamic acid] handle (**32**), which can then be quickly cleaved in basic media, after treatment with anhydrous morpholine in DMF.[58,89] The absence of piperidine results in the prevention of aspartimide-based byproduct formation (**4**–**8**).

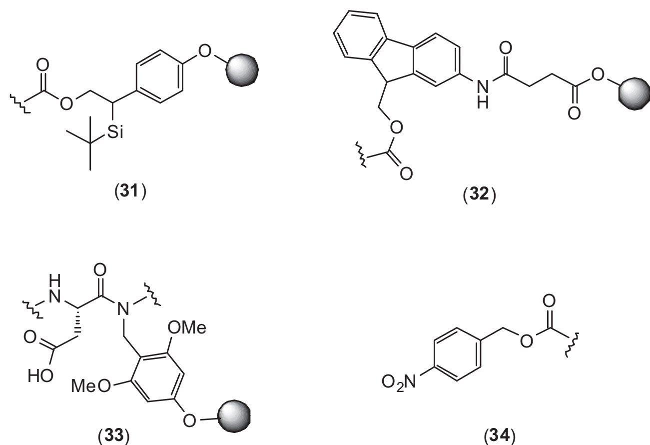



**Fig. 3.** Structure of non-basic cleaved PTMSEL and HMFS linkers, backbone amide anchoring groups and Sn(II) labile pNZ $N^{\alpha}$-protecting group.

In addition, amide backbone linkers (**33**) also have applications in the prevention of generation of Asi (**4**) units.[93] The advantage of this type of handle is that the growing peptide chain remains anchored to the resin by one of the backbone amide nitrogen atoms and therefore the Fmoc SPPS of C-terminal modified peptides can be achieved as result of the possibility of bidirectional growth.[93,94] If this nitrogen atom corresponds to the residue preceding Asp (which initiates the unwanted cyclization), aspartimides (**4**) are not formed.

[hydrogenation or, more conveniently, Sn(II) chloride], which do not give rise to Asi (**4**), isoaspartyl-β-peptide (**5**) or piperidides (**7** and **8**).[82] A recommended strategy consists of the introduction of pNZ-protected amino acids in the residues following Asp.[82]

### 4.3. Pseudo-Pro and Asp-X backbone amide-protecting groups

Asp-Pro sequences do not undergo the undesired cyclization that leads to Asi (**4**) units because the backbone amide nitrogen atom of the preceding residue that initiates this reaction is *N*,*N*-alkylated and therefore not activated towards nucleophilic attack.[54] On the basis of this observation, pseudoproline (PP) building blocks featuring an oxazolidine ring were introduced, representing one of the most attractive approaches to completely suppress Asi (**4**) formation (Fig. 4).[95] These cyclic constructs, available as aa-PP dipeptides, which also disrupt secondary structures, are derived from Ser [Ser($\psi^{me,me}$pro), **35**], Thr [Thr($\psi^{me,me}$pro), **36**] and Cys [Cys($\psi^{me,me}$pro), **37**] and function additionally as side chain-protecting groups, regenerating the original residue after peptide elongation under TFA treatment.[95] Alternatively, backbone amide-protecting groups, which mimic a pseudo-Pro effect, have been used for this purpose and to disrupt β-sheet aggregation (Fig. 4).[54,84,96] The first of these to be applied in the prevention of aminosuccinyl-peptide (**4**) generation was Hmb (2-hydroxyl-4-methoxybenzyl) (**38**), which in combination with Asp(O$^t$Bu) side chain protection (**19**) completely suppresses this side reaction.[52,54,97] Interestingly, the Asp-(Hmb)-Gly dipeptide building block can be directly introduced into the peptide chain.[52,84] However, many drawbacks have been associated with the use of Hmb, such as depsipeptide formation, incomplete removal after treatment with concentrated aqueous TFA, low yield and purity, and high cost.[33,62,71,83,98–100] Furthermore, Hmb is not compatible with the Boc/Bzl scheme and decreases the coupling rates once in the peptide chain as a result of its high bulkiness.[57,58] For this reason, the use of this compound is impractical for peptide synthesis.[77]

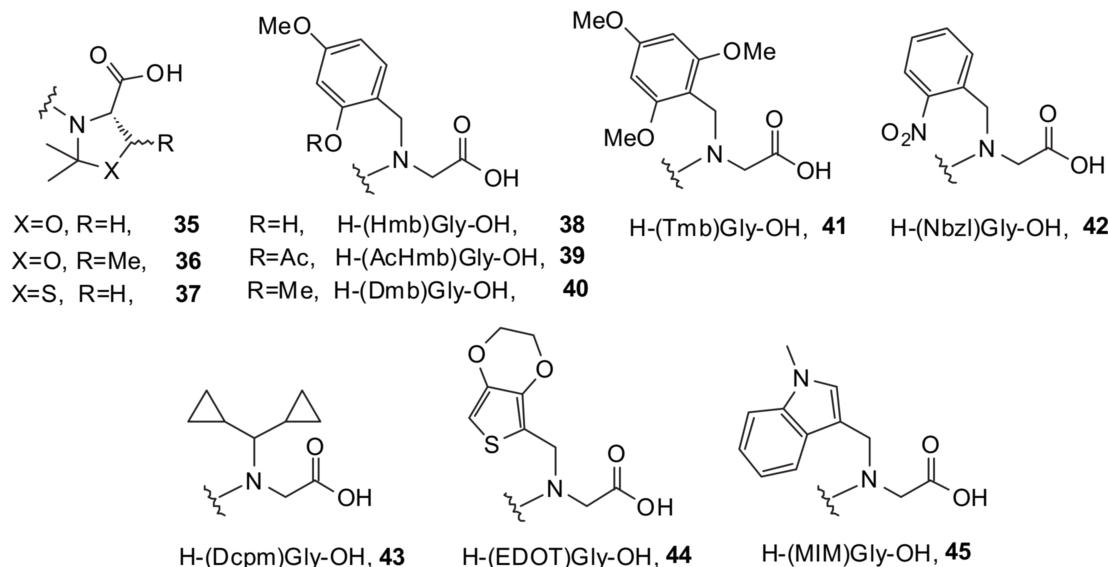



**Fig. 4.** Pseudoproline and Gly-linked amide backbone-protecting groups designed to suppress aspartimide formation.

Finally, some $N^{\alpha}$-amino protecting groups have been specifically designed to prevent this side reaction and are removed under safe conditions.[82] Thus, the pNZ (*p*-nitrobenzyloxycarbonyl) amino-protecting group (**34**), totally orthogonal to Fmoc, Boc and Alloc groups, is selectively eliminated under mild neutral conditions

Some alternatives, such as AcHmb (**39**), Tmb (**41**), Nbzl (**42**) or Dmb (**40**), were proposed in the following years.[33,54,76] Of these, only Dmb showed significant advantages over its predecessor.[33] With this backbone-protecting group, no Asi (**4**) was observed. Moreover, yields and acid lability were increased.[33] Dmb (**40**) is also reported to

be more easily introduced.[81,84] Nevertheless, its removal (along with final cleavage) requires 95% TFA and sometimes prolonged treatments, conditions that are known enhance the formation of Asi (**4**).[80,83] Alternatively, backbone protection can be effected with Dcpm (**43**), EDOT (**44**) and MIM (**45**).[80,83,101] The former (*N*-dicyclopropylmethyl), introduced as synthon [H-(Dcpm)-Gly] (**43**) or as dipeptide, efficiently suppresses this side reaction and is more reactive than Hmb (**38**) and Dmb (**40**) analogues.[80,101] In contrast to Hmb (**38**), Dcpm is inert to acylations and can be removed with mild 5% TFA in chloroform.[80] EDOT (3,4-ethylenedioxy-2-thienyl, **44**) and MIM (1-methyl-3-indolylmethyl, **45**) are, like Dcpm (**43**), more acid labile than Dmb (**40**), and, in addition, they are commercially available and easily synthesized.[83] Remarkably, EDOT (**44**) is also introduced in higher yield than Dmb **40** (97 vs 60%), because of its lower steric hindrance.[83]

### 4.4. Asp(OMe) conversion

As previously mentioned, the amino-succinyl ring (**4**) can be opened with a variety of nucleophiles.[55] When methanol is used, then the methyl ester of the α- and β-peptide (**9** and **10**) is formed.[55,62] The appearance of such byproducts was envisaged after ESI-MS detection of an [M+14] molecular ion, which supposedly derives from the combination of the methanol used in the resin washings, and traces of DIEA from the coupling cocktail.[51,62] However, this ring opening of the Asi (**4**) can be turned into an advantage, as purification of the target peptide is facilitated when evolution to the β-peptide (**5**) is prevented.[34,51] Thus, complete conversion from Asi (**4**) into a mixture of the methyl esters (**9** and **10**) is achieved by treatment of the aspartimide-containing peptide chain with 2% DIEA in methanol.[51] The suitability of using secondary alcohols was rejected, since these require high temperature to yield the esters.[34]

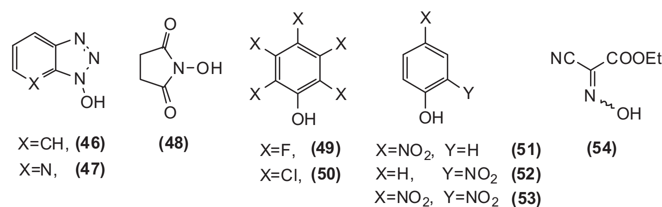
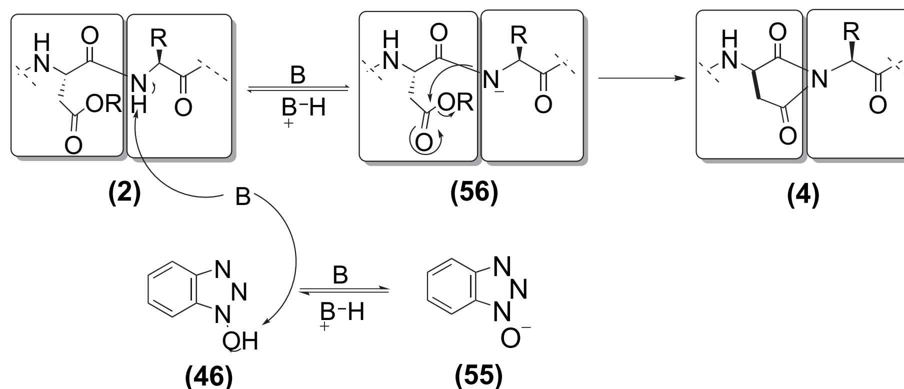



**Fig. 5.** *N*-Hydroxylamine and phenol-type additives.

*N*-Hydroxylamine-based additives also contribute to the wide arsenal of approaches to prevent the formation of Asi (**4**) and derived byproducts (**5**, **7**, **8**). This beneficial effect is observed in base-catalyzed Asi (**4**) cyclization, during coupling or Fmoc removal with secondary/tertiary amines. The common belief is that the unique acidic properties of *N*-hydroxylamines used as additives in peptide synthesis (p$K_a$=2−10) are responsible for this behaviour.[32] The abstraction of the amide backbone proton has been proposed as the crucial step in the cyclization that leads to Asi (**4**). Thus, addition of a relative strong acid, such as HOBt (**46**), results in competition with the Asp-X amide backbone for the base present in the medium (Scheme 3; exemplified with HOBt, **46**).[32] When HOBt (**46**) is used as additive, conversion into its anion (**55**) by the effect of the base would decrease the percentage of negatively charged amide backbone nitrogen (**56**), which is responsible for initiating Asi (**4**) formation (Scheme 3). Such minimization of the unwanted base-catalyzed cyclization, through acidic buffering of the medium, is not surprising given the similar effect induced by unprotected Glu, Asp or Tyr when these precede Asp in the sequence.[50,58,70] Moreover, the acidity of *N*-hydroxylamines lies in a well-suited area, since lower p$K_a$ would result in complete abstraction by the base, thereby decreasing the efficiency of the coupling/deprotection reaction.[32]




**Scheme 3.** Competition between *N*-hydroxylamine and amide backbone proton abstraction.

## 5. *N*-Hydroxylamines as aspartimide suppressants

*N*-hydroxylamine-based compounds and coupling reagents are widely used as amide bond-forming agents (Fig. 5).[31,102–105] In addition, additives are beneficial in order to reduce the extent of racemization and guanidylation of the N-terminus of the growing peptide chain and to increase coupling efficiency.[106,107] This substantial contribution to coupling strategies prompted their evaluation in the prevention of other non-coupling-derived side reactions. In some cases, like pyrrolidonecarboxylic acid (pca) generation in Glu residues, the addition of *N*-hydroxylamines causes an increase in the rate of byproduct formation.[20] However, in other unwanted reactions, usually occurring under basic conditions, their addition can be advantageous. This is the case of Pro over coupling or trifluoroacetylation of the amino group after Boc removal during the following coupling.[108,109]

### 5.1. Minimization during coupling in solution

When studying the extent of Asi (**4**) formation during coupling, the effect of additives to carbodiimides was examined in an Asp(OBzl)-Gly dipeptide model, a relatively prone sequence (Scheme 3).[32] Surprisingly, it was observed that these compounds, mainly *N*-hydroxylamines, do not accelerate the cyclization kinetics in the presence of tertiary amines, but delay them (see Scheme 3 for the proposed mechanism).[32] In detail, *N*-hydroxysuccinimide (**48**, p$K_a$=5.1) resulted in a three-fold decrease in the cyclization rate of Asp (**1**) to Asi (**4**), whereas 1-hydroxybenzotriazole (**46**, p$K_a$=4.3) induced inhibition by 20-fold, compared to the experiment without additive.[32]

Strongly acidic non-hydroxylamine additives, namely pentachlorophenol (**50**, p$K_a$=5.3) and 2,4-dinitrophenol (**53**, p$K_a$=4.1), are the most efficient in preventing the side reaction.[32]

Unexpectedly, no direct correlation between acidity and suppression could be found. In an example, pentafluorophenol (**49**, p$K_a$=5.3) is as acidic as its chloro analogue (**50**), but considerably less efficient. Similarly, HOBt (**46**) delays Asi (**4**) formation to a lesser extent than the less acidic pentachlorophenol (**50**), probably because of the higher bulkiness of the former.[32] Another hypothesis is that the presence of salt-like adducts when phenols are mixed with $Et_3N$ or DIEA affects the side reacion.[32] Interestingly, an equimolar combination of any of the most acidic phenols (**50** and **53**) with HOBt (**46**) maintains the high efficiency.[32] The concentration of the additives is also is a key factor in the suppression of Asi (**4**). The optimal ratio is an equimolar amount of base and additive, since a higher percentage of the latter does not result in improved performance.[32]

The evaluation of *N*-hydroxylamine and phenol-based additives was further tested in tri- and tetrapeptide models including the Asp(OBzl)-Asn sequence.[32] As in the previous models, activated phenols (**50** and **53**) show enhanced inhibition, in comparison to HOBt (**46**). Remarkably, the combination of pentachlorophenol (**50**) and HOBt (**46**) affords only traces of Asi (**4**), a stronger performance than any of the additives alone, and similar to that of 2,4-dinitrophenol (**53**).[32] Other studies showed the advantages of the simultaneous use of HOBt (**46**) and triethylamine ($Et_3N$) as base. Although this strategy is less efficient in the prevention of Asi-peptide (**4**) formation than 4-DMAP, acylation is much faster (3 min vs hours), thus resulting in a more adequate choice.[43] Interestingly, it was also observed than DIC/HOBt (**46**) gives rise to a lower percentage of byproducts than HOBt-based TBTU/HOBt and DIEA, presumably due to the absence of base in the coupling mixture.[62] An excess of coupling reagents is also reported to increase the extent of this side reaction.[82]

### 5.2. Minimization during β-carboxyl activation

As previously pointed out, during β-carboxyl activation of the side chain of Asp (**1**)/Asn (**3**) in *N*-glycopeptide (**15**) chemistry or cyclizations, significant aspartimide (**4**) formation is observed.[54,56] Many authors have proposed the presence of competition between the desired coupling and aspartic acid cyclization to Asi (**4**) units.[35,63,64] In this context, low-rate coupling reagents, such as PyBOP, result in enhanced percentage of byproducts, whereas more efficient agents, like DEPBT, are the most well-suited choice.[35,63]

Furthemore, in contrast to DCM, the addition of HOBt (**46**) partially solves the negative effect of THF, increasing the yield to 80% and the percentage of *N*-glycopeptide (**15**) from 29 to 66%.[56] Supposedly, the *N*-hydroxylamine accelerates the rate of glycosamine attachment, thereby decreasing the impact of Asi (**4**) formation. In contrast, the less acidic pentafluorophenol (**49**) increases the amount of byproducts (7–50% Asi).[56]

### 5.3. Minimization during Fmoc basic removal

For the last ten years, efforts have been directed towards the prevention of this side reaction in Fmoc/$^t$Bu chemistry, the currently predominant protection strategy, because its repetitive removal substantially decreases the purity of the peptide.[47,77] Piperidides of the α- and β-peptide (**7**, **8**) are usually formed, plus racemized versions.[45] Furthermore, the formation of byproducts under base catalysis is more severe than in acidic media as a result of the enhanced kinetics.[34,42,51,58] In view of the positive effect of the additives in solution coupling, their inclusion in piperidine solutions has been evaluated.[47,48,69]

Similar to the effect induced in solution, equimolar amounts of weakly acidic additives, with respect to the base, decrease Asi (**4**) formation.[47] Interestingly, 2% of the additives in 20% piperidine in DMF results in an increase in the target peptide from 40 to 60–71%.[69] Contrary to the relative performance in solution, *N*-hydroxylamine HOBt (**46**) is more efficient than pentachlorophenol (**50**) (67 vs 60% peptide).[48] The highly deactivated 2- or 4-mononitrophenol (**51** and **52**) and 2,4-dinitrophenol (**53**) are the most suitable choice of additive (70–71% peptide).[69] Other authors have reported the combination of HOBt (**46**) and 2,4-dinitrophenol (**53**) as a highly efficient methodology to prevent Asi (**4**) formation.[70]

During assembly of the D-Leu-D-Ala analogue of CRH hormone, which may adopt a favourable conformation towards cyclization of Asp, addition of 0.1 M solution to the piperidine solution results in a decrease in the side reaction to only traces, as detected by ESI-MS.[48,69] Increasing amounts of additive have been shown to induce higher suppression.[69,109] Recently, HOAt (**47**), the 7-aza-analogue of HOBt (**46**), proved to be equally efficient.[109] Optimization of the inclusion of the additives is achieved by the presence of Oxyma (**54**).[109]

## 6. Conclusions

Aspartimide and piperidide formation is problematic in peptide synthesis. Although the inconveniences caused by these intramolecular cyclizations are well known, a solution has yet to be found. Many factors contribute to accelerating or delaying the extent of this side reaction, such as the type of base, acid, protecting group, resin and solvent used. Research on some of these influential parameters has been focused on enhancing the steric hindrance of the base or protecting group, thus hampering amide backbone hydrogen abstraction, or on designing linkers/resin/protecting groups, which do not require the use of a strong acid or base known to promote aspartimide formation. These strategies are generally simple and cost saving, but do not offer complete prevention in demanding sequences or experimental conditions. Thus, even when using Asp(ODie), piperazine or microwave irradiation, small percentages of byproducts are still observed.[77,79] Hindering the size of the protecting group or base can also result in slower coupling and Fmoc-removal steps, thereby decreasing the global efficiency of the synthesis.[43,79] Moreover, the syntheses of these compounds might be complex and require two additional steps.[79,87] Furthermore, the introduction of non-conventional or unavailable protecting groups or linkers results in additional synthetic steps.

Full suppression of Asi (**4**) formation can be achieved by eliminating the amide backbone hydrogen of the residue preceding Asp, either using pseudoproline or amide backbone protectants. However, these building blocks also have substantial drawbacks that preclude their consideration as the ultimate solution to this side reaction.[62,80] To begin with, pseudoproline strategies are limited to Asp-Ser, Asp-Thr and Asp-Cys sequences. Similarly, the use of backbone amide-protecting groups as dipeptide building blocks is restricted to Asp-Gly sequences (the synthesis with chiral residues would result in a large extent of racemization).[80] Moreover, their introduction as synthons [H-(X)-Gly] is difficult and slow. The removal of these backbone-protecting groups, with the exception of EDOT, MIM and Dcpm, often leads to strong acidic conditions, which might cause the unwanted cyclization.[62,84] In addition, most of these groups are rather expensive. Therefore, research efforts must continue in order to pursue straightforward, affordable and complete elimination of this detrimental side reaction and the derived ring-opening byproducts.

While this troublesome side reaction remains unsolved, an attractive, cost- and time-saving, and widely available alternative to the previously mentioned strategies has emerged as a result mainly of the work of Bodanszky and Dölling, namely acidic *N*-hydroxylamines and phenols. Consistently with reduction of aspartimide formation when acidic-side chain residues precede Asp,

benzotriazoles and deactivated phenols cause a similar effect. A combination of some of these additives to carbodiimides in Fmoc-removal cocktails greatly reduces the appearance of aspartimides. Furthermore, this combination is independent of the synthetic strategy used and does not require additional introduction/removal steps for protecting groups. Excellent protocols including *N*-hydroxylamines have been reported, such as the combination of HOBt (**14**), piperazine and microwave irradiation or the use of the cocktail hexamethyleneimine/*N*-methylpyrrolidine/HOBt/NMP/DMSO 4:50:4:71:71 (v/v/w/v/v), which minimize Asi (**4**) formation to 1–5%.[62,70,71,77,91] Recently, novel oximes have been added to the arsenal of available *N*-hydroxylamines, thus contributing to improving options available to prevent aspartimide formation.

## Acknowledgements

The work in the laboratory of the authors has been partially financed by the Secretaría de Estado de Cooperación Internacional (AECI), the CICYT (CTQ2009-07758), the Generalitat de Catalunya (2009SGR 1024), the Institute for Research in Biomedicine Barcelona (IRB Barcelona), and the Barcelona Science Park. R.S.-F. thanks the Ministerio de Educación y Ciencia for a Ph.D. fellowship.

## References and notes


1. Lloyd-Williams, P.; Albericio, F.; Giralt, E. CRC, Boca Raton, Fla., USA, 1997, 297 p.
2. König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 2024–2033.
3. Bayer, E.; Gil-Av, E.; Konig, W. A.; Nakaparksin, S.; Oro, J.; Parr, W. *J. Am. Chem. Soc.* **1970**, *92*, 1738–1740.
4. du Vigneaud, V.; Behrens, O. K. *J. Biol. Chem.* **1937**, *117*, 27–36.
5. Gausepohl, H. P.; Pieles, U.; Frank, R. W. *Pept.: Chem. Biol., Proc. Am. Pept. Symp., 12th* **1992**, 523–524.
6. Brown, T.; Jones, J. H. *J. Chem. Soc., Chem. Commun.* **1981**, 648–649.
7. Künzi, H.; Manneberg, M.; Studer, R. O. *Helv. Chim. Acta* **1974**, *57*, 566–574.
8. Wu, Y.; Matsueda, G. R.; Bernatowicz, M. *Synth. Commun.* **1993**, *23*, 3055–3060.
9. Paul, R.; Kende, A. S. *J. Am. Chem. Soc.* **1964**, *86*, 4162–4166.
10. Kashelikar, D. V.; Ressler, C. *J. Am. Chem. Soc.* **1964**, *86*, 2467–2473.
11. Kisfaludy, L.; Schon, I.; Renyei, M.; Gorog, S. *J. Am. Chem. Soc.* **1975**, *97*, 5588–5589.
12. Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 1637–1640.
13. Erickson, B. W.; Merrifield, R. B. *J. Am. Chem. Soc.* **1973**, *95*, 3750–3756.
14. Neumann, N. P.; Moore, S.; Stein, W. H. *Biochemistry* **1962**, *1*, 68–75.
15. Guttmann, S.; Boissonnas, R. A. *Helv. Chim. Acta* **1958**, *41*, 1852–1867.
16. Yajima, H.; Akaji, K.; Funakoshi, S.; Fujii, N.; Irie, H. *Chem. Pharm. Bull.* **1980**, *28*, 1942–1945.
17. Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson, D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730–3743.
18. Riniker, B.; Hartmann, A. *Pept.: Chem., Struct. Biol., Proc. Am. Pept. Symp., 11th* **1990**, 950–952.
19. Feinberg, R. S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1975**, *97*, 3485–3496.
20. Dimarchi, R. D.; Tam, J. P.; Kent, S. B.; Merrifield, R. B. *Int. J. Pept. Protein Res.* **1982**, *19*, 88–93.
21. Gairi, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron Lett.* **1994**, *35*, 175–178.
22. Lenard, J.; Hess, G. P. *J. Biol. Chem.* **1964**, *239*, 3275–3281.
23. Lenard, J.; Schally, A. V.; Hess, G. P. *Biochem. Biophys. Res. Commun.* **1964**, *14*, 498–502.
24. Partridge, S. M.; Davis, H. F. *Nature* **1950**, *165*, 62–63.
25. Sakakibara, S.; Shin, K. H.; Hess, G. P. *J. Am. Chem. Soc.* **1962**, *84*, 4921–4928.
26. Gisin, B. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1972**, *94*, 3102–3106.
27. Fischer, P. M.; Solbakken, M.; Undheim, K. *Tetrahedron* **1994**, *50*, 2277–2288.
28. Atherton, E.; Sheppard, R. C., IRL, Oxford, UK, 1989, 203 p.
29. Lukszo, J.; Patterson, D.; Albericio, F.; Kates, S. A. *Lett. Pept. Sci.* **1996**, *3*, 157–166.
30. Hofmann, K. H.; Haas, W.; Smithers, M. J.; Wells, R. D.; Wolman, Y.; Yanaihara, N.; Zanetti, G. *J. Am. Chem. Soc.* **1965**, *87*, 620–631.
31. Subirós-Funosas, R.; El-Faham, A.; Albericio, F. *N-hydroxylamines for Peptide Synthesis, Patai's Chemistry of Functional Groups*; John Wiley & Sons: 2011; Vol. 2, pp 623–730.
32. Martinez, J.; Bodanszky, M. *Int. J. Pept. Protein Res.* **1978**, *12*, 277–283.
33. Zahariev, S.; Guarnaccia, C.; Pongor, C. I.; Quaroni, L.; Cemazar, M.; Pongor, S. *Tetrahedron Lett.* **2006**, *47*, 4121–4124.
34. Stathopoulos, P.; Papas, S.; Kostidis, S.; Tsikaris, V. *J. Pept. Sci.* **2005**, *11*, 658–664.
35. Taichi, T.; Yamazaki, T.; Kimura, T.; Nishiuchi, Y. *Tetrahedron Lett.* **2010**, *50*, 2377–2380.
36. Carlescu, I.; Osborn, H. M. I.; Desbrieres, J.; Scutaru, D.; Popa, M. *Carbohydr. Res.* **2010**, *345*, 33–40.
37. Ittel, S. D. Gridnev, A. A. Preparation and uses of bis(*n*-silylalkyl)aspartimides. In U.S. Pat. Appl. Publ., vol. US20090165676, 2009, 14 pp.
38. Rivera-Fillat, M. P.; Reig, F.; Martinez, E. M.; Grau-Oliete, M. R. *J. Pept. Sci.* **2010**, *16*, 315–321.
39. Schon, I.; Kisfaludy, L. *Int. J. Pept. Protein Res.* **1979**, *14*, 485–494.
40. Capasso, S.; Mazzarella, L.; Sica, F.; Zagari, A.; Cascarano, G.; Giacovazzo, C. *Acta Crystallogr. B* **1992**, *48*, 285–290.
41. Capasso, S.; Mazzarella, L.; Zagari, A. *Chirality* **1995**, *7*, 605–609.
42. Nicolas, E.; Pedroso, E.; Giralt, E. *Tetrahedron Lett.* **1989**, *30*, 497–500.
43. Bodanszky, M.; Tolle, J. C.; Deshmane, S. S.; Bodanszky, A. *Int. J. Pept. Protein Res.* **1978**, *12*, 57–68.
44. Bodanszky, M.; Sigler, G. F.; Bodanszky, A. *J. Am. Chem. Soc.* **1973**, *95*, 2352–2357.
45. Yang, Y.; Sweeney, W. V.; Schneider, K.; Thörnqvist, S.; Chait, B. T.; Tam, J. P. *Tetrahedron Lett.* **1994**, *33*, 9689–9692.
46. Bannwarth, W.; Kitas, E. A. *Helv. Chim. Acta* **1992**, *75*, 707–714.
47. Dölling, R.; Beyermann, M.; Hänel, J.; Kernchen, F.; Krause, E.; Franke, P.; Brudel, M.; Bienert, M. 3rd International Innovation and Perspectives in Solid-Phase Synthesis Symposium, 31th Aug–4th Sept., Oxford, UK, 1993, Poster P21.
48. Dölling, R.; Beyermann, M.; Hänel, J.; Kernchen, F.; Krause, E.; Franke, P.; Brudel, M.; Bienert, M. *J. Chem. Soc., Chem. Commun.* **1994**, 853–854.
49. Flora, D.; Mo, H.; Mayer, J. P.; Khan, M. A.; Yan, L. Z. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1065–1068.
50. Bodanszky, M.; Kwei, J. Z. *Int. J. Pept. Protein Res.* **1978**, *12*, 69–74.
51. Kostidis, S.; Stathopoulos, P.; Chondrogiannis, N.-I.; Sakarellos, C.; Tsikaris, V. *Tetrahedron Lett.* **2003**, *44*, 8673–8676.
52. Mergler, M.; Dick, F.; Sax, B.; Weiler, P.; Vorherr, T. *J. Pept. Sci.* **2003**, *9*, 36–46.
53. Wagner, M.; Kunz, H. *Angew. Chem., Int. Ed.* **2002**, *41*, 317–321.
54. Michael, K. Frontiers in Modern Carbohydrate Chemistry; ACS Symposium Series 960, American Chemical Society: Washington, DC, 2007; pp 328–353.
55. Schon, I.; Rill, A. *Collect. Czech. Chem. Commun.* **1989**, *54*, 3360–3373.
56. Kaneshiro, C. M.; Michael, K. *Angew. Chem., Int. Ed.* **2006**, *45*, 1077–1081.
57. Karlstroem, A.; Unden, A. *Tetrahedron Lett.* **1996**, *37*, 4243–4246.
58. Rabanal, F.; Pastor, J. J.; Nicolas, E.; Albericio, F.; Giralt, E. *Tetrahedron Lett.* **2000**, *41*, 8093–8096.
59. Suli-Vargha, H.; Schlosser, G.; Ilas, J. *J. Pept. Sci.* **2007**, *13*, 742–748.
60. Cebrian, J.; Domingo, V.; Reig, F. *J. Pept. Res.* **2003**, *62*, 238–244.
61. Tam, J. P.; Wong, T.-W.; Riemen, M. W.; Tjoeng, F. S.; Merrifield, P. W. *Tetrahedron Lett.* **1979**, *42*, 4033–4036.
62. Ruczynski, J.; Lewandowska, B.; Mucha, P.; Rekowski, P. *J. Pept. Sci.* **2008**, *14*, 335–341.
63. Yamamoto, N.; Takayanagi, A.; Sakakibara, T.; Dawson, P. E.; Kajihara, Y. *Tetrahedron Lett.* **2006**, *47*, 1341–1346.
64. Chen, R.; Tolbert, T. J. *J. Am. Chem. Soc.* **2010**, *132*, 3211–3216.
65. Aswad, D. W. *Ann. N. Y. Acad. Sci.* **1990**, *613*, 26–36.
66. Inaba, M.; Gupta, K. C.; Kuwabara, M.; Takahashi, T.; Benz, E. J., Jr.; Maede, Y. *Blood* **1992**, *79*, 3355–3361.
67. Payan, I. L.; Chou, S. J.; Fisher, G. H.; Man, E. H.; Emory, C.; Frey, W. H. *Neurochem. Res.* **1992**, *17*, 187–191.
68. Desfougères, Y.; Jardin, J.; Lechevalier, V.; Pezennec, S.; Nau, F. *Biomacromolecules* **2011**, *12*, 156–166.
69. Dölling, R.; Beyermann, M.; Hänel, J.; Kernchen, F.; Krause, E.; Franke, P.; Brudel, M.; Bienert, M. 23rd EPS, 4–10th Sept., Braga, Portugal, Poster, 1994, P061.
70. Lauer, J. L.; Fields, C. G.; Fields, G. B. *Lett. Pept. Sci.* **1995**, *1*, 197–205.
71. Mergler, M.; Dick, F.; Sax, B.; Stahelin, C.; Vorherr, T. *J. Pept. Sci.* **2003**, *9*, 518–526.
72. Tam, J. P.; Riemen, M. W.; Merrifield, R. B. *Pept. Res.* **1988**, *1*, 4033–4036.
73. Dixon, M. J.; Nathubhai, A.; Andersen, O. A.; van Aalten, D. M. F.; Eggleston, I. M. *Org. Biomol. Chem.* **2009**, *7*, 259–268.
74. Albericio, F.; Andreu, D.; Giralt, E.; Navalpotro, C.; Pedroso, E.; Ponsati, B.; Ruiz-Gayo, M. *Int. J. Pept. Protein Res.* **1989**, *34*, 124–128.
75. Dimarchi, R. D.; Tam, J. P.; Merrifield, R. B. *Int. J. Pept. Protein Res.* **1982**, *19*, 270–279.
76. Offer, J.; Quibell, M.; Johnson, T. *J. Chem. Soc., Perkin Trans. 2* **1996**, 175–182.
77. Palasek, S. A.; Cox, Z. J.; Collins, J. M. *J. Pept. Sci.* **2007**, *13*, 143–148.
78. Liu, M.; Hamilton, D. W.; Barany, G. *Molecules* **2010**, *15*, 6399–6410.
79. Mergler, M.; Dick, F. *J. Pept. Sci.* **2005**, *11*, 650–657.
80. Roeder, R.; Henklein, P.; Weisshoff, H.; Muegge, C.; Paetzel, M.; Schubert, U.; Carpino, L. A.; Henklein, P. *J. Pept. Sci.* **2010**, *16*, 65–70.
81. Ali, A. M.; Taylor, S. D. *J. Pept. Sci.* **2010**, *16*, 190–199.
82. Isidro-Llobet, A.; Guasch-Camell, J.; Álvarez, M.; Albericio, F. *Eur. J. Org. Chem.* **2005**, 3031–3039.
83. Isidro-Llobet, A.; Just-Baringo, X.; Alvarez, M.; Albericio, F. *Biopolymers* **2008**, *90*, 444–449.
84. Cardona, V.; Eberle, I.; Barthelemy, S.; Beythien, J.; Doerner, B.; Schneeberger, P.; Keyte, J.; White, P. D. *Int. J. Pept. Res. Ther.* **2008**, *14*, 285–292.
85. Yang, C. C.; Merrifield, R. B. *J. Org. Chem.* **1976**, *41*, 1032–1041.
86. Yue, C.; Thierry, J.; Potier, P. *Tetrahedron Lett.* **1993**, *34*, 323–326.
87. Boyd, J. G.; Lin, W. Peptides: The Wave of the Future, Proceedings of the Second International and the Seventeenth American Peptide Symposium, San Diego, CA, United States, June 9–14, 2001, pp 61–62.
88. Michael, K.; Hogenauer, T.; Cai, Z.; Kaneshiro, C. M.; Dirk, C.; Franz, A. H. Abstracts, 65th Southwest Regional Meeting of the American Chemical Society, El Paso, TX, United States, November 4–7, 2009.

89. Rabanal, F.; Pastor, J. J.; Nicolas, E.; Albericio, F.; Giralt, E. Peptides 2000, Proceedings of the European Peptide Symposium, 26th, Montpellier, France, Sept. 10–15, 2000, 2001, pp 265–266.
90. Wade, J.; Mathieu, M.; Macris, M.; Tregear, G. *Lett. Pept. Sci.* **2000**, *7*, 107–112.
91. Collins, J. M. Microwave enhanced N-Fmoc deprotection in peptide synthesis using piperazine. In Eur. Pat. Appl, vol. EP 1818339, 2007, 13 pp.
92. Subirós-Funosas, R.; Acosta, G. A.; El-Faham, A.; Albericio, F. *Tetrahedron Lett.* **2009**, *50*, 6200–6202.
93. Alsina, J.; Kates, S. A.; Barany, G.; Albericio, F. *Methods Mol. Biol.* **2005**, *298*, 195–208.
94. Jensen, K. J.; Alsina, J.; Songster, M. F.; Vagner, J.; Albericio, F.; Barany, G. *J. Am. Chem. Soc.* **1998**, *120*, 5441–5452.
95. Haack, T.; Mutter, M. *Tetrahedron Lett.* **1992**, *33*, 1589–1592.
96. Ueki, M.; Horino, H.; Senoh, C.; Sakata, T.; Oyamada, H. *Pept. Chem.* **1990**, *27*, 23–28.
97. Quibell, M.; Owen, D.; Peckman, L. C.; Johnson, T. *J. Chem. Soc., Chem. Commun.* **1994**, 2343–2344.
98. Nicolas, E.; Pujades, M.; Bacardit, J.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 2317–2320.
99. Sampson, W. R.; Patsiouras, H.; Ede, N. J. *J. Pept. Sci.* **1999**, *5*, 403–409.
100. Johnson, T.; Packman, L. C.; Hyde, C. B.; Owen, D.; Quibell, M. *J. Chem. Soc., Perkin Trans. 1* **1996**, 719–728.
101. Carpino, L. A.; Nasr, K.; Abdel-Maksoud, A. A.; El-Faham, A.; Ionescu, D.; Henklein, P.; Wenschuh, H.; Beyermann, M.; Krause, E.; Bienert, M. *Org. Lett.* **2009**, *11*, 3718–3721.
102. Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397–4398.
103. Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1927–1930.
104. Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, *16*, 1219–1222.
105. Subirós-Funosas, R.; Moreno, J. A.; Bayó-Puxan, N. ,; Abu-Rabeah, K.; Ewenson, A.; Atias, D.; Marks, R. S.; Albericio, F. *Chimi. Oggi, Chemistry Today* **2008**, *26*, 10–12.
106. Subirós-Funosas, R.; Prohens, R.; Barbas, R.; El-Faham, A.; Albericio, F. *Chem.—Eur. J.* **2009**, *15*, 9394–9403.
107. El-Faham, A.; Subirós-Funosas, R.; Prohens, R.; Albericio, F. *Chem.—Eur. J.* **2009**, *15*, 9404–9416.
108. Fletcher, G. A.; Low, M.; Young, G. T. *J. Chem. Soc. Perkin 1* **1973**, 1162–1164.
109. Subirós-Funosas, R.; El-Faham, A.; Albericio, F. unpublished results.

## Biographical sketch

**Ramon Subirós-Funosas** was born in Barcelona, Spain, in 1983. Before receiving his B.Sc. in Chemistry in 2007, he moved for a period of one year to GlaxoSmithKline, S.L. in 2005, in Stevenage, UK, where he joined the Medicinal Chemistry Department as Industrial Placement Student, working on synthetic organic chemistry. In 2007, he joined the group of Professor Fernando Albericio in the University of Barcelona, Barcelona, Spain, where he obtained his Ph.D. in Organic Chemistry in 2011, developing a new family of coupling reagents based on ethyl 2-cyano-2-hidroxiiminoacetate (Oxyma) as additive to carbodiimides. In 2010, he visited the group of Phillip E. Dawson at The Scripps Research Institute, La Jolla, CA, USA, for a 4-months internship, learning ligation techniques for the assembly of proteins. His major research interests include novel applications of *N*-hydroxylamines and methodology of native chemical ligation.

**Professor Ayman El-Faham** received his B.Sc. degree in Chemistry in 1980 and his M.Sc. in 1985 in Physical Organic Chemistry, from Faculty of Science, University of Alexandria, Egypt. In 1991 he received his Ph.D. in organic chemistry as a joint project between the University of Alexandria and the University of Massachusetts, Amherst, MA, U.S.A. under the supervision of Professor L.A. Carpino, where he worked on the synthesis of new protecting groups for both solution and solid-phase peptide synthesis. He worked on new coupling reagents during his postdoctoral work (1992–1999) at the University of Massachusetts in Professor Carpino's Lab. He received the University of Alexandria award in Chemistry in 1999. Following a position as Head of the Chemistry Department, Beirut Arab University, Lebanon (2000–2004), and as a Professor of Organic Chemistry and Direct Manager of both the NMR Lab and the Central Lab at the Faculty of Science, Alexandria University, Egypt (2004–2008), he worked at King Saud University, as a Professor of Organic Chemistry, Saudi Arabia (2008–2010). In 2010 he received his D.Sc. in Chemistry back at University of Alexandria, Egypt, where he is currently a Professor of Organic Chemistry. His research interests include the synthesis of peptides under solution and solid-phase conditions, natural products, heterocylic synthesis, and biologically active synthetic targets.

**Professor Fernando Albericio** was born in Barcelona, Spain in 1953. He received his Ph.D. in Chemistry at the University of Barcelona, in 1981. Following postdoctoral work at Tufts University (Boston), at the Université d'Aix-Marseille (France), and at the University of Minnesota (1981–1984), he returned to Barcelona as an Associate Professor. During the 1992–1994 period, he was Director of Peptide Research with Milligen/Biosearch at Boston. He rejoined the University of Barcelona, where he was promoted to Professor in 1995. Nowadays, he is holding various appointments: General Director of the Barcelona Science Park, Professor at the University of Barcelona, and Group Leader at the Institute for Research in Biomedicine, Barcelona, Spain. Professor Albericio is deeply involved in the development of the third mission of the University, the transference of knowledge and technology to the society. He has founded several biotech companies and is acting in the board of directors of several foundations and companies. Furthermore, he is consultant for several companies in the chemical and pharmaceutical areas. Professor Albericio's major research interests cover practically all aspects of peptide synthesis and combinatorial chemistry methodologies, as well as synthesis of peptides and small molecules with therapeutic activities. Recently, Professor Albericio has honoured with a Doctorate Honoris Causa by the Universidad de Buenos Aires (Argentina) and the Vincent du Vigneaud Award (American Peptide Society).