Tetrahedron 68 (2012) 3056–3062

Contents lists available at SciVerse ScienceDirect

Tetrahedron

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

Cyanoacetamide-based oxime carbonates: an efficient, simple alternative for the introduction of Fmoc with minimal dipeptide formation

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ARTICLE INFO

Article history:
Received 17 December 2011
Received in revised form 7 February 2012
Accepted 8 February 2012
Available online 14 February 2012

Keywords:
Amino acids
9-Fluorenylmethyloxycarbonyl
Oxime
N-Protecting group
Peptide synthesis

ABSTRACT

Nowadays, most peptides are chemically achieved by using the Fmoc/*t*Bu protection strategy, due to its fully orthogonal character, mild temporary group removal and resin cleavage steps. However, its introduction into *N*-unprotected amino acids is not exempt of synthetic inconveniences, such as dipeptide formation. Lately, novel oxime carbonates were introduced in the arsenal of reagents for the introduction of Fmoc, presenting almost negligible percentage of side-products. Herein, an enforced version of this family of Fmoc-carbonates is presented, containing stable and highly acidic cyanoacetamide-based oximes as leaving group. Such reactive species, affordable in only two steps from simple, readily available starting materials, show unusual ability to obtain the corresponding Fmoc-protected residues in high yield and minimal impact of detrimental side-products, mainly Fmoc-dipeptides.

1. Introduction

Peptides are increasingly gaining recognition as potential bioactive ingredients in the pharmaceutical industry.[1–4] Consequently, their purity is required to meet with the highest standard within compounds amenable to be employed as drugs.[5,6] In order to accomplish such requirements, the choice of an appropriate protection strategy, which allows selective and safe introduction and removal of the masking group, is fundamental for the success of the whole process.[7,8] Currently, Fmoc/*t*Bu-based peptide synthesis is the preferred option over classical Boc/Bzl strategy for the majority of synthetic chemists.[9,10] Therefore, a complete and clean introduction of this base-labile group into the *N*-terminus of any amino acid building block is of outmost importance.

In spite of the vast number of reagents reported to date for the attachment of the Fmoc group into the *N*-terminal amino group of amino acid building blocks in the form of carbamate, there is still no ultimate active species capable to provide optimal Fmoc-introduction (Fig. 1). The traditional chloroformate strategy (**1**) represents an extremely powerful approach, providing fast amino protection.[10,11] However, these reagents display high instability and are not suitable for sterically low hindered residues, such as Glycine, where the amount of dipeptide (and even tripeptide) formation may reach up to 20% of the crude material (Scheme 1).[12]

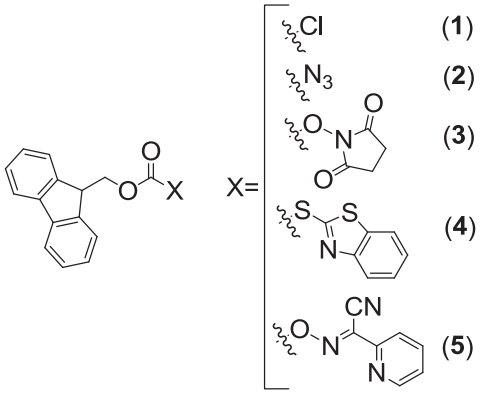

Fig. 1. Most relevant approaches described for amino Fmoc-protection.

Abbreviations: Boc, *tert*-butoxycarbonyl; DCM, dichloromethane; ESI-MS, electrospray ionization mass spectrometry; Fmoc, fluorenylmethyloxycarbonyl; NMR, nuclear magnetic resonance; Oxyma, ethyl 2-cyano-2-hydroxyiminoacetate; SPPS, solid-phase peptide synthesis; UV, ultraviolet.

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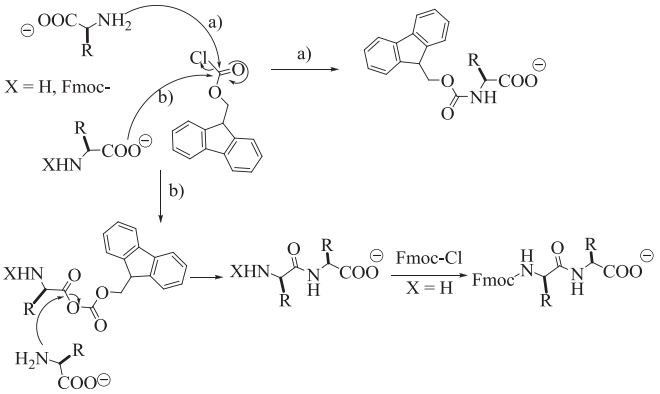
doi:10.1016/j.tet.2012.02.020



Scheme 1. Amino acid dimerization during Fmoc-protection.

Less reactive approaches, namely azidocarbonates (**2**) or succinimidocarbonates (**3**), minimize to a great extent dimerization, but either produce other side-products or have notorious explosive potential.[6,11,13] Protection forming the azide *in situ* is a safer alternative to the above mentioned method.[14] Few years ago, 2-mercaptobenzotiazole (**4**) templates have also been employed with reasonable success during Fmoc-protection, but separation of byproducts during purification hampers practical application.[15] Less prevalent approaches consist in employing symmetrical pyrocarbonates,[16] trimethylsilyl esters,[17,18] or 1,2,2,2-tetrachloroethyl,[19,20] 5-norbornene-2,3-dicaroximido,[21] pentafluorophenyl,[22] perfluorophenyl,[23] and 1-hydroxybenzotriazole[24,25] mixed carbonates. In the last year, benzotriazole-based reagents in the *N*-acyl form have been described for the efficient introduction, not only of Fmoc, but also Boc and Alloc protecting groups with low or undetectable dipeptide formation.[26] However, this polynitrogenated heterocyclic core has a consistent record on explosive potential, similarly to the parent 1-hydroxy analogues, which has had great impact in methodology of peptide synthesis.[27–29]

Recently, in a preliminary communication, we reported a series of Fmoc-introducing carbonates, containing various oxime moieties with unusual stability due to the absence of α-hydrogen atoms.[30] The high acidity of such oximes (p*Ka*=4–8), bearing a series of electron-withdrawing substituents, play a key part in the reactivity of the corresponding carbonates, generally affording the Fmoc-protected amino acid in yields close to 90%, after 14 h reaction time.[30] In addition, the content of unwanted dipeptide material in the reaction crude remained below 0.1%, even when Glycine (the most prone residue to dimerization) was selected as Fmoc-protection model system. Among all Fmoc-oxime carbonates tested for that purpose, most remarkable results were obtained with the one featuring the least acidic oxime: the *N*-hydroxypicolinimidoyl cyanide derivative (*N*-{[(9*H*-fluoren-9-yl)methoxy] carbonyloxy}picolinimidoylcyanide) (**5**, Fig. 1), which afforded Fmoc-Gly-OH in 92% yield and only 0.01% Fmoc-Gly-Gly-OH. Nonetheless, in spite of this extraordinary performance, routinely application of this oxime-based carbonate in peptide synthesis laboratories seems impractical as result of the high production cost of the starting cyano-2-pyridylacetonitrile.

In view of the promising features of the precedent Fmoc-oxime templates, a second generation has been synthesized and evaluated, aiming at solving the practical drawbacks of the previous carbonates and simultaneously conserving or increasing their efficiency. Thus, seeking an optimal balance of reactivity/oligomerization, the ester moiety contained in the highly reactive ethyl 2-cyano-2-hydroxyiminoacetate (Oxyma) template was replaced with several amide functionalities.[31] Although apparently a minor structural modification, shifting an ester for an amide bond in the cyanooxime series is able to reduce 0.6 units its acidity and introduce conformational restriction due to existence of C–N rotational barrier.[32,33] Consequently, the potential amount of side-products is predicted to decrease, maintaining at the same time a valuable reactivity.

Various linear and cyclic carboxamido groups have been investigated ranging from the simplest unsubstituted amide (**6**) to analogues containing *N*-ethyl (mimicking the Oxyma template, **7**), *N*-piperidinyl (**8**) and *N*-morpholinyl (**9**, aiming at higher solubility) chains (Fig. 2). Such cyanoacetamide-based oximes (**15**–**18**, Scheme 2) are highly appreciated in organometallic chemistry, serving as excellent bivalent ligands to bind various metallic ions.[33–36] Thus, Silver (I) and Tin (IV) complexes of unsubstituted carboxamide **15**, the most studied in metal coordination among these oximes, are employed for biomedical purposes as antimicrobial additives (in odontological devices) or as cytotoxic agents (showing greater activity than cisplatin against human cervical cancer cells) and also with industrial applications as gas sensor.[34–36] Growing interest on this particular cyanooxime (**15**) has led to the calculation and measurement of its O–H bond dissociation enthalpy in gas-phase and also to a complete X-ray crystallographic and thermal analysis.[37,38] Recently, considerable cell antiproliferative activity of palladium (II) and platinum (II) complexes, based also on piperidino and morpholino–cyanoacetamide oximes (**17** and **18**), was reported.[33] Furthermore, piperidino oxime **17** has been employed as building block to synthesize iminolactones.[39] All oximes described herein, including the *N*-ethyl analogue (**16**) have been employed to build carbamates displaying detoxifying action of agricultural pesticides.[40] Exhaustive structural and spectroscopic studies on cyanoacetamide oximes **15**–**18** and the corresponding anionic states have been described.[33,35] Despite the vast existing literature on metal-complexed derivatives of these oximes, their properties have not yet been applied to peptide chemistry, with the exception of unsubstituted carboxamide **15**. Similarly to Oxyma, this oxime (**15**) was first reported as additive to carbodiimides with reasonable activity, but further studies were surprisingly abandoned.[32,41] To our knowledge, this is the first report on Fmoc-carbonates including cyanoacetamide oximes **15**–**18**.

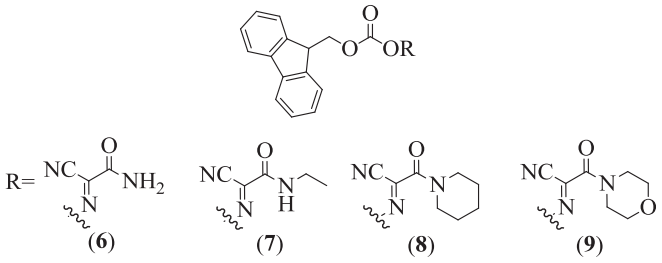
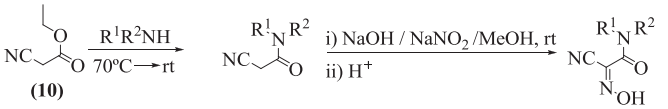


Fig. 2. Proposed cyanocarboxamido family of Fmoc-oxime carbonates.



R^1 = H, R^2 = H,	**(11)**	R^1 = H, R^2 = H,	**(15, AmOX)**
R^1= H, R^2= CH_3CH_2,	**(12)**	R^1= H, R^2= CH_3CH_2,	**(16, N-Oxyma)**
R^1, R^2 = $(CH_2)_5$,	**(13)**	R^1, R^2 = $(CH_2)_5$,	**(17,PipOX)**
R^1, R^2 = $(CH_2)_4O$,	**(14)**	R^1, R^2 = $(CH_2)_4O$,	**(18, MorOX)**

Scheme 2. Two-step synthesis of cyanocarboxamide oximes.

A further advantage of the cyanoacetamido series of Fmoc-oxime carbonates (**6**–**9**) is that retrosynthetic analysis of the corresponding oximes (**15**–**18**) show that these could be easily accessed from ethyl cyanoacetate by amidation with subsequent nitrosation. Although alternative synthetic pathways have been

proposed for some of these oximes (**16**), involving hydration of the cyano group, the amidation/nitrosation sequence of reactions is a general and more consistent, extensively reported approach.[33,36,37,39,42] Based on information from various commercial sources, selection of ethyl cyanoacetate as starting material would save approximately 5–10 times production costs, in comparison with 2-pyridylacetonitrile, thereby resulting in a cost-effective and powerful family of Fmoc-introducing reagents.

2. Results and discussion

2.1. Synthesis of cyanoacetamide oximes 15–18

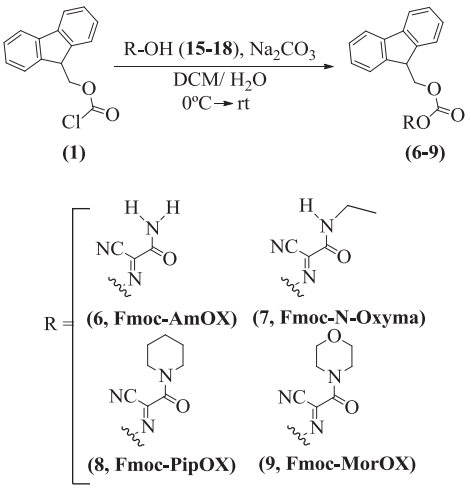
Cyanoacetamide-containing oximes (**15**–**18**), as the reactive core contained in the novel Fmoc-carbonates, were conveniently prepared from ethyl cyanoacetate (**10**) in only two steps (Scheme 2), following previously described procedures.[33,34,39] In a first stage, excluding commercially available 2-amino-*N*-hydroxy-2-oxoacetimidoyl cyanide (**11**), ethyl cyanoacetate (**10**) was converted to the corresponding intermediate 2-cyano *N*-alkyl acetamide derivatives **12**–**14** after overnight reaction with various primary or secondary amines (namely *N*-ethylamine, piperidine and morpholine). This procedure involved heating at 70 °C in the neat amine as solvent, which resulted in enhanced efficiency than use of protic solvents or KF-alumina solvent-free conditions.[33,39,42] Beige or white-off solids were obtained after precipitation of the product, with high purity according to previously described spectroscopic and melting point data.[33,39]

The second and last step consisted in transformation of these cyanoacetamide intermediates (**11**–**14**) into the corresponding oximes (**15**–**18**) using the Meyer nitrosation reaction.[43] Several more convenient variations of this reaction are known, using alkyl nitrites, such as iBuONO, iPrONO and MeONO as source of nitrous acid.[33,34,36] Among these modified procedures, in situ generation of gaseous methyl nitrite (MeONO) was selected in view of the efficacy in previous preparation of oximes.[34,44] Thus, nitrosation took place smoothly by this methodology in presence of sodium hydroxide, followed by acidification (Scheme 2).[44,45] Whereas AmOX (**15**) and *N*-Oxyma (**16**) precipitated immediately after the acidic treatment, other oximes, such as PipOX (**17**) and MorOX (**18**), needed addition of cold ether (**17**,**18**), affording white crystalline, stable solids in all cases.

Overall yields from the starting material (**10**) ranged between 77 and 87%, a significant improvement on the efficacy of these two steps according to previous publications.[33,34,39] Among all cyanoacetamide oximes, the piperidinyl derivative PipOX (**17**) was obtained in the highest yield. During the preparation of these new cyanoacetamide-based oximes, we noticed their high solubility in water, especially in the case of piperidino and morpholino-containing counterparts PipOX (**17**) and MorOX (**18**), accordingly to previous literature, which is of great impact on facilitating byproduct removal during aqueous work-up.[33] Among these two counterpats, highest solubility was achieved by MorOX (**18**), selected to meet that purpose. This behaviour is in sharp contrast to *N*-hydroxypicolinimidoyl cyanide (**5**), which required precipitation in a mixture of AcOH and water, revealing its less polar character.

2.2. Synthesis of Fmoc-carbonates 6–9

Successful isolation and purification of all cyanoacetamide oximes (**15**–**18**) permitted the preparation of the envisaged second generation of Fmoc-oxime carbonates (**6**–**9**). Using established procedures for Fmoc attachment into highly acidic components, Fmoc-Cl (**1**) was employed to build the target Fmoc-oximes under Schotten–Bauman conditions (Scheme 3).[46,47] After only 2 h of mixing the reagents, and subsequent recrystallization from CH_2Cl_2/hexane, *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyloxy)-2-amino-2-oxoacetimidoyl cyanide **6** (Fmoc-AmOX), *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyloxy)-2-(ethylamino)-2-oxoacetimidoyl cyanide **7** (Fmoc-*N*-Oxyma), *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyloxy)-2-oxo-2-(piperidin-1-yl)acetimidoyl cyanide **8** (Fmoc-PipOX), and *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyloxy)-2-morpholino-2-oxoacetimidoyl cyanide **9** (Fmoc-MorOX), were produced in 79–91 yield% (Table 1).



Scheme 3. Preparation of cyanocarboxamide-based Fmoc-oxime carbonates.

Table 1
Yield, mp, and exact mass analysis of Fmoc-cyanoacetamide oxime carbonates (**6**–**9**) herein prepared

Product	Yield (%)	mp (°C)	Exact mass (*m*/*z*)	
			Calculated	Experimental
Fmoc-AmOX (**6**)	91	152–153	*M*=335.09	$[M+H]^+$=336.10 $[M+Na]^+$=358.08 $[2M+Na]^+$=693.17
Fmoc-*N*-Oxyma (**7**)	84	160–161	*M*=363.12	$[M+H]^+$=364.13 $[M+Na]^+$=386.11 $[2M+H]^+$=727.25 $[2M+Na]^+$=749.23
Fmoc-PipOX (**8**)	79	—[a]	*M*=403.15	$[M+H]^+$=404.16 $[M+Na]^+$=426.14 $[2M+Na]^+$=829.30
Fmoc-MorOX (**9**)	88	135–136	*M*=405.13	$[M+H]^+$=406.14 $[M+Na]^+$=428.12 $[2M+H]^+$=811.27

[a] A waxy solid, its melting point could not be measured.

With the sole exception of the piperidino analogue **8** (a waxy solid), the rest of Fmoc-cyanoacetamideoxime carbonates were achieved as white crystalline solids, which greatly facilitated their handling in the subsequent evaluation. Furthermore, exact mass analysis of the obtained novel carbonates provided clear evidence of the high purity of the reagents (Table 1).

2.3. N_α-Protection of H-Gly-OH

Evaluation of the suitability of cyanoacetamideoxime-based Fmoc-carbonates **6**–**9** was undertaken on the protection of N^α-unsubstituted residues. In terms of Fmoc-dipeptide formation, H-Gly-OH is an excellent platform to check the performance of a certain Fmoc-introducing reagent, since its low sterical hindrance favours a high percentage of oligomerization.[14,15,30] Thus, previously synthesized Fmoc-oxime carbonates (**6**–**9**) were let stand overnight in a homogoneous acetone/aqueous bicarbonate solvent mixture, after careful dropwise addition.[15] Fmoc-protected Glycine samples were obtained after removing unreacted starting carbonates and acidifying with 10% HCl. N_α-protection employing Fmoc-cyanoacetamideoximes (**6**–**9**) resulted in yields exceeding 90% in all experiments, a superior performance to that observed in the previous generation of oxime-based Fmoc-carbonates (Table 2). Particularly, in terms of yield Fmoc-*N*-Oxyma (**7**), Fmoc-PiPOX (**8**) and Fmoc-MorOX (**9**) were more efficient than the previously reported (*N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyloxy}picolinimidoylcyanide) (**5**) in the preparation of Fmoc-Gly-OH.

Table 2
Yield, mp, purity of Fmoc-Gly-OH and dipeptide formation using Fmoc-cyanoacetamide oxime carbonates (**6**–**9**)

Reagent used	Yield (%)	mp (°C)[a]	Purity (%)[b]	Fmoc-Gly–Gly-OH (%)[b]
Fmoc-AmOX (**6**)	90.8	176–177	99.03	0.06
Fmoc-*N*-Oxyma (**7**)	91.8	175–176	98.56	0.17
Fmoc-PipOX (**8**)	92.1	176–177	99.94	0.06
Fmoc-MorOX (**9**)	93.5	177–178	99.92	0.08

[a] Mp of a 99.8% pure commercial sample of Fmoc-Gly-OH = 177–178 °C.
[b] Purity and content of Fmoc-Gly–Gly-OH was determined after HPLC analysis (for a more detailed description, see Experimental Section).

Regarding Fmoc-Gly-OH purity, two distinct behaviours were observed for Fmoc-cyanoacetamide oxime carbonates (**6**–**9**). On one hand, Fmoc-AmOX (**6**) and Fmoc-*N*-Oxyma (**7**) provided less pure crudes than the first generation of Fmoc-oximes (Table 2).[30] On the other hand, piperidino- (**8**) and morpholino (**9**)-based analogues rendered N^α-protected Glycine of outstanding purity (above 99.9%), superseeding the quality of the crudes provided by the cyano-2-pyridil counterpart (**5**).[30] The higher water solubility of PipOX (**17**) and MorOX (**18**) over the rest of oximes probably accounts for such difference in performance of the corresponding Fmoc-oximes, since this feature is basic to efficiently remove the oximes from the target carbonates in the final acidification step. However, all cyanoacetamideoxime derivatives **6**–**9** minimized the impact of Fmoc-dipeptide to a content below 0.1%, a higher oligomerization reduction to that of the majority of the previously reported generation of Fmoc-oxime carbonates.[30] The identity of Fmoc-Gly–Gly-OH was confirmed after coinjection of crude materials with a pure sample of the dipeptide, obtained after elongation and cleavage from 2-chlorotrityl resin (Fig. 3).

2.4. N^α-Protection of H-Ala-OH

Although the low bulkiness of H-Gly-OH is optimal to check the tendency of a Fmoc-introducing reagent towards dimerization, it may not be realistic in terms of protection yield when other more hindered Fmoc-residues need to be prepared. Therefore, for a more rigorous evaluation of cyanoacetamide-based Fmoc-oxime carbonates **6**–**9**, protection efficiency was further tested using H-Ala-OH, representative model for C^α-substituted amino acids. Acetone/aqueous bicarbonate solvent system was also employed to conduct the N^α-protection reaction, followed by precipitation of Fmoc-Ala-OH samples in acidic media. Protection yields were not greatly affected in comparison to H-Gly-OH analogous experiment, maintaining an excellent yield at range 89–93% (Table 3). Depending on the Fmoc-oxime employed, efficiency was slightly increased or decreased, being Fmoc-PipOX (**8**) the carbonate that provided the highest yield (93.2%). This result provided further evidence on the outstanding Fmoc-introduction efficiency of the second generation of Fmoc-oxime carbonates, even in residues other than Glycine. Melting point and HPLC analysis demonstrated that the AmOX derivative (**6**) offered the least pure Fmoc-Ala-OH sample among all Fmoc-oximes tested, whereas the piperidino-containing analogue (**8**) produced an almost 99.9% pure material (Table 3).

Furthermore, Fmoc-PipOX (**8**) also gave rise to the lowest impact of Fmoc-Ala–Ala-OH, close to 0.1%. In this model, the least efficient Fmoc-oximes, AmOX (**6**) and MorOX (**9**) derivatives, additionally produced crudes with greatest content of Fmoc-dipeptide, whereas Fmoc-*N*-Oxyma (**7**) performed at a similar level to Fmoc-PipOX (**8**). In an analogous manner to the previous protection system, Fmoc-Ala–Ala-OH was synthesized and coinjected with crude samples to unambiguously identify the dipeptide in the mixture (Fig. 4).

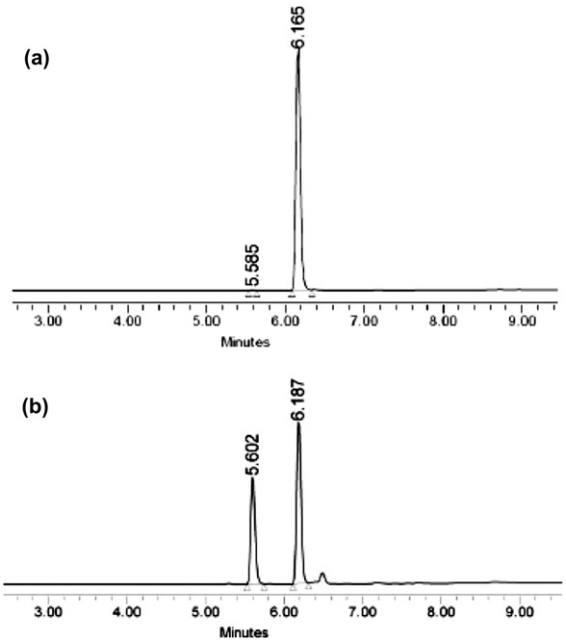


Fig. 3. HPLC analysis of (a) Fmoc-Gly-OH obtained with Fmoc-MorOX (**9**) and (b) co-injection with pure Fmoc-Gly–Gly-OH (for more detail, see Experimental Section).

Table 3
Yield, mp, purity of Fmoc-Ala-OH and dipeptide formation using Fmoc-cyanoacetamide oxime carbonates (**6**–**9**)

Reagent used	Yield (%)	mp (°C)[a]	Purity(%)[b]	Fmoc-Ala-Ala-OH (%)[b]
Fmoc-AmOX (**6**)	89.5	141–142	98.43	0.72
Fmoc-*N*-Oxyma (**7**)	92.6	137–138	99.60	0.38
Fmoc-PipOX (**8**)	93.2	138–139	99.86	0.13
Fmoc-MorOX (**9**)	88.9	138–139	99.28	0.69

[a] Mp of commercial Fmoc-Ala-OH=147–153 °C.
[b] Purity and content of Fmoc-Ala–Ala-OH was determined after HPLC analysis (for a more detailed description, see Experimental Section).

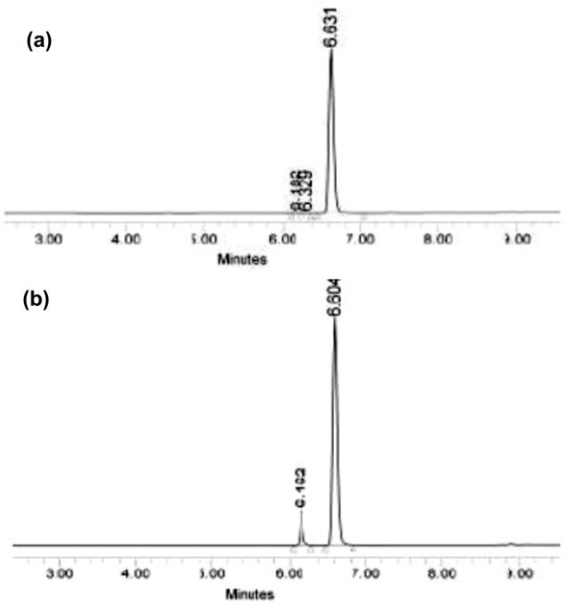


Fig. 4. HPLC analysis of (a) Fmoc-Ala-OH obtained with Fmoc-PipOX (**8**) and (b) co-injection with pure Fmoc-Ala–Ala-OH (for more detail, see Experimental Section).

3. Conclusions

A novel family of acidic and stable oximes, based on a cyanoacetamide scaffold, has been designed with the aim of building a second generation of Fmoc-oxime carbonates, combining minimal dipeptide formation and low production costs. Synthetic approaches to oximes featuring either unsubstituted or *N*-piperidinyl, *N*-morpholinyl and *N*-ethyl substituted amides were successfully accomplished in only two simple steps consisting in amidation and nitrosation with in situ generated gaseous methyl nitrite, improving the efficacy of previously described approaches. The selection of ethyl cyanoacetate as starting material for the diversified strategy towards cyanoacetamide oximes causes great impact on reducing the production costs of the Fmoc-oxime carbonates at an industry level, which prevented the implementation of the most relevant members of the previous generation of this class of compounds. The corresponding Fmoc-cyanoacetamideoxime carbonates were prepared from the various oximes in high yield and purity using Fmoc-Cl in presence of sodium carbonate under Schotten–Bauman conditions.

The performance of cyanoacetamideoxime-containing Fmoc-carbonates was evaluated in the N_{α}-protection of H-Gly-OH and H-Ala-OH, chosen as models for the minimization ability of Fmoc-dipeptides and protection efficiency, respectively. Among these novel Fmoc-oximes, the piperidino analogue (**8**) stands as the derivative affording the highest yield (92–93%) and greatest minimization of Fmoc-oligomers, regardless of amino acid tested. Both Fmoc-PipOX (**8**) and Fmoc-MorOX (**9**) afforded Fmoc-Gly-OH in purity above 99.9%, superseding the performance of the best derivative from the previous family of Fmoc-oxime carbonates, (*N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyloxy}picolinimidoylcyanide) (**5**). A remarkable feature of the cyanoacetamide family of acidic oximes is their unprecedented solubility in water, which greatly facilitates their removal when using the corresponding carbonates, consequently enhancing the Fmoc-aa-OH purity. In summary, cyanoacetamide-based oximes proved to be useful in building cost-effective, suitable Fmoc-carbonates and stand as a promising scaffold for the design of active reagents in peptide synthesis.

4. Experimental section

4.1. Instrumentation and chemicals

All solvents used for recrystallization, extraction, HPLC analysis and TLC were of commercial grade, distilled before use, and stored under dry conditions. Melting points were determined with a Buchi B-540 apparatus and are uncorrected. Magnetic resonance spectra (^{1}H NMR and ^{13}C NMR spectra) were recorded on a Varian Mercury 400 MHz spectrometer at room temperature. Tetramethylsilane (TMS) was used as reference for all NMR spectra, with chemical shifts reported in δ units (parts per million) relative to TMS. Follow-up of the reactions and checks of the purity of the compounds was performed by TLC on silica gel-protected aluminium sheets (Type 60 GF254, Merck) and the spots were detected by exposure to UV-lamp at λ 254 nm for a few seconds. For analytical separation and characterization, a reverse-phase Waters 2695 HPLC separation module, coupled to a Waters 2998 PDA UV detector, was used. Chromatograms were processed with Empower software. Peptide mass was detected by means of an HPLC-PDA system as described above, coupled to a Waters Micromass ZQ mass detector, using the MassLynx 4.1 software. Exact masses were determined with a Thermo Scientific *LQT-FT Ultra* mass spectrometer (ESI positive polarity, Xcalibur 2.0SR2 analyzer, 1750 V capillary voltage, 180 °C desolvation and source temperature, 40 V sample cone, 100–1500 m/z), introducing the sample by direct infusion.

4.2. General procedure for the preparation of cyanoacetamide oximes (15–18)[45]

Ethyl cyano acetic ester (11.3 g, 100 mmol) and amines (morpholine, piperidine, ethyl amine) (150 mmol) were stirred at 70 °C for 6 h, then left to stir at room temperature overnight. The product precipitated, was filtered and washed with diethyl ether to give the desired 2-cyanoacetamide derivatives, which were used without further purification in the next nitrosation step. Into an ice cooled solution of 2-acetonitrile derivatives (100 mmol) and sodium hydroxide (4 g, 100 mmol) in methanol (30 mL) was introduced gaseous methyl nitrite,[45] which was generated from a suspension of sodium nitrite (8.3 g) in a mixture of methanol (10 mL) and water (10 mL) by dropwise addition of a mixture of concentrated sulfuric acid (5 mL) and water (10 mL). The mixture was stirred for 2 h at room temperature and concentrated to dryness. The residue was dissolved in the least amount of water (5 mL), ice cooled, and acidified with concentrated hydrochloric acid to precipitate. In some cases precipitation occurs only by the addition of diethyl ether.

*4.2.1. 2-Amino-N-hydroxy-2-oxoacetimidoyl cyanide (**15**).* The product was obtained as a white powder, mp 199–200 °C in yield 84%. ^{1}H NMR ($CDCl_3$): δ 7.07, 7.37 (2 br s, 2H, NH_2), 13.38 (s, 1H, OH, D_2O exchangeable). ^{13}C NMR (C_2D_6SO): δ 109.45, 128.58, 160.32.

*4.2.2. 2-(Ethylamino)-N-hydroxy-2-oxoacetimidoyl cyanide (**16**).* The product was obtained as white crystals, mp 174–175 °C in yield 77% (from ethyl cyanoacetic ester). ^{1}H NMR (C_3D_6O): δ 1.14 (t, 3H, CH_3, J=7.2 Hz), 3.34 (q, 2H, CH_2, J=7.2 Hz), 7.83 (br s, 1H, NH), 13.59 (s, 1H, OH, D_2O exchangeable). ^{13}C NMR (C_3D_6O): δ 14.21, 34.47, 108.39, 157.96, 158.01.

*4.2.3. N-Hydroxy-2-oxo-2-(piperidin-1-yl) acetimidoyl cyanide (**17**).*[33,39] The product was obtained as a white powder, mp 159–160 °C (lit. 159–162 °C)[1] in yield 87% (from ethyl cyanoacetic ester). ^{1}H NMR (C_3D_6O): δ 1.59–1.71 (m, 6H, $3CH_2$), 3.60–3.64 (m,

4H, $2CH_2$). ^{13}C NMR (C_2D_6SO): δ 24.14, 25.59, 26.19, 43.66, 48.03, 109.79, 127.44, 157.73.

*4.2.4. N-Hydroxy-2-morpholino-2-oxoacetimidoyl cyanide (**18**).*[33] The product was obtained as white crystals, mp 193–194 °C (lit. 155–160)[1] in yield 79% (from ethyl cyanoacetic ester). ^1H NMR (C_3D_6O): δ 3.66–3.75 (m, 8H, $4CH_2$), 13.12 (s, 1H, OH, D_2O exchangeable). ^{13}C NMR (C_2D_6SO): δ 43.13, 47.62, 66.22, 66.51, 109.79, 127.31, 158.23.

4.3. General method for preparation of Fmoc-oxime derivatives (6–9)

A solution of (9*H*-fluoren-9-yl)methyl carbonochloridate (10 mmol) in 30 mL CH_2Cl_2 was added slowly to a solution of (10 mmol) of oxime (**15**–**18**) and sodium carbonate (20 mmol) in 20 mL of H_2O with stirring at 0 °C. The resulting clear mixture was stirred at 0 °C for 30 min and then at room temperature for 2 h. After dilution with CH_2Cl_2 (50 mL), the organic phase was collected and washed with water and saturated aqueous NaCl (30 mL), dried over anhydrous $MgSO_4$. Filtered and the solvent was removed with a rotary evaporator, the residue was recrystallized from CH_2Cl_2/hexane to give Fmoc-oxime derivatives.

*4.3.1. N-(((9H-fluoren-9-yl)methoxy)carbonyloxy)-2-amino-2-oxoacetimidoyl cyanide (**6**).* The product was obtained as white crystals, mp 152–153 °C in yield 91%. ^1H NMR (C_2D_6SO): δ 4.46 (t, 1H, CH, J=6.4 Hz), 4.83 (d, 2H, CH_2, J=6.4 Hz), 7.36 (t, 2H, Ar–H, J=7.6 Hz), 7.44–7.47 (m, 3H, Ar–H+NH), 7.73 (d, 2H, Ar–H, J=7.2 Hz), 7.89–7.91 (m, 3H, Ar–H+NH). ^{13}C NMR: δ 46.72, 71.68, 107.19, 120.40, 125.26, 127.51, 128.26, 134.07, 141.56, 143.26, 151.08, 157.82. A sample for exact mass determination was prepared, dissolving in H_2O/CH_3CN 1:1 and diluting in H_2O/CH_3CN/1%TFA: m/z=336.09841 $[M+H]^+$, 358.08030 $[M+Na]^+$, 693.17150 $[2M+Na]^+$.

*4.3.2. N-(((9H-fluoren-9-yl)methoxy)carbonyloxy)-2-(ethylamino)-2-oxoacetimidoyl cyanide (**7**).* The product was obtained as white crystals, mp 160–161 °C in yield 84%. ^1H NMR ($CDCl_3$): δ 1.24 (t, 3H, CH_3, J=7.2 Hz), 3.42–3.49 (m, 2H, CH_2), 4.35 (t, 1H, CH, J=7.6 Hz), 4.63 (d, 2H, CH_2, J=7.6 Hz), 7.35 (t, 2H, Ar–H, J=7.6 Hz), 7.44 (t, 2H, Ar–H, J=7.6 Hz), 7.61 (d, 2H, Ar–H, J=6.8 Hz), 7.79 (d, 2H, Ar–H, J=6.8 Hz). ^{13}C NMR: δ 14.64, 35.46, 46.60, 72.64, 106.54, 120.51, 125.28, 127.63, 128.52, 133.48, 141.57, 142.61, 151.41, 155.53. A sample for exact mass determination was prepared, dissolving in H_2O/CH_3CN and diluting in H_2O/CH_3CN/1%TFA: m/z=364.12973 $[M+H]^+$, 386.11162 $[M+Na]^+$, 727.2516 $[2M+H]^+$, 749.23387 $[2M+Na]^+$.

*4.3.3. N-(((9H-fluoren-9-yl)methoxy)carbonyloxy)-2-oxo-2-(piperidin-1-yl)acetimidoyl cyanide (**8**).* The product was obtained as waxy product in yield 79%. ^1H NMR ($CDCl_3$): δ 1.66–1.70 (m, 6H, $3CH_2$), 3.58, 3.67 (2t, 4H, $2CH_2$, J=5.6 Hz), 4.34 (t, 1H, CH, J=7.6 Hz), 4.59 (d, 2H, CH_2, J=7.6 Hz), 7.32–7.37 (m, 2H, Ar–H), 7.44 (t, 2H, Ar–H, J=7.6 Hz), 7.61 (d, 2H, Ar–H, J=6.8 Hz), 7.79 (d, 2H, Ar–H, J=6.8 Hz). ^{13}C NMR: δ 24.33, 25.48, 26.47, 44.44, 46.65, 48.59, 72.33, 107.17, 120.46, 125.33, 127.59, 128.44, 133.06, 141.55, 142.78, 151.57, 155.31. A sample for exact mass determination was prepared, dissolving in H_2O/CH_3CN and diluting in H_2O/CH_3CN/1%TFA: m/z=404.16274 $[M+H]^+$, 426.14243 $[M+Na]^+$, 829.29634 $[2M+Na]^+$.

*4.3.4. N-(((9H-fluoren-9-yl)methoxy)carbonyloxy)-2-morpholino-2-oxoacetimidoyl cyanide (**9**).* The product was obtained as white crystals, mp 135–136 °C in yield 88%. ^1H NMR ($CDCl_3$): δ 3.71–3.79 (m, 8H, $4CH_2$), 4.34 (t, 1H, CH, J=7.2 Hz), 4.62 (d, 2H, CH_2, J=7.2 Hz), 7.35 (t, 2H, Ar–H, J=6.4 Hz), 7.43 (t, 2H, Ar–H, J=7.6 Hz), 7.62 (d, 2H, Ar–H, J=7.2 Hz), 7.79 (d, 2H, Ar–H, J=7.2 Hz). ^{13}C NMR: δ 43.71, 46.64, 47.75, 66.56, 66.77, 72.47, 107.01, 120.49, 125.28, 127.60, 128.48, 141.56, 142.68, 151.41, 155.67. A sample for exact mass determination was prepared, dissolving in H_2O/CH_3CN and diluting in H_2O/CH_3CN/1%TFA: m/z=406.14105 $[M+H]^+$, 428.12304 $[M+Na]^+$, 811.27424 $[2M+H]^+$.

4.4. Synthesis of *N*-(9-fluorenylmethyloxycarbonyl)glycine (Fmoc-Gly-OH)

A solution of the Fmoc-OX derivatives (20 mmol) in 100 mL acetone was added dropwise to a stirred solution of Glycine (20 mmol) and $NaHCO_3$ (50 mmol) in 100 mL water. After stirring overnight the reaction mixture was concentrated under reduced pressure and then extracted with CH_2Cl_2 (50 mL) to remove the unreacted Fmoc-OX derivatives. The reaction mixture was cooled and acidified with 10% HCl to congo red litmus paper to give a white solid, which was filtered and washed with water several times, dried to give a white solid, with mp=175–178 °C (mp of an authentic commercial sample of 99.8% purity=177–178 °C; literature[14]=174–176 °C (Table 2). The purity of Fmoc-Gly-OH was determined by injection of 5 μl of a sample prepared from Fmoc-Gly-OH in acetonitrile onto HPLC using the following conditions: Waters SunFire C_{18} column (2.5 μm, 4.6×75 mm); linear gradient 10–100% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min; flow=1.0 ml/min; PDA detection at 254 nm t_R=Fmoc-Gly-OH=6.2; t_R=Fmoc-Gly-Gly-OH=5.6 min and coinjection with an authentic sample of Fmoc-Gly-OH and Fmoc-Gly-Gly-OH.

4.5. Synthesis of *N*-(9-fluorenylmethyloxycarbonyl)alanine (Fmoc-Ala-OH)

A solution of the Fmoc-OX derivatives (20 mmol) in 100 mL acetone was added dropwise to a stirred solution of Alanine (20 mmol) and $NaHCO_3$ (50 mmol) in 100 mL water. After stirring overnight the reaction mixture was concentrated under reduced pressure and then extracted with CH_2Cl_2 (50 mL) to remove the unreacted Fmoc-OX derivatives. The reaction mixture was cooled and acidified with 10% HCl to congo red litmus paper to give a white solid, which was filtered and washed with water several times, dried to give a white solid, mp 137–142 °C (authentic commercial sample mp 147–153 °C (Table 2). The purity of Fmoc-Ala-OH was determined by injection of 5 μl of a sample prepared from Fmoc-Ala-OH in acetonitrile onto HPLC using the following conditions: Waters SunFire C_{18} column (2.5 μm, 4.6×75 mm); linear gradient 10–100% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min; flow=1.0 ml/min; PDA detection at 254 nm t_R=Fmoc-Ala-OH=6.6 min; t_R=Fmoc-Ala-Ala-OH=6.2 min and coinjection with an authentic sample of Fmoc-Ala-OH and Fmoc-Ala-Ala-OH (mp: 188–189 °C).

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. RSF thanks the Ministerio de Educación y Ciencia for a FPU PhD fellowship. Yoav Luxembourg (Luxembourg Biotechnologies) is thanked for his support.

Supplementary data

^1H and ^{13}C NMR spectra of compounds **6**–**9** and **15**–**18**. Supplementary data related to this article can be found online at doi:10.1016/j.tet.2012.02.020.

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