Research Article

Journal of PeptideScience

Received: 2 January 2012 Revised: 2 January 2013 Accepted: 2 January 2013 Published online in Wiley Online Library: 14 March 2013

(wileyonlinelibrary.com) DOI 10.1002/psc.2503

α-*N*-Protected dipeptide acids: a simple and efficient synthesis via the easily accessible mixed anhydride method using free amino acids in DMSO and tetrabutylammonium hydroxide

G. Verardo[a]* and A. Gorassini[b]

The importance of dipeptides both in medicinal and pharmacological fields is well documented and many efforts have been made to find simple and efficient methods for their synthesis. For this reason, we have investigated the synthesis of α-*N*-protected dipeptide acids by reacting the easily accessible mixed anhydride of α-*N*-protected amino acids with free amino acids under different reaction conditions. The combination of TBA-OH and DMSO has been found to be the best to overcome the low solubility of amino acids in organic solvents. Under these experimental conditions, the homogeneous phase condensation reaction occurs rapidly and without detectable epimerization. The present method is also applicable to side-chain unprotected Tyr, Trp, Glu, and Asp but not Lys. This latter residue is able to engage two molecules of mixed anhydride giving the corresponding isotripeptide. Moreover, the applicability of this protocol for the synthesis of tri- and tetrapeptides has been tested. This approach reduces the need for protecting groups, is cost effective, scalable, and yields dipeptide acids that can be used as building blocks in the synthesis of larger peptides. Copyright © 2013 European Peptide Society and John Wiley & Sons, Ltd.

Characterization data of all compounds synthesized are given in the Supporting Information.

Keywords: α-*N*-protected dipeptide acids; mixed carbonic carboxylic anhydride; free amino acids; tetrabutylammonium hydroxide; epimerization; isotripeptides

Introduction

Dipeptides and small peptides have wide applications both in medicinal and synthetic chemistry. For example, they are enzyme inhibitors [1–3], prodrugs [4–12], peroxisome proliferator-activated receptor gamma antagonist [13], anti-HIV-1 [14], and chemotherapeutic metallopharmaceuticals [15–21]. Dipeptides are also used as additional supplementation of single important amino acid nutrients like glutamine [22–29], tyrosine [25,30,31], and cysteine [25]. Moreover, they provide one-dimensional nanostructured materials [32–35], ecofriendly anionic surfactants [36], and catalysts for the direct asymmetric aldol reaction [37–40].

In the solution phase synthesis of a peptide bond, the α-amino and the α-carboxyl groups of the *N*-terminal and *C*-terminal, respectively, amino acid residues must be protected before activating the carboxyl group. The elongation of the peptide chain involves the selective deprotection of the functional group that is to enter into formation of the subsequent peptide bond [41–45]. All these sequential steps are time-consuming, reduce the overall yield, and sometimes may cause a small degree of epimerization [46,47]. The possibility of using free amino acids as the nucleophile may reduce some of these drawbacks.

There are many reports on the coupling reaction between free and α-*N*-protected amino acids with different α-carboxyl activating agents. The active ester has been by far the most used: both phenols and *N*-hydroxy compounds such as 4-nitro- [13,48], 2,3,4,5,6-pentafluorophenol [49–51], 1-hydroxybenzotriazole [52–56], benzisoxazolium salts [57,58], *N*-hydroxysuccinimide [59,60], and *N*-hydroxy-3-azaspiro[5,5]undecane-2,4-dione [61] have been employed to this purpose. These intermediates are stable and the coupling reaction can be carried out under the experimental conditions suitable to dissolve the free amino acids (water with or without an organic cosolvent, presence of an inorganic or organic base). To avoid the use of water, some authors proposed a system consisting of a strong acid [62] or a neutral salt [63,64] in the presence of a tertiary amine to increase the solubility of free amino acids in DMF.

Always with the aim to increase the solubility of amino acids in organic solvents (MeCN, DMF, $CHCl_3$, EtOAc), methods employing phosphazene bases (Schwesinger bases) [65], phase-transfer reagents [66–68], alkaline earth metal ions [69], and ultrasound in the presence of TEA [70] have also been reported. These

* *Correspondence to: Giancarlo Verardo, Dipartimento di Chimica, Fisica e Ambiente, Università di Udine, Via del Cotonificio 108, I-33100 Udine, Italy. E-mail: giancarlo.verardo@uniud.it*

a *Dipartimento di Chimica, Fisica e Ambiente, Università di Udine, Via del Cotonificio 108, I-33100 Udine, Italy*

b *Dipartimento di Storia e Tutela dei Beni Culturali, Università di Udine, Palazzo Caiselli, Vicolo Florio 2, I-33100 Udine, Italy*

experimental protocols allow to carry out the coupling reaction of free amino acids with *N*-carboxyamino acid anhydrides [70] or with α-*N*-protected amino acids activated as esters [65,67–70] or acyl azides [66,68] in a water-free system. Recently, Brown *et al*. [71] reported the synthesis of some Fmoc-dipeptides combining Fmoc-amino acid fluorides with an excess of unprotected amino acids dissolved in hexafluoropropanol.

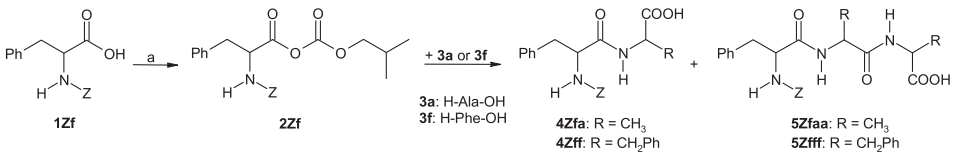
In all the reported methods, the *N*-protected-carboxyl-activated amino acid must be isolated and purified from the reaction by-products and some require expensive and/or toxic reagents (e.g., phosphazene bases, diethylaminosulfur trifluoride, and triphosgene). Moreover, the use of phase-transfer reagents or alkaline earth metal ions require the lyophilization of the amino acids salts obtained thus resulting in more expensive and time-consuming processes.

It is noteworthy that the easily accessible mixed carbonic carboxylic anhydride method has received little attention in recent years. In fact, the few entries found in literature report the reaction of basic aqueous solutions of amino acids only with *N*-protected proline [17,37] (low yields) and, more recently, with *N*-protected phenylalanine [72]. The last one seemed the most appealing for our purposes, but we have encountered some problems (shown in later text) to reproduce the yields reported for some of the reactions described and to extend the protocol to other *N*-protected amino acids. All these reasons prompted us to develop a more versatile approach to the synthesis of *N*-protected dipeptide acids using the mixed anhydride method to activate the carboxyl group of *N*-protected amino acids.

Results and Discussion

The first approach to this study was the synthesis of Z-Phe-Phe-OH (**4Zff**) according to the method proposed by Noguchi *et al*. [72]: a mixture of Z-Phe-OH (**1Zf**, 1.0 mmol), ethyl chloroformate (1.4 mmol), and TEA (3.0 mmol) in THF (20 ml) was stirred for 30 min at 0 °C. After this time, a solution of H-Phe-OH (**3f**, 1.5 mmol) in H_2O (20 ml) was added to the resulted suspension and the stirring was continued for 30 min at 0 °C. After suitable work-up [72], the crude product (0.457 g) was crystallized [72] to afford only 0.281 g (63% yield) of **4Zff**. ^{1}H NMR analysis of the concentrated crystallization mother liquor (0.175 g) evidenced the presence of Z-Phe-OH (**1Zf**), *N*-ethoxycarbonylphenylalanine, and **4Zff** in 63%, 32%, and 5% yields, respectively. The large amount of water, the presence of TEA, and the excess of ethyl chloroformate used during the reaction were responsible for the formation of **1Zf** and *N*-ethoxycarbonylphenylalanine. Similar or worse results were obtained for the synthesis of Fmoc-Phe-Val-OH (**4Ffv**), Boc-Leu-Phe-OH (**4Blf**), and Fmoc-Leu-Ala-OH (**4Fla**). On the basis of these results, we have investigated, with the aid of ESI–MS, the reaction of H-Phe-OH (**3f**) with the mixed anhydride **2Zf** obtained from Z-Phe-OH (**1Zf**, 1.0 mmol) and isobutyl chloroformate (IBCF, 1.03 mmol) prepared in THF (10 ml) in the presence of NMM (1.03 mmol) at 10 °C (Table 1). First of all, we reduced the amount of water to dissolve **3f** (1.2 mmol) using 1.6 M NaOH (1.2 mmol). The addition of this solution to the mixed anhydride **2Zf** caused the partial precipitation of sodium phenylalaninate, and the ESI–MS analysis of the crude mixture after 30 min at 0 °C showed the presence of the tripeptide Z-Phe-Phe-Phe-OH (**5Zfff**, 42%), the starting Z-Phe-OH (**1Zf**, 20%), and the desired dipeptide **4Zff** which was isolated in 15% yield (Table 1, entry 1). The low concentration of phenylalaninate in THF, because of its partial precipitation and the presence of the strong base NaOH, allowed the displacement of the isobutyloxycarbonyl group from the unreacted **2Zf** to the sodium salt of dipeptide **4Zff** giving the corresponding mixed anhydride **6Zff** which competed with **2Zf** for **3f** producing the tripeptide **5Zfff** (Scheme 1). In fact, when tetrabutylammonium

Table 1. Experimental conditions optimization for the synthesis of α-*N*-protected dipeptide acids **4** by the reaction of mixed anhydride **2Zf** and free amino acids **3**



Entry	Solvent[b] (ml)	Base (mmol)	**3** (mmol)	Additive (mmol)	Reaction time (min)	Yield[c] of **1Zf** (%)	Yield[c] of **5** (%)		Yield[d] of **4** (%)
1	H_2O (0.75)	NaOH (1.2)	**3f** (1.2)		30	20	**5Zfff**	42	**4Zff** 15
2	H_2O (0.75)	NaOH (1.2)	**3f** (1.2)	TBA-Br (1.2)	30	10	**5Zfff**	20	**4Zff** 42
3	H_2O (5.24)	NaOH (1.1)	**3f** (1.1)	KH_2PO_4 (5.4)	120	8	**5Zfff**	4	**4Zff** 73
4	H_2O (5.24)	NaOH (1.1)	**3a** (1.1)	KH_2PO_4 (5.4)	120	14	**5Zfaa**	5	**4Zfa** 64
5	H_2O (4.38)	TBA–OH (1.1)	**3a** (1.1)	KH_2PO_4 (5.4)	60	7	**5Zfaa**	4	**4Zfa** 74
6	DMSO (3.50) H_2O (0.52)	TBA–OH (1.1)	**3f** (1.2)		30	3	**5Zfff**	4	**4Zff** 82
7	DMSO (3.50) H_2O (0.52)	TBA–OH (1.1)	**3f** (1.2)		30	3	**5Zfff**	no detect.	**4Zff** 91

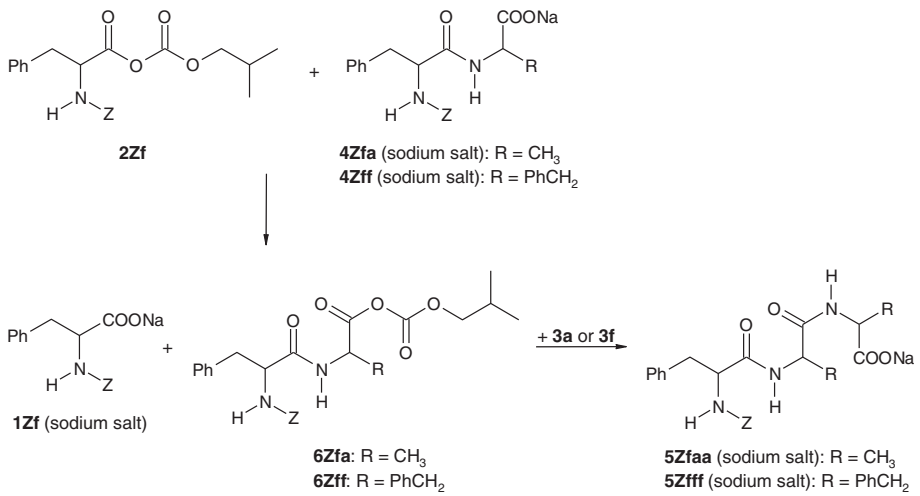
[a]The synthesis of the mixed anhydride **2Zf** was carried out by adding IBCF (1.03 mmol) to a THF (10 ml) solution of **1Zf** (1.0 mmol) and NMM (1.03 mmol) at −15 °C. The reaction mixture was stirred for 10 min at −15 °C before adding **3**.

[b]Type and amount of solvent used to dissolve **3**.

[c]Determined by ESI–MS analysis.

[d]Isolated yield.

Journal of PeptideScience



Scheme 1. Formation of tripeptides **5Zfaa** and **5Zfff**.

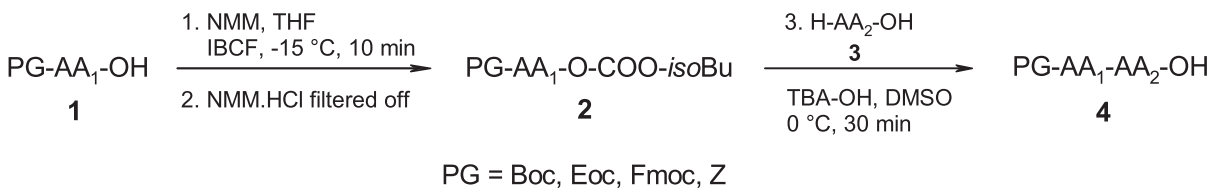
bromide (TBA-Br, 1.2 mmol) was added to increase the solubility of phenylalaninate in THF, the yields of tripeptide **5Zfff** and Z-Phe-OH (**1Zf**) decreased to 20% and 10%, respectively, and Z-Phe-Phe-OH (**4Zff**) was isolated in 42% yield (Table 1, entry 2). However, also in this case, the presence of NaOH could cause the formation of Z–Phe–Phe–Phe–OH (**5Zfff**) promoting the route described in Scheme 1. To verify this hypothesis, the coupling reaction was carried out at pH 6.5 by adding a mixture of H-Phe-OH (**3f**, 1.1 mmol), 0.8 M NaOH (1.1 mmol), and 1.4 M KH_2PO_4 (5.4 mmol) to the preformed mixed anhydride **2Zf**. The ESI–MS analysis of the intact reaction mixture showed longer reaction times for the complete disappearance of **2Zf** (2 h), but the amount of Z-Phe-Phe-Phe-OH (**5Zfff**) was reduced to 4% (Table 1, entry 3) and Z-Phe-Phe-OH (**4Zff**) was isolated in 73% yield. Under these experimental conditions, the substitution of H-Phe-OH (**3f**) with the more water soluble H-Ala-OH (**3a**) caused an increase in the hydrolysis product Z-Phe-OH (**1Zf**, 14% yield) affording the corresponding dipeptide Z-Phe-Ala-OH (**4Zfa**) in 64% yield (Table 1, entry 4). To overcome the low solubility of **3a** in THF at pH 6.5, NaOH was substituted with 55% aqueous TBA–OH (1.1 mmol). In this case (Table 1, entry 5), the coupling reaction of **3a** with the preformed mixed anhydride **2Zf** was faster (1 h), the extent of hydrolysis was reduced (7% yield), and the dipeptide **4Zfa** was isolated in 74% yield. Although this method (Method A) provided better yields compared with those obtained with the protocol of Noguchi *et al.* [72], some tripeptide Z-Phe-Ala-Ala-OH (**5Zfaa**) was still present (Table 1, entry 5), and the reaction with the high water-soluble aspartic (**3d**) and glutamic acid (**3e**) gave the corresponding dipeptide **4** in low yield (Table 3). The observation that DMSO was able to dissolve a mixture of H-Phe-OH (**3f**, 1.2 mmol) and 55% aqueous TBA–OH (1.1 mmol) allowed to carry out the reaction in the presence of a large amount of **3f** in the organic phase (THF–DMSO) favoring the coupling mechanism leading to **4Zff** (reaction time 30 min, 82% yield) over the hydrolysis of the mixed anhydride **2Zf** (3% yield; Table 1, entry 6). Finally (Table 1, entry 7), the removal by filtration of the *N*-methylmorpholinium chloride formed in the activation step gave a crude product with no detectable amount of tripeptide **5Zfff** (ESI–MS) and 91% of isolated yield of the desired dipeptide **4Zff** (Method B). On the basis of these experimental findings, Method B was extended to the synthesis of other dipeptides **4** (Table 2).

The obtained results as well as some properties of dipeptides **4** prepared are reported in Table 3. Some dipeptides **4** were also synthesized according to Method A and the yields were compared with those obtained from Method B (Table 3).

With the aid of ESI–MS analysis, we observed that the reaction rate (30 min) was not influenced by the nature of both the mixed anhydride **2** and the α-amino acid **3** involved. However, when the reaction was carried out with α-*N*-protected amino acids such as valine (**1Bv**, **1Fv**), 2-methylalanine (**1Bu**, **1Zu**), and proline (**1Zp**), the *N*-isobutyloxycarbonyl **7** of the amino acid **3** was present in 7%–9% yield. The absence of unreacted *N*-protected amino acid **1** in the corresponding mixed anhydrides **2Bv**, **2Fv**, **2Bu**, **2Zu**, and **2Zp** highlighted the complete consumption of IBCF during the synthesis of **2**. Therefore, both the steric hindrance of the amino acid side chain (Val, Aib, Pro) and the high reactivity of the TBA salt of **3** (**3-TBA**) resulted in the formation of isobutylcarbamate **7** via the reaction of **3-TBA** with the carboxyl carbon of the carbonic acid ester moiety of the mixed anhydride (Scheme 2) [74,75]. In fact, the substitution of IBCF with the more sterically hindered isopropyl chloroformate [76] produced less isopropylcarbamate **8** (3%–4%) and increased the yield of the corresponding dipeptide **4** (Scheme 2; Table 3).

The amount of TBA–OH employed was critical because even a slight excess produced the *N*-isobutyloxycarbonyl **9** [77] of the starting *N*-protected amino acid **1** via the corresponding mixed anhydride **2** (Scheme 3). For this reason, less than one equivalent of TBA–OH with respect to **3** was used even with amino acids bearing acidic functional groups such as tyrosine (**3y**), aspartic (**3d**), and glutamic (**3e**) acid. When the reaction between the mixed anhydride of *N*-benzyloxycarbonylphenylalanine (**2Zf**; 1.0 equiv) and tyrosine (**3y**; 1.2 equiv) was carried out in the presence of an excess of TBA–OH (2.4 equiv), the ESI–MS analysis of the intact reaction mixture showed, in addition to the dipeptide Z-Phe-Tyr-OH (**4Zfy**; 55% yield) and the expected *N*-isobutyloxycarbonyl-*N*-benzyloxycarbonylphenylalanine (**9Zf**; 10% yield), the presence of *N,O*-bis-(carbobenzyloxyphenylalanyl)-tyrosine (**10**; 35% yield) because of the reaction of two molecules of **2Zf** with both the α-amino and 4-hydroxyphenol groups of tyrosine (**3y**). The yield of **10** raised to 72% (isolated yield 32%) when the reaction was performed using **3y**, TBA–OH, and **2Zf** in the molar ratio of 0.5 : 1.0 : 1.0, respectively.

Table 2. Synthesis of α-*N*-protected dipeptide acids **4**



1, 2	PG-AA_1-	**3**	H-AA_2-OH	**4**	PG-AA_1-AA_2-OH
Bf	Boc-Phe-	**a**	H-Ala-OH	**Bfa**	Boc-Phe-Ala-OH
Bf	Boc-Phe-	**y**	H-Tyr-OH	**Bfy**	Boc-Phe-Tyr-OH
Bf	Boc-Phe-	**d**	H-Asp-OH	**Bfd**	Boc-Phe-Asp-OH
Bf	Boc-Phe-	**e**	H-Glu-OH	**Bfe**	Boc-Phe-Glu-OH
Ba	Boc-Ala-	**y**	H-Tyr-OH	**Bay**	Boc-Ala-Tyr-OH
Bl	Boc-Leu-	**f**	H-Phe-OH	**Blf**	Boc-Leu-Phe-OH
Bl	Boc-Leu-	**q**	H-Gln-OH	**Blq**	Boc-Leu-Gln-OH
Bu	Boc-Aib-	**f**	H-Phe-OH	**Buf**	Boc-Aib-Phe-OH
Bv	Boc-Val-	**f**	H-Phe-OH	**Bvf**	Boc-Val-Phe-OH
Bv	Boc-Val-	**w**	H-Trp-OH	**Bvw**	Boc-Val-Trp-OH
Eb	Eoc-Phg-	**a**	H-Ala-OH	**Eba**	Eoc-Phg-Ala-OH
Ff	Fmoc-Phe-	**a**	H-Ala-OH	**Ffa**	Fmoc-Phe-Ala-OH
Ff	Fmoc-Phe-	**f**	H-Phe-OH	**Fff**	Fmoc-Phe-Phe-OH
Ff	Fmoc-Phe-	**l**	H-Leu-OH	**Ffl**	Fmoc-Phe-Leu-OH
Ff	Fmoc-Phe-	**v**	H-Val-OH	**Ffv**	Fmoc-Phe-Val-OH
Ff	Fmoc-Phe-	**b**	H-Phg-OH	**Ffb**	Fmoc-Phe-Phg-OH
Fl	Fmoc-Leu-	**a**	H-Ala-OH	**Fla**	Fmoc-Leu-Ala-OH
Fl	Fmoc-Leu-	**b**	H-Phg-OH	**Flb**	Fmoc-Leu-Phg-OH
Fl	Fmoc-Leu-	**u**	H-Aib-OH	**Flu**	Fmoc-Leu-Aib-OH
Fv	Fmoc-Val-	**d**	H-Asp-OH	**Fvd**	Fmoc-Val-Asp-OH
Fm	Fmoc-Met-	**e**	H-Glu-OH	**Fme**	Fmoc-Met-Glu-OH
Fw	Fmoc-Trp-	**t**	H-Thr-OH	**Fwt**	Fmoc-Trp-Thr-OH
Zf	Z-Phe-	**a**	H-Ala-OH	**Zfa**	Z-Phe-Ala-OH
Zf	Z-Phe-	**y**	H-Tyr-OH	**Zfy**	Z-Phe-Tyr-OH
Zf	Z-Phe-	**f**	H-Phe-OH	**Zff**	Z-Phe-Phe-OH
Zf	Z-Phe-	**q**	H-Gln-OH	**Zfq**	Z-Phe-Gln-OH
Zf	Z-Phe-	**l**	H-Leu-OH	**Zfl**	Z-Phe-Leu-OH
Zf	Z-Phe-	**u**	H-Aib-OH	**Zfu**	Z-Phe-Aib-OH
Za	Z-Ala-	**y**	H-Tyr-OH	**Zay**	Z-Ala-Tyr-OH
Za	Z-Ala-	**e**	H-Glu-OH	**Zae**	Z-Ala-Glu-OH
Zu	Z-Aib-	**f**	H-Phe-OH	**Zuf**	Z-Aib-Phe-OH
Zw	Z-Trp-	**b**	H-Phg-OH	**Zwb**	Z-Trp-Phg-OH
Zp	Z-Pro-	**f**	H-Phe-OH	**Zpf**	Z-Pro-Phe-OH

On the basis of these results, we tested the behavior of lysine hydrochloride (**3k**) with the mixed anhydride of Boc-Phe- (**2Bf**), Fmoc-Val- (**2Fv**), and Z-Leu- (**2Zl**) using a 0.5 : 1.0 : 1.0 ratio of **3k** : TBA–OH : **2**. As expected, the two amino groups of lysine showed the same reactivity towards **2** affording the corresponding isotripeptide **11** (Scheme 4) in 55%–76% overall yield.

In order to exclude any incursion of racemization under our experimental conditions, Eoc-L-Phg-L-Ala-OH (**4Eba**) was prepared by the reaction of *N*-ethoxycarbonyl-L-phenylglycine (**1Eb**) with alanine (**3a**) and its HPLC–ESI–MS profile as well as ^{1}H and ^{13}C NMR spectra were compared with those of the diastereoisomeric mixture of Eoc-DL-Phg-L-Ala-OH (**diast-4Eba**) obtained from the reaction of racemic *N*-ethoxycarbonyl-DL-phenylglycine (**rac-1Eb**) with **3a**. The HPLC–ESI–MS analysis of **diast-4Eba** evidenced two distinct peaks at $R_t = 40.1$ and 41.3 min of the same area. In addition, the ^{1}H and ^{13}C NMR spectra showed, with the exception of the ethoxycarbonyl moiety, two sets of peaks for each proton and carbon signal, respectively. On the other hand, when the reaction was carried out with **1Eb**, the HPLC–ESI–MS profile of **4Eba** pointed out the presence of only one peak at $R_t = 41.3$ min and the corresponding ^{1}H and ^{13}C NMR spectra did not exhibit a similar complexity. The analogous chromatographic and NMR behaviour found in all the synthesized α-*N*-protected dipeptides **4** confirmed that the present protocol proceeds with retention of configuration. It is worth to point out that when we attempted to synthesize **4Eba** following the method proposed by Noguchi *et al.* [72], the HPLC–ESI–MS analysis of the crude product evidenced the presence of a mixture of Eoc-L-Phg-Ala-OH (**4Eba**) and Eoc-D-Phg-Ala-OH (**epi-4Eba**) in the approximate ratio 80 : 20.

Journal of PeptideScience

Table 3. Yields and some properties of α-*N*-protected dipeptide acids **4** prepared

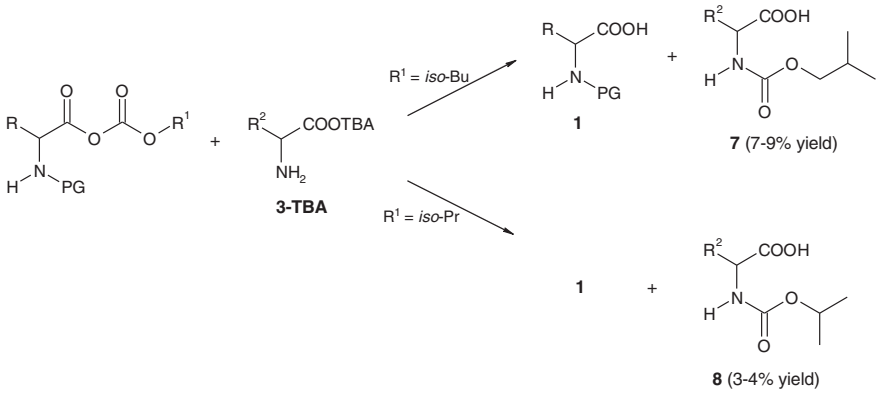
4	Product	Recrystallization solvent	Yield (%)	mp (°C)	$[\alpha]_D^{20}$ (*c*, solvent)
Bfa	Boc-Phe-Ala-OH	Toluene-Hexane	81[a]	124–126	−4.7 (0.6, MeOH)
Bfy	Boc-Phe-Tyr-OH	Toluene-Hexane	87[a]	95–97	+5.3 (0.8, MeOH)
Bfd	Boc-Phe-Asp-OH	Toluene-Hexane	80[a]	78–80	+4.9 (0.6, MeOH)
Bfe	Boc-Phe-Glu-OH	Toluene-Hexane	84[a]; 58[b]	167–169	−7.7 (0.9, MeOH)
Bay	Boc-Ala-Tyr-OH	$CHCl_3$-Hexane	91[a]	78–80	+6.1 (0.9, MeOH)
Blf	Boc-Leu-Phe-OH	$CHCl_3$-Hexane	82[a]	120–122	−9.4 (0.8, MeOH)
Blq	Boc-Leu-Gln-OH	$CHCl_3$-Hexane	87[a]	91–93	−20.1 (0.7, MeOH)
Buf	Boc-Aib-Phe-OH	Cyclohexane	69[a]; 79[c]	135–137	+16.1 (0.6, MeOH)
Bvf	Boc-Val-Phe-OH	$CHCl_3$-Hexane	70[a]; 80[c]	134–136	−15.5 (0.6, MeOH)
Bvw	Boc-Val-Trp-OH	Toluene-Hexane	73[a]; 83[c]	77–79	−5.8 (0.7, MeOH)
Eba	Eoc-Phg-Ala-OH	$CHCl_3$-Hexane	82[a]; 75[b]	98–100	−98.9 (0.8, MeOH)
Ffa	Fmoc-Phe-Ala-OH	Toluene-Hexane	81[a]	214–216[d]	−23.6 (0.2, MeOH)[d]
Fff	Fmoc-Phe-Phe-OH	Toluene-Hexane	86[a]; 74[b]	166–168	−18.0 (0.6, MeOH)
Ffl	Fmoc-Phe-Leu-OH	Toluene-Hexane	95[a]	194–196	−24.7 (0.3, MeOH)
Ffv	Fmoc-Phe-Val-OH	Toluene-Hexane	88[a]	221–223	−13.9 (0.5, MeOH)
Ffb	Fmoc-Phe-Phg-OH	Toluene-Hexane	79[a]	205–207	−48.6 (0.5, DMSO)
Fla	Fmoc-Leu-Ala-OH	$CHCl_3$-Hexane	88[a]	194–196[e]	−28.2 (0.8, MeOH)[e]
Flb	Fmoc-Leu-Phg-OH	$CHCl_3$-Hexane	88[a]	204–206	−42.9 (0.2, MeOH)
Flu	Fmoc-Leu-Aib-OH	$CHCl_3$-Hexane	71[a]	196–198[f]	−10.3 (1.0, DMF)[f]
Fvd	Fmoc-Val-Asp-OH	Toluene-Hexane	77[a], 59[b]; 88[c]	182–184	−19.7 (0.9, MeOH)
Fme	Fmoc-Met-Glu-OH	Toluene-Hexane	89[a]; 51[b]	131–133[g]	−18.9 (0.9, MeOH)[g]
Fwt	Fmoc-Trp-Thr-OH	$CHCl_3$-Hexane	94[a]	114–116	−13.9 (0.7, MeOH)
Zfa	Z-Phe-Ala-OH	$CHCl_3$-Hexane	85[a]; 74[b]	156–158	−14.0 (0.5, MeOH)
Zfy	Z-Phe-Tyr-OH	Toluene-EtOAc	84[a]	189–191	−4.4 (0.5, MeOH)
Zff	Z-Phe-Phe-OH	$CHCl_3$-Hexane	91[a]; 79[b]	160–162	−17.0 (0.5, MeOH)
Zfq	Z-Phe-Gln-OH	Toluene-Hexane	90[a]	185–187	−10.8 (0.6, MeOH)
Zfl	Z-Phe-Leu-OH	Toluene-Hexane	81[a]	139–141	−23.5 (0.4, MeOH)
Zfu	Z-Phe-Aib-OH	$CHCl_3$-Hexane	70[a]	159–161	−8.0 (0.5, MeOH)
Zay	Z-Ala-Tyr-OH	Toluene-Hexane	84[a]; 70[b]	152–154	+10.7 (0.6, MeOH)
Zae	Z-Ala-Glu-OH	Toluene-EtOAc	86[a]	143–145[h]	−17.4 (0.5, MeOH)[h]
Zuf	Z-Aib-Phe-OH	Toluene-Hexane	70[a]; 77[c]	78–80	+23.6 (0.5, MeOH)
Zwb	Z-Trp-Phg-OH	Toluene-Hexane	90[a]	190–192	−61.8 (0.5, MeOH)
Zpf	Z-Pro-Phe-OH	$CHCl_3$-Hexane	75[a]; 80[c]	132–134[i]	−13.8 (0.5, MeOH)[i]

[a]Isolated yield. The reaction was carried out according to Method B.
[b]Isolated yield. The reaction was carried out according to Method A.
[c]Isolated yield. The reaction was carried out according to Method B but using isopropylchloroformate during the synthesis of the mixed anhydride of **1**.
[d]Lit. [55]: mp 208.7–210.6 °C; $[\alpha]_D^{24}$ −10.2 (1.5, DMF).
[e]Lit. [55]: mp 179.0–179.8 °C; $[\alpha]_D^{24}$ −6.5 (1.5, DMF).
[f]Lit [73]: mp 173–176 °C; $[\alpha]_D^{25}$ −10.0 (1.0, DMF).
[g]Lit. [54]: mp 148–150 °C; $[\alpha]_D^{25}$ −18.1 (1.5, DMF).
[h]Lit. [54]: mp 121–123 °C; $[\alpha]_D^{25}$ +0.7 (1.5, DMF).
[i]Lit: [58]: mp 129–130 °C; $[\alpha]_D^{25}$ −46.1 (1.0, EtOH).

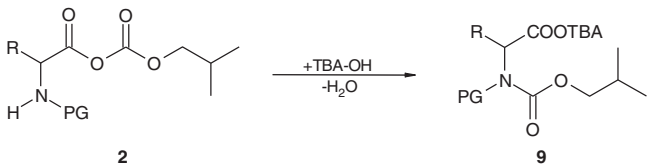
Finally, in an effort to apply this protocol to the synthesis of tripeptides **5**, we studied the amidation of the mixed anhydride **12** of Fmoc-Leu-Phg-OH (**4Flb**) with valine (**3v**). The ESI–MS analysis of the intact reaction mixture obtained after the formation (10 min at −15 °C) of **12** showed the complete conversion of **12** into the corresponding racemization-sensitive 2,4-disubstituted-5(4*H*)-oxazolone **13** [78–80]. The HPLC–ESI–MS profile of the crude product obtained after the reaction of **13** with **3v** evidenced the presence of a mixture of the desired tripeptide Fmoc-L-Leu-L-Phg-L-Val-OH (**5Flbv**) and its epimer Fmoc-L-Leu-D-Phg-L-Val-OH (**epi-5Flbv**) in the approximate ratio of 91 : 9 (Scheme 5). The reaction carried out using the crude dipeptide **4Flb** obtained from the first coupling reaction afforded, after crystallization from toluene, the pure tripeptide **5Flbv** in 63% overall yield after the two steps. In a similar way were synthesized the tripeptides Fmoc-Leu-Phe-Val-OH (**5Flfv**) and Boc-Phe-Val-Ala-OH (**5Bfva**) and the tetrapeptide Boc-Leu-Phe-Val-Ala-OH (**14**) in 68%, 66%, and 48% overall yield, respectively.

Conclusions

We have developed an efficient and simple synthesis of α-*N*-protected dipeptide acids **4** by the coupling reaction of the easily accessible mixed anhydride of an α-*N*-protected amino acid **2** with a DMSO solution of the free amino acid **3** and aqueous TBA–OH.



Scheme 2. Formation of carbamates **7** and **8** when the mixed anhydride of sterically hindered amino acids (Val, Aib, Pro) was used.



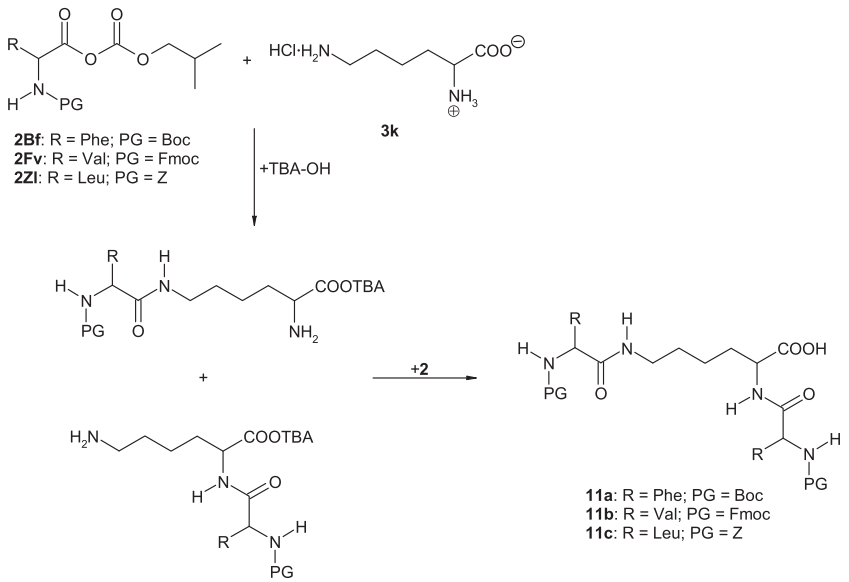
Scheme 3. Formation of *N*-protected-*N*-isobutyloxycarbonyl amino acid **9** in the presence of excess TBA–OH.

The present protocol has been successfully applied also to amino acids bearing unprotected hydroxyl and acidic side chains (H-Thr-OH, H-Tyr-OH, H-Asp-OH, and H-Glu-OH). Under these same experimental conditions, lysine (**3k**) reacted with two molecules of mixed anhydride **2** affording the corresponding isotripeptide **11**. The homogeneous phase reaction proceeds rapidly and with retention of configuration as evidenced by HPLC–ESI–MS, ^{1}H and ^{13}C NMR analysis. We are currently investigating the possibility of suppressing the incursion of epimerization during the synthesis of polypeptide with the present mixed anhydride method.

Materials and Methods

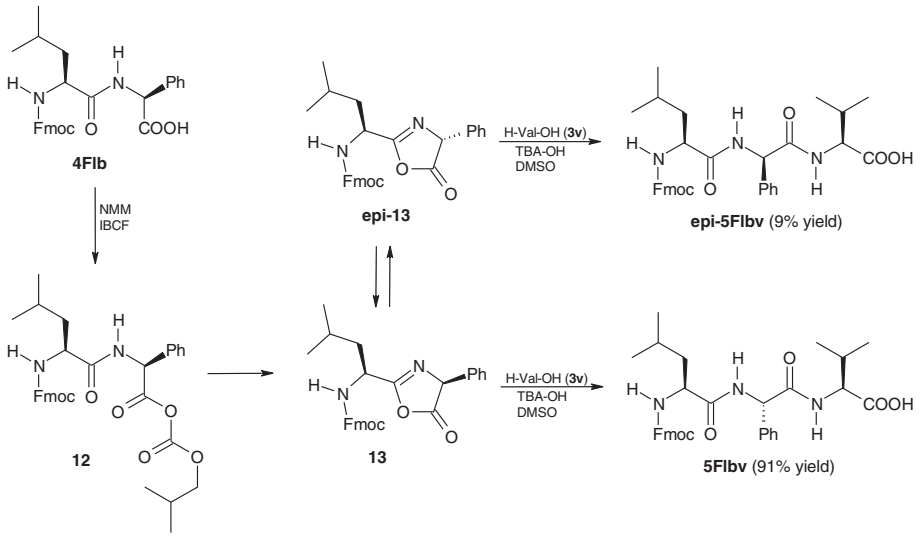
General

N-Ethoxycarbonyl-L-phenylglycine (**1Eb**) and *N*-ethoxycarbonyl-DL-phenylglycine (**rac-1Eb**) were prepared by reported procedure [81]. All solvents and reagents were purchased from Aldrich Chemical Company and used without further purification. The reactions were monitored by ESI–MS in the positive and negative ion mode with a Finnigan LXQ (linear trap) (Thermo Electron Corporation, Madison, WI, USA) by simply diluting the intact reaction mixture with methanol and directly infusing the obtained solution into the ion source with the aid of a syringe pump. HPLC–ESI–MS analyses were performed with the same instrument coupled with a Finnigan Surveyor LC Pump Plus (Thermo Electron Corporation, Madison, WI, USA) and equipped with a Finnigan Surveyor Autosampler Plus (Thermo Electron Corporation, Madison, WI, USA). The LC separations were performed on a Hypersil AA-ODS column (200 mm × 2.1 mm, 5 μm) from Agilent Technologies (Spinea, Italy) operating at 30 °C at a flow rate of 0.2 ml/min with the mobile phase composed of (i) water modified with 0.1% (v/v) of formic acid and (ii) methanol. The eluent was maintained at



Scheme 4. Synthesis of the isotripeptides **11a-c**.

Journal of PeptideScience



Scheme 5. Formation of the tripeptide Fmoc-L-Leu-L-Phg-L-Val-OH (**5Flbv**) and its epimer Fmoc-L-Leu-D-Phg-L-Val-OH (**epi-5Flbv**) via the racemization-sensitive 2,4-disubstituted-5(*4H*)-oxazolone **13**.

94% A for the first 20 min after injection then the proportion of solvent B was linearly increased to 50% over a period of 10 min and kept at this concentration until the end of the run. Infrared spectra were obtained with a Bruker Vector 22 spectrophotometer (Bruker Corporation, MA, USA) using the KBr technique for solids and recorded in the range 4000–400 cm^{-1}. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-F 200 spectrometer (Bruker Corporation, MA, USA) at 200 and 50 MHz, respectively, using DMSO-d_6 at 40 °C as solvent. NMR peak locations are reported as δ values from TMS. Some ^{1}H multiplets are characterized by the term *app* (apparent): this refers only to their appearance and may be an oversimplification. Optical rotations were determined on suitable solutions (g/100 ml) at 20 °C using an AP-300 automatic polarimeter purchased from ATAGO (Japan). Elemental analyses were performed with a Carlo Erba Mod. 1106 elemental analyser (Carlo Erba Strumentazione, Milan, Italy). Melting points were determined with an automatic Mettler Mod. FP61 (Mettler Instrumente AG, Greifensee, CH) melting point apparatus and are not corrected.

Peptide Synthesis

*Synthesis of α-N-protected dipeptide acids **4**: Method A.*

NMM (0.227 ml, 2.06 mmol) was slowly added to a stirred solution of α-*N*-protected amino acid **1** (2.00 mmol) in THF (20 ml) at room temperature. After 5 min, IBCF (0.267 ml, 2.06 mmol) was slowly added to the reaction mixture cooled down to −15 °C and stirring was continued for 10 min at the same temperature. The reaction mixture was subsequently warmed up to 0 °C and a solution (ca. pH 6.5) of amino acid **3** (2.20 mmol), 55% aqueous TBA–OH (1.04 ml, 2.20 mmol) and 1.4 M KH_2PO_4 (7.71 ml, 10.80 mmol) was added in one lot. The mixture was allowed to warm to room temperature under vigorous stirring and, after additional 1 h, THF was removed under reduced pressure. The residue was acidified to pH 2–3 with 5% HCl, the solid formed was dissolved in AcOEt (70 ml) and the organic phase washed with H_2O (2 x 20 ml), brine (15 ml), and finally dried over Na_2SO_4. After filtration and evaporation of the solvent in vacuo, the crude product was recrystallized from suitable solvents affording the α-*N*-protected dipeptide acid **4** in 58%–79% overall yield (Table 3).

*Synthesis of α-N-protected dipeptide acids **4**: Method B.*

NMM (0.227 ml, 2.06 mmol) was slowly added to a stirred solution of α-*N*-protected amino acid **1** (2.00 mmol) in THF (20 ml) at room temperature. After 5 min, IBCF (0.267 ml, 2.06 mmol) was slowly added to the reaction mixture cooled down to −15 °C and stirring was continued for 10 min at the same temperature. After this time, *N*-methylmorpholinium hydrochloride was filtered off and washed with THF (2–3 ml). The mother liquor was cooled to 0 °C and a solution of amino acid **3** (2.40 mmol) and 55% aqueous TBA–OH (1.04 ml, 2.20 mmol) in DMSO (7.0 ml) was added in one lot and the reaction mixture was vigorous stirred for 30 min at the same temperature. THF was removed under reduced pressure and the residue, after acidification to pH 2–3 with 5% HCl, was dissolved in AcOEt (70 ml) and the organic phase washed with H_2O (2 × 20 ml), brine (15 ml), and finally dried over Na_2SO_4. After filtration and evaporation of the solvent in vacuo, the crude product was recrystallized from suitable solvents affording the α-*N*-protected dipeptide acid **4** in 69%–94% overall yield (Table 3).

The synthesis of Boc-Aib-Phe-OH (**4Buf**), Boc-Val-Phe-OH (**4Bvf**), Boc-Val-Trp-OH (**4Bvw**), Fmoc-Val-Asp-OH (**4Fvd**), Z-Aib-Phe-OH (**4Zuf**), and Z-Pro-Phe-OH (**4Zpf**) was also carried out by substituting IBCF with 1 M solution of isopropyl chloroformate in toluene (2.06 ml, 2.06 mmol) for the preparation of the mixed anhydride intermediate of **1Bu**, **1Bv**, **1Fv**, **1Zu** and **1Zp**, respectively. Method B was followed with only this variation affording the α-*N*-protected dipeptide acid **4** in 70%–88% overall yield (Table 3).

*Synthesis of Fmoc-Leu-Phg-Ala-OH (**5Flba**), Fmoc-Leu-Phe-Val-OH (**5Flfv**), and Boc-Phe-Val-Ala-OH (**5Bfva**)*

NMM (1.03 equiv) was slowly added to a stirred solution of all the previously synthesized crude α-*N*-protected dipeptide acid **4** (1.00 equiv) in THF (10 ml $mmol^{-1}$) at room temperature. After 5 min, IBCF (1.03 equiv) was slowly added to the reaction mixture cooled down to −15 °C and stirring was continued for 10 min at the same temperature. After this time, *N*-methylmorpholinium hydrochloride was filtered off and washed with THF (2–3 ml). The mother liquor was cooled to 0 °C and a solution of amino acid **3** (1.20 equiv) and 55% aqueous TBA–OH (1.10 equiv) in DMSO (3.5 ml $mmol^{-1}$) was added in one lot and the reaction mixture

was vigorous stirred for 30 min at the same temperature. THF was removed under reduced pressure and the residue, after acidification to pH 2–3 with 5% HCl, was dissolved in AcOEt (50 ml $mmol^{-1}$) and the organic phase washed with H_2O (2 × 15 ml $mmol^{-1}$), brine (10 ml $mmol^{-1}$), and finally dried over Na_2SO_4. After filtration and evaporation of the solvent in vacuo, the crude product was recrystallized from toluene affording the α-*N*-protected tripeptide acids **5** in 63%–68% overall yield after the two steps.

*Synthesis of Boc-Leu-Phe-Val-Ala-OH (**14**).*

NMM (1.03 equiv) was slowly added to a stirred solution of all the previously synthesized crude tripeptide Boc-Leu-Phe-Val-OH (**5Blfv**, 1.00 equiv) in THF (10 ml $mmol^{-1}$) at room temperature. After 5 min, IBCF (1.03 equiv) was slowly added to the reaction mixture cooled down to −15 °C and stirring was continued for 10 min at the same temperature. After this time, *N*-methylmorpholinium hydrochloride was filtered off and washed with THF (2–3 ml). The mother liquor was cooled to 0 °C and a solution of alanine (**3a**, 1.20 equiv) and 55% aqueous TBA–OH (1.10 equiv) in DMSO (3.5 ml $mmol^{-1}$) was added in one lot and the reaction mixture was vigorously stirred for 30 min at the same temperature. THF was removed under reduced pressure and the residue, after acidification to pH 2–3 with 5% HCl, was dissolved in AcOEt (50 ml $mmol^{-1}$) and the organic phase washed with H_2O (2 × 15 ml $mmol^{-1}$), brine (10 ml $mmol^{-1}$), and finally dried over Na_2SO_4. After filtration and evaporation of the solvent in vacuo, the crude product was recrystallized from toluene affording the tetrapeptide Boc-Leu-Phe-Val-Ala-OH (**14**) in 48% overall yield after the three steps.

*Synthesis of N,O-bis-(carbobenzyloxyphenylalanyl)-tyrosine (**10**).*

NMM (0.227 ml, 2.06 mmol) was slowly added to a stirred solution of Z-Phe-OH (**1Zf**; 0.548 g, 2.00 mmol) in THF (20 ml) at room temperature. After 5 min, IBCF (0.267 ml, 2.06 mmol) was slowly added to the reaction mixture cooled down to −15 °C and stirring was continued for 10 min at the same temperature. After this time, *N*-methylmorpholinium hydrochloride was filtered off and washed with THF (2–3 ml). The mother liquor was cooled to 0 °C and a solution of tyrosine (**3y**; 0.1812 g, 1.00 mmol) and 55% aqueous TBA–OH (0.948 ml, 2.00 mmol) in DMSO (7.0 ml) was added in one lot and the reaction mixture was vigorously stirred for 90 min at the same temperature. THF was removed under reduced pressure and the residue, after acidification to pH 2–3 with 5% HCl, was dissolved in AcOEt (90 ml) and the organic phase washed with H_2O (2 × 20 ml), brine (15 ml), and finally dried over Na_2SO_4. After filtration and evaporation of the solvent in vacuo, the crude product was recrystallized from toluene affording *N,O*-bis-(carbobenzyloxyphenylalanyl)-tyrosine (**10**) in 32% overall yield.

*Synthesis of Boc-Phe-Lys(Boc-Phe)-OH (**11a**), Fmoc-Val-Lys(Fmoc-Val)-OH (**11b**) and Z-Leu-Lys(Z-Leu)-OH (**11c**)*

NMM (0.227 ml, 2.06 mmol) was slowly added to a stirred solution of α-*N*-protected amino acid **1** (2.00 mmol) in THF (20 ml) at room temperature. After 5 min, IBCF (0.267 ml, 2.06 mmol) was slowly added to the reaction mixture cooled down to −15 °C and stirring was continued for 10 min at the same temperature. After this time, *N*-methylmorpholinium hydrochloride was filtered off and washed with THF (2–3 ml). The mother liquor was cooled to 0 °C and a solution lysine hydrochloride (**3k**, 0.1827 g, 1.00 mmol) and 55% aqueous TBA–OH (0.948 ml, 2.00 mmol) in DMSO (7.0 ml) was added in one lot and the reaction mixture was vigorously stirred for 90 min at room temperature. THF was removed under reduced pressure and the residue, after acidification to pH 2–3 with 5% HCl, was dissolved in AcOEt (90 ml) and the organic phase washed with H_2O (2 × 20 ml), brine (15 ml), and finally dried over Na_2SO_4. After filtration and evaporation of the solvent in vacuo, the crude product was recrystallized from $CHCl_3$-hexane affording the isotripeptides **11a–c** in 55%–76% overall yield.

Acknowledgements

The authors are grateful to Dr. P. Martinuzzi and Dr. S. Turco for recording ^1H and ^{13}C NMR spectra.

References

1 Sharma SK, Al-Hourani BJ, Wuest M, Mane JY, Tuszynski J, Baracos V, Suresh M, Wuest F. Synthesis and evaluation of fluorobenzoylated di- and tripeptides as inhibitors of cyclooxygenase-2 (COX-2). *Bioorg. Med. Chem.* 2012; **20**: 2221–2226.
2 Tokunaga KH, Yoshida C, Suzuki KM, Maruyama H, Futamura Y, Araki Y, Mishima S. Antihypertensive effect of peptides from royal jelly in spontaneously hypertensive rats. *Biol. Pharm. Bull.* 2004; **27**: 189–192.
3 Narai-Kanayama A, Aso K. Angiotensin I-converting enzyme inhibitory oligo-tyrosine peptides synthesized by α-chymotrypsin. *Enzyme Microb. Tech.* 2009; **44**: 235–241.
4 Omkvist DH, Trangbaek DJ, Mildon J, Paine JS, Brodin B, Begtrup M, Nielsen CU. Affinity and translocation relationships via hPEPT1 of H-X_{aa}-Ser-OH dipeptides: evaluation of H-Phe-Ser-OH as a pro-moiety for ibuprofen and benzoic acid prodrugs. *Eur. J. Pharm. Biopharm.* 2011; **77**: 327–331.
5 Diez-Torrubia A, Garcia-Aparicio C, Cabrera S, De Meester I, Balzarini J, Camarasa M-J, Velazquez S. Application of the dipeptidyl peptidase IV (DPPIV/CD26) based prodrug approach to different amine-containing drugs. *J. Med. Chem.* 2010; **53**: 559–572.
6 Burke PJ, Senter PD, Meyer DW, Miyamoto JB, Anderson M, Toki BE, Manikumar G, Wani MC, Kroll DJ, Jeffrey SC. Design, synthesis, and biological evaluation of antibody-drug conjugates comprised of potent camptothecin analogues. *Bioconjugate Chem.* 2009; **20**: 1242–1250.
7 Talluri RS, Samanta SK, Gaudana R, Mitra AK. Synthesis, metabolism and cellular permeability of enzymatically stable dipeptide prodrugs of acyclovir. *Int. J. Pharm.* 2008; **361**: 118–124.
8 Santos C, Morais J, Gouveia L, de Clercq E, Pannecouque C, Nielsen CU, Steffansen B, Moreira R, Gomes P. Dipeptide derivatives of AZT: synthesis, chemical stability, activation in human plasma, hPEPT1 affinity, and antiviral activity. *ChemMedChem* 2008; **3**: 970–978.
9 Nashed YE, Mitra AK. Synthesis and characterization of novel dipeptide ester prodrugs of acyclovir. *Spectrochim. Acta A* 2003; **59**: 2033–2039.
10 Friedrichsen GM, Jakobsen P, Taub M, Begtrup M. Application of enzymatically stable dipeptides for enhancement of intestinal permeability. Synthesis and in vitro evaluation of dipeptide-coupled compounds. *Bioorg. Med. Chem.* 2001; **9**: 2625–2632.
11 Chung MC, Goncalves MF, Colli W, Ferreira EI, Miranda MTM. Synthesis and in vitro evaluation of potential antichagasic dipeptide prodrugs of primaquine. *J. Pharm. Sci.* 1997; **86**: 1127–1131.
12 Cheng S, Zhang X, Wang W, Zhao M, Zheng M, Chang HW, Wu J, Peng S. A class of novel *N*-(3*S*-1,2,3,4-tetrahydroisoquinoline-3-carbonyl)-L-amino acid derivatives: their synthesis, anti-thrombotic activity evaluation, and 3D QSAR analysis. *Eur. J. Med. Chem.* 2009; **44**: 4904–4919.
13 Deng G, Liu Z, Ye F, Luo X, Zhu W, Shen X, Liu H, Jiang H. Tryptophan-containing dipeptide derivatives as potent PPAR gamma antagonists: design, synthesis, biological evaluation, and molecular modeling. *Eur. J. Med. Chem.* 2008; **43**: 2699–2716.
14 Parra A, Rivas F, Lopez PE, Garcia-Granados A, Martinez A, Albericio F, Marquez N, Munoz E. Solution- and solid-phase synthesis and anti-HIV activity of maslinic acid derivatives containing amino acids and peptides. *Bioorg. Med. Chem.* 2009; **17**: 1139–1145.
15 Metzler-Nolte N Biomedical applications of organometal-peptide conjugates. In Medicinal Organometallic Chemistry, Jaouen G, Metzler-Nolte N (eds). Springer-Verlag: Heidelberg, 2010; 195–217.
16 Nath M, Pokharia S, Eng G, Song XQ, Kumar A. New triorganotin (IV) derivatives of dipeptides as models for metal-protein interactions: synthesis, structural characterization and biological studies. *Spectrochim. Acta A.* 2006; **63**: 66–75.

17 Martynova KV, Andreeva LA, Klimova PA, Kirillova YG, Shevchenko VP, Nagaev IY, Shram SI, Shvets VI, Myasoedov NF. Structural–functional study of glycine-and-proline-containing peptides (glyprolines) as potential neuroprotectors. *Bioorg. Khim.* 2009; **35**: 165–171 (Engl. Transl., 150–156).
18 Nath M, Singh H, Eng G, Song X. New diorganotin(IV) derivatives of dipeptides: synthesis and characteristic spectral studies. *Spectrochim. Acta A.* 2008; **71**: 529–536.
19 Kaluderovic GN, Schmidt H, Paschke R, Kalinowski B, Dietrich A, Mueller T, Steinborn D. Platinum(II) complexes with L-methionylglycine and L-methionyl-L-leucine ligands: synthesis, characterization and in vitro antitumoral activity. *J. Inorg. Biochem.* 2007; **101**: 543–549.
20 Nath M, Pokharia S, Eng G, Song XQ, Kumar A. Diorganotin(IV) derivatives of dipeptides containing at least one essential amino acid residue: synthesis, characteristic spectral data, cardiovascular, and anti-inflammatory activities. *Synth. React. Inorg. Met. Org. Chem.* 2004; **34**: 1689–1708.
21 Nath M, Pokharia S, Eng G, Song XQ, Kumar A, Gielen M, Willem R, Biesemans M. New trimethyltin(IV) derivatives of dipeptides: synthesis, characteristic spectral studies and biological activity. *Appl. Organomet. Chem.* 2004; **18**: 460–470.
22 Ligthart-Melis GC, van de Poll MCG, Vermeulen MAR, Boelens PG, van den Tol MP, van Schaik C, de Bandt J-P, Deutz NEP, Dejong CHC, van Leeuwen PAM. Enteral administration of alanyl-[2-^{15}N]glutamine contributes more to the de novo synthesis of arginine than does intravenous infusion of the dipeptide in humans. *Am. J. Clin. Nutr.* 2009; **90**: 95–105.
23 Yagasaki M, Hashimoto S. Synthesis and application of dipeptides; current status and perspectives. *Appl. Microbiol. Biotechnol.* 2008; **81**: 13–22.
24 Müehling J, Burchert D, Langefeld TW, Matejec R, Harbach H, Engel J, Wolff M, Welters ID, Fuchs M, Menges T, Kruell M, Hempelmann G. Pathways involved in alanyl-glutamine-induced changes in neutrophil amino- and alpha-keto acid homeostasis or immunocompetence. *Amino Acids* 2007; **33**: 511–524.
25 Furst P, Kuhn KS. Amino-acid substrates in new bottles: implications for clinical nutrition in the 21st century. *Nutrition* 2000; **16**: 603–606.
26 Ortiz de Urbina JJ, Jorquera F, Culebras JM, Villares C, Gonzalez-Gallego J, Tunon MJ. Effects of parenteral nutrition supplemented with alanyl-glutamine on nutrition status in rats. *J. Parenter. Enteral Nutr.* 2005; **29**: 262–265.
27 Tjader I, Rooyackers O, Forsberg AM, Vesali RF, Garlick PJ, Wernerman J. Effects on skeletal muscle of intravenous glutamine supplementation to ICU patients. *Intensive Care Med.* 2004; **30**: 266–275.
28 Furst P, Alteheld B, Stehle P. Why should a single nutrient - glutamine - improve outcome? The remarkable story of glutamine dipeptides. *Clin. Nutr.* 2004; **1**: 3–15.
29 Wischmeyer PE, Jayakar D, Williams U, Singleton KD, Riehm J, Bacha EA, Jeevanandam V, Christians U, Serkova N. Single dose of glutamine enhances myocardial tissue metabolism, glutathione content, and improves myocardial function after ischemia-reperfusion injury. *J. Parenter. Enteral Nutr.* 2003; **27**: 396–403.
30 Daabees TT, Stegink LD. L-Alanyl-L-tyrosine as a tyrosine source during intravenous nutrition of the rat. *J. Nutr.* 1978; **108**: 1104–1113.
31 Daabees TT, Stegink LD. L-Alanyl-L-tyrosine as a tyrosine source during total parenteral nutrition. Infusion at 0.5 and 2 mmoles/kg/day in adult rats. *Pediatr. Res.* 1979; **13**: 894–899.
32 Park JS, Han TH, Oh JK, Kim SO. Size-dependent isotropic/nematic phase transition behavior of liquid crystalline peptide nanowires. *Macromol. Chem. Phys.* 2009; **210**: 1283–1290.
33 Park JS, Han TH, Oh JK, Kim SO. Morphology control of one-dimensional peptide nanostructures. *J. Nanosci. Nanotechnol.* 2008; **8**: 5547–5550.
34 Görbitz CH. The structure of nanotubes formed by diphenylalanine, the core recognition motif of Alzheimer's β-amyloid polypeptide. *Chem. Commun.* 2006; 2332–2334.
35 Reches M, Gazit E. Casting metal nanowires within discrete self-assembled peptide nanotubes. *Science* 2003; **300**: 625–627.
36 Kushwaha SSS, Katiyar A. Synthesis, characterization and surface-active properties of surfactants having dipeptidyl linkage as hydrophilic group. *Indian J. Chem., Sect A* 2008; **47**: 1818–1822.
37 Chen Y-H, Sung P-H, Sung K. Synthesis of proline-derived dipeptides and their catalytic enantioselective direct aldol reactions: catalyst, solvent, additive and temperature effects. *Amino Acids* 2010; **38**: 839–845.
38 Hammar P, Cordova A, Himo F. Density functional theory study, of the stereoselectivity in small peptide-catalyzed intermolecular aldol reactions. *Tetrahedron Asym.* 2008; **19**: 1617–1621.
39 Lei M, Shi L, Li G, Chen S, Fang W, Ge Z, Cheng T, Li R. Dipeptide-catalyzed direct asymmetric aldol reactions in the presence of water. *Tetrahedron* 2007; **63**: 7892–7898.
40 Tsogoeva S, Jagtap SB. Dual catalyst control in the chiral diamine-dipeptide-catalyzed asymmetric Michael addition. *Synlett* 2004; **14**: 2624–2626.
41 Bodanszky M. Principles of Peptide Synthesis. Springer-Verlag: Berlin, 1993.
42 Lloyd-Williams P, Albericio F, Giralt E. Chemical Approaches to the Synthesis of Peptides and Proteins. CRC Press: New York, 1997.
43 Tsuda Y, Okada Y. Amino Acids, Peptides and Proteins in Organic Chemistry. Building Blocks, Catalysis and Coupling Chemistry, Vol. **3**, Hughes AB (ed.). Wiley-VCH Verlag GmbH & Co. KgaA: Weinheim, 2011.
44 Benoiton NL. Chemistry of Peptide Synthesis. CRC Press (Taylor & Francis Group): New York, 2005.
45 Benoiton NL. Methods of Organic Chemistry. Synthesis of Peptides and Peptidomimetics. Goodman M, Felix A, Moroder L, Toniolo C (eds). Houben-Weyl: New York, 2002.
46 Salam SMA, Kagawa K, Matsubara T, Kawashiro K. Protease-catalyzed dipeptide synthesis from N-protected amino acid carbamoylmethyl esters and free amino acids in frozen aqueous solutions. *Enzyme Microb. Technol.* 2008; **43**: 537–543.
47 Wang N, Huang YB, Xu L, Wu XX, Zhang XZ. Trypsin-catalyzed kinetically controlled synthesis of a precursor dipeptide of thymopentin in organic solvents, using a free amino acid as nucleophile. *Prep. Biochem. Biotechnol.* 2004; **34**: 45–56.
48 Gagnon P, Huang XC, Therrien E, Keillor JW. Peptide coupling of unprotected amino acids through in situ *p*-nitrophenyl ester formation. *Tetrahedron Lett.* 2002; **43**: 7717–7719.
49 Tsogoeva SB, Jagtap SB, Ardemasova ZA, Kalikhevich VN. Trends in asymmetric Michael reactions catalysed by tripeptides in combination with an achiral additive in different solvents. *Eur. J. Org. Chem.* 2004; 4014–4019.
50 Babu VVS, Rao RVR. Microwave irradiated high-speed solution synthesis of peptide acids employing Fmoc-amino acid pentafluorophenyl esters as coupling agents. *Indian J. Chem., Sect B* 2005; **44**: 2328–2332.
51 Meneses C, Nicoli SL, Trembleau L. Multigram-scale synthesis of short peptides via a simplified repetitive solution-phase procedure. *J. Org. Chem.* 2010; **75**: 564–569.
52 Katritzky AR, Meher G, Narindoshvili T. Efficient synthesis of peptides by extension at the N- and C-terminii of arginine. *J. Org. Chem.* 2008; **73**: 7153–7158.
53 Katritzky AR, Todadze E, Angrish P, Draghici B. Efficient peptide coupling involving sterically hindered amino acids. *J. Org. Chem.* 2007; **72**: 5794–5801.
54 Katritzky AR, Todadze E, Cusido J, Angrish P, Shestopalov AA. Selective peptide chain extension at the N-terminus of aspartic and glutamic acids utilizing 1-(*N*-protected-α-aminoacyl)benzotriazoles. *Chem. Biol. Drug Des.* 2006; **68**: 37–41.
55 Katritzky AR, Yoshioka M, Narindoshvili T, Chung A, Khashab NM. *N*-Fmoc-protected(α-dipeptidoyl)benzotriazoles for efficient solid-phase peptide synthesis by segment condensation. *Chem. Biol. Drug Des.* 2008; **72**: 182–188.
56 Katritzky AR, Angrish P, Todadze E, Ghiviriga I. Expedient synthesis of *N*-Z-pyroglutamyl-amino acid derivatives. *Bioorg. Med. Chem. Lett.* 2007; **17**: 6000–6002.
57 Kemp DS, Wang S-W, Rebek JJr, Mollan RC, Banquer C, Subramanyam G. Peptide synthesis with benzisoxazolium salts – II activation chemistry of 2-ethyl-7-hydroxybenzisoxazolium fluoborate; coupling chemistry of 3-acyloxy-2-hydroxy-N-ethylbenzamides. *Tetrahedron* 1974; **30**: 3955–3967.
58 Kemp DS, Wrobel SJJr, Wang S-W, Bernstein Z, Rebek JJr. Peptide synthesis with benzisoxazolium salts – III utility of 7-hydroxy-2-ethylbenzisoxazolium fluoroborate in the synthesis of peptides. *Tetrahedron* 1974; **30**: 3969–3980.
59 Anderson GW, Zimmerman JE, Callahan FM. The use of esters of *N*-hydroxysuccinimide in peptide synthesis. *J. Am. Chem. Soc.* 1964; **86**: 1839–1842.
60 Katoh T, Kurauchi M. Process for the production of glutamine derivatives. *Eur. Pat.* 1989; 311057; *Chem. Abstr.* 1989; **111**: 97736.
61 Nowshuddin S, Reddy AR. Synthesis of dipeptides from *N*-hydroxy-3-azaspiro[5,5]undecane-2,4-dione activated alpha-amino acids. *Tetrahedron Asym.* 2011; **22**: 22–25.
62 Mitin YV. An effective organic solvent system for the dissolution of amino acids. *Int. J. Pept. Protein Res.* 1996; **48**: 374–376.
63 Ryadnov MG, Klimenko LV, Mitin YV. An effective water-free aprotic system for dissolving free amino acids. *Bioorg. Khim.* 1999; **25**: 323–328.

64 Ryadnov MG, Kashparova NY, Kashparov IA, Mitin YV. Synthesis of peptides using free amino acids. The effect of inorganic compounds on the solubility of amino acids in aprotic solvents. *Bioorg. Khim.* 1998; **24**: 408–411.
65 Palomo C, Palomo AL, Palomo F, Mielgo A. Soluble α-amino acid salts in acetonitrile: practical technology for the production of some dipeptides. *Org. Lett.* 2002; **4**: 4005–4008.
66 Nagase T, Fukami T, Urakawa Y, Kumagai U, Ishikawa K. Application of the tetrabutylammonium salt of an amino-acid for liquid-phase peptide-synthesis - an easy way to prepare endothelin antagonistic linear peptides via an azide method. *Tetrahedron Lett.* 1993; **34**: 2495–2496.
67 Chen ST; Wang KT. Phase-transfer reagents as C-terminal protecting groups - facile incorporation of free amino-acids or peptides into peptide sequences. *J. Chem. Soc., Chem. Commun.* 1990; 1045–1047.
68 Andreeva LA, Alfeeva LY, Potaman NN, Nezavibatko VN. Use of tetrabutylammonium salts of amino acids in peptide synthesis. *Int. J. Pept. Protein Res.* 1992; **39**: 493–496.
69 Hashimoto C, Takeguchi K, Kodomari M. An efficient synthetic method of *N*-protected dipeptide acids using amino acid calcium carboxylates in an organic solvent. *Synlett* 2011; **10**: 1427–1430.
70 Anuradha MV, Ravindranath B. Acylation of unprotected amino acids using ultrasound. *Tetrahedron* 1997; **53**: 1123–1130.
71 Brown ZZ, Schafmeister CE. Exploiting an inherent neighboring group effect of α-amino acids to synthesize extremely hindered dipeptides. *J. Am. Chem. Soc.* 2008; **130**: 14382–14383.
72 Noguchi T, Tehara N, Uesugi Y, Jung S, Imai N. Convenient peptide synthesis without protection of C-terminals. *Chem. Lett.* 2012; **41**: 42–43.
73 Tantry SJ, Babu VVS. Synthesis of N^{α}-protected peptide acids by the N→C chain extension employing *O,N-bis*-trimethylsilyl-amino acids using the mixed anhydride method. *Indian J. Chem., Sect B* 2004; **43**: 1282–1287.
74 Chaudhary A, Girgis M, Prashad M., Hu B, Har D, Repič O, Blacklock TJ. Using mixed anhydrides from amino acids and isobutyl chloroformate in *N*-acylations: a case study on the elucidation of mechanism of urethane formation and starting amino acid liberation using carbon dioxide as the probe. *Tetrahedron Lett.* 2003; **44**: 5543–5546.
75 Chen FMF, Lee Y, Steinauer R, Benoiton NL. Mixed anhydrides in peptide synthesis. A study of urethane formation with a contribution on minimization of racemization. *Can. J. Chem.* 1987; **65**: 613–618.
76 Benoiton NL, Lee Y, Chen FMF. Isopropyl chloroformate as a superior reagent for mixed anhydride generation and coupling in peptide synthesis. *Int. J. Protein Res.* 1988; **31**: 577–580.
77 Chen FMF, Benoiton NL. *N,N*-dialkoxycarbonylamino acid formation from a mixed anhydride through intramolecular urethane acylation. *Can. J. Chem.* 1987; **65**: 1228–1229.
78 Joullié MM, Lassen KM. Evolution of amide bond formation. *Arkivoc* 2010; **viii**: 189–250.
79 Slebioda M, St-Amand MA, Chen FMF, Benoiton NL. Studies on the kinetics of racemization of 2,4-disubstituted-5(4*H*)-oxazolones. *Can. J. Chem.* 1988; **66**: 2540–2544.
80 Chen FMF, Benoiton NL, Slebioda M. Mixed carboxylic-carbonic acid anhydrides of acylamino acids and peptides as a convenient source of 2,4-dialkyl-5(4*H*)-oxazolones. *Int. J. Protein Res.* 1988; **31**: 339–344.
81 Chen FMF, Benoiton NL. Diisopropylethylamine eliminates dipeptide formation during the acylation of amino acids using benzoyl chloride and some alkyl chloroformates. *Can. J. Chem.* 1987; **65**: 1224–1227.

