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α-Aminoxy Acids as Building Blocks for the Oxime and Hydroxylamine Pseudopeptide Links. Application to the Synthesis of Human Elastase Inhibitors

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Abstract: The aminoxy acids NH_2–O–C^{α}HR–CO_2H are much more easily obtained in the enantiomerically pure form than the analogous hydrazino acids NH_2–NH–C^{α}HR–CO_2H, and it has been shown that the isosteric amidoxy ψ[CO–NH–O] and hydrazide ψ[CO–NH–NH] amide surrogates induce two quite similar γ-like folded structures. An aminoxy acid can also be *N*-coupled to a peptide aldehyde to give the aldoxime ψ[CH=N–O] link or to a peptide ketone to form the ketoxime ψ[CR=N–O] link. The former can be further reduced into the hydroxylamine ψ[CH_2–NH–O] link which gives rise to reduced amidoxy peptides. The structural properties induced by these amide surrogates were studied, using IR and NMR spectroscopy, paying particular attention to the *Z*/*E*-isomerism of the oxime link. In order to investigate their inhibitory potency, the three amide surrogates were introduced in the Pro^3-Val^4 and Val^4-Ala^5 position of Z-Ala^1-Ala^2-Pro^3-Val^4-Ala^5-Ala^6-NHiPr, a substrate which is cleaved in the Val^4-Ala^5 position by human leukocyte elastase (HLE). The [Val^4ψ[CO–NH–O]Ala^5] analogue was still a substrate, while the [Pro^3ψ[CO–NH–O]Val^4] and [Val^4ψ[CH=N–*O*]Ala^5] pseudopeptides acted as HLE competitive inhibitors. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hydroxylamine peptides; oxime peptides; reduced amidoxy peptides; HLE; elastase; inhibition

INTRODUCTION

The design of amide surrogates as pseudopeptide links provides the opportunity of modulating separately the backbone and the side chains in peptide analogues, and thus of adapting the conformation of the backbone with retention of the side chains which may be essential for bioactivity. For example, the incorporation of an α-hydrazino acid (NH_2–NH–CHR–CO_2H) in a peptide chain results in the hydrazide ψ[CO–NH–NH] link which has been shown to induce a very stable γ-like turn with a bifurcate *H*-bond involving both the hydrazide carbonyl and the α-nitrogen lone-pair [1,2]. However, both the synthesis and regioselective coupling of optically pure hydrazino acids pose various difficulties [3–6].

It has been shown that the isosteric amidoxy ψ[CO–NH–O] and hydrazide ψ[CO–NH–NH] links have the same structural properties and stabilize quite similar γ-like folded structures [7–11]. Moreover, the synthesis of optically pure α-aminoxy acids (NH_2–O–CHR–CO_2H) from amino acids is well documented and easier than that of the α-hydrazino

Abbreviations: α_1-PI, α_1-protease inhibitor; HLE, human leukocyte elastase.

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acids [12,13]. Therefore, the possibility offered by the α-aminoxy acids for the design of other amide surrogates, and the structural properties of the resulting pseudopeptide fragments were investigated. For example, an α-aminoxy group can be coupled to an aldehyde or a ketone to create the aldoxime ψ[CH=N–O] or ketoxime ψ[CR=N–O] link, respectively [11]. The oxime junction ψ[CH=N–O] is commonly used for ligation of bioactive peptides to carriers [14–18] but has not been considered as a possible amide surrogate in pseudopeptide analogues yet. Furthermore, the double bond in the oxime group can be reduced to give an hydroxylamine [19], and it is proposed to call the resulting ψ[CH_2–NH–O] a reduced amidoxy link with reference to the so-called reduced amide link ψ[CH_2–NH]. The ψ[CH=N–O], ψ[CR=N–O] and ψ[CH_2–NH–O] surrogates have been introduced in the middle position of model dipeptides having the general formula RCO-Xaa-Xbb-NHR'. The resulting pseudopeptides have been investigated by IR and NMR spectroscopy in order to determine the structural properties induced by these amide surrogates, and especially the *Z*/*E*-isomerism of the oxime link.

Elastin degradation by elastase, a serine protease, is regulated in humans by the plasma inhibitor α_1-PI, but a functional or genetic deficiency of α_1-PI may induce various chronic inflammatory diseases such as pulmonary emphysema [20]. Various hydrazide analogues of the human leukocyte elastase (HLE) substrate Z-Ala^1-Ala^2-Pro^3-Val^4-Ala^5-Ala^6-NHiPr have been investigated for potential HLE inhibition. While the [Val^{4}ψ[CO–NH–NH]Ala^5] hydrazide link is cleaved, the [Pro^{3}ψ[CO–NH–NH]Val^4] and [Ala^{2}ψ[CO–NH–NH]Pro^3] analogues act as competitive HLE inhibitors [6,21]. In order to test their properties as potential, non-cleavable amide surrogates, the ψ[CO–NH–O], ψ[CH=N–O] and ψ[CH_2–NH–O] links have been substituted for the amide bond in position Pro^3-Val^4 and Val^4-Ala^5 of Z-Ala^1-Ala^2-Pro^3-Val^4-Ala^5-Ala^6-NHiPr. The six pseudohexapeptides have been obtained and tested for their recognition by HLE and their inhibition properties.

MATERIALS AND METHODS

Synthesis of the Oxime and Reduced Amidoxy Dipeptides

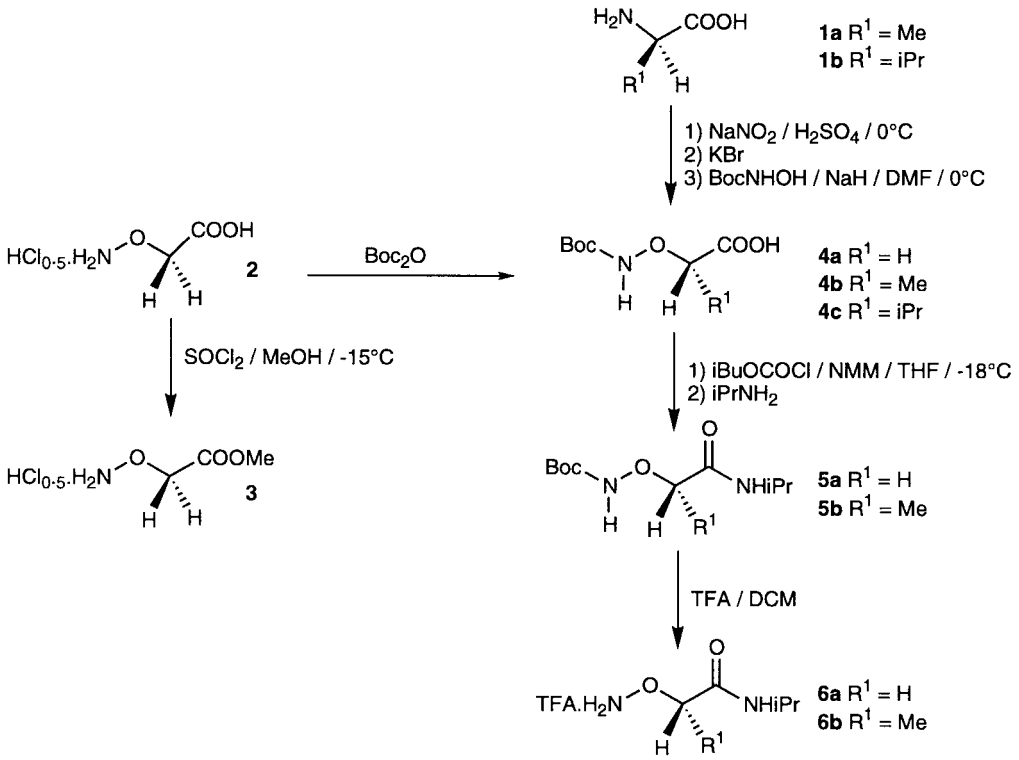
The α-aminoxy acids have been isolated from natural products having antibacterial properties in some cases [22–24]. The synthesis of an α-aminoxy acid was first reported in 1893 [25,26], and reinvestigated in the early 1960s [27–31]. Later, *N*-protected α-aminoxy acid derivatives were prepared by action of the sodium salt of an *N*-protected hydroxylamine with an α-bromo acid, obtained *via* HNO_2 deamination of the corresponding α-amino acid [13,22,31]. It is to be noted that the chirality of the α-carbon of the α-amino acid is inverted. The procedure after Briggs and Morley [13] has been used for the preparation of the (*S*)–Boc–NH–O–CHR^1–CO_2H derivatives with R^1 = Me **4b** from D-alanine or R^1 = iPr **4c** from D-valine (Scheme 1). Boc–NH–O–CH_2–CO_2H **4a** (now commercially available from Novabiochem) was obtained from $HCl_{0.5}$.H_2N–O–CH_2–CO_2H **2** by treatment with Boc_2O (Scheme 1). The corresponding isopropylamides **5a** [11] and **5b** were prepared using the classical mixed anhydride with ICF.

(S)-Boc-NH–O–CHMe–CO_2H (4b). (53% yield from **1a**). White powder M.p.: 91 °C; *m*/*z* [ES] calcd 205.1, found 204.1 for $[M-H]^-$. ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 1.51 (s, 9H, Boc-(CH_3)$_3$), 1.53 (d, 3H, *J* = 8.4 Hz, C$^\beta H_3$), 4.47 (q, 1H, *J* = 8.4 Hz, C$^\alpha H$), 7.67 (s, 1H, N*H*).

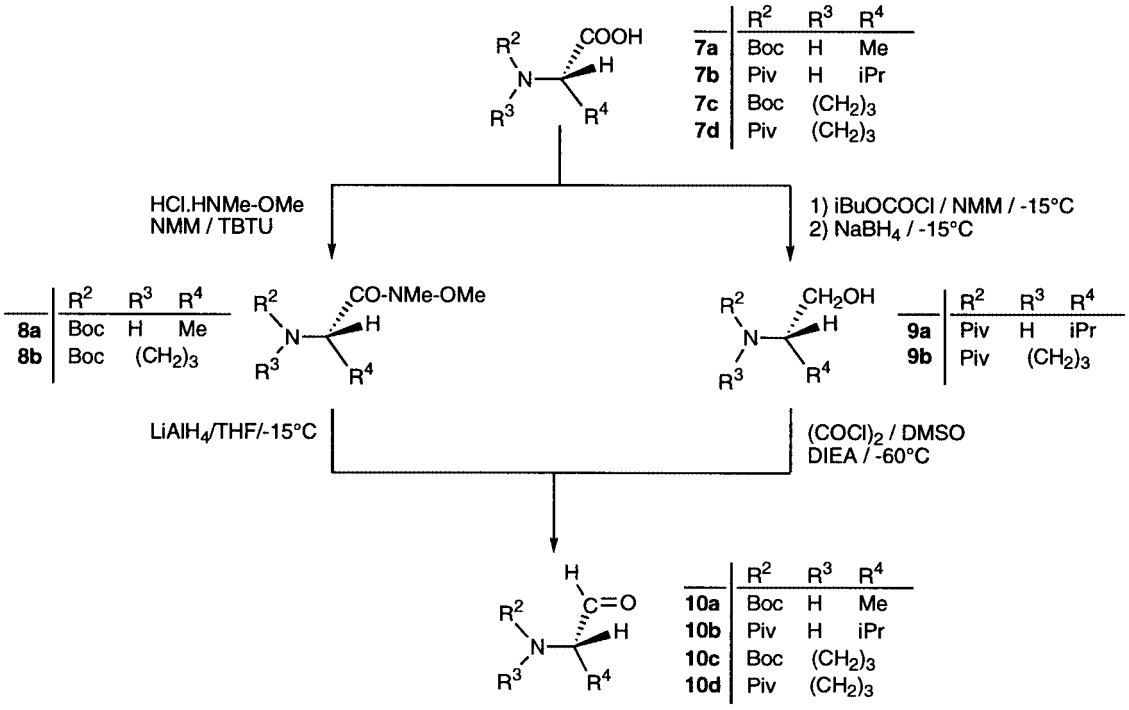
(S)-Boc-NH–O–CHiPr–CO_2H (4c). (26% yield from **1b**). Gummy solid; *m*/*z* [ES] calcd 233.1, found 232.1 for $[M-H]^-$. ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 0.98 and 1.09 (2d, 6H, *J* = 6.9 Hz and *J* = 7.3 Hz, (C$^\gamma H_3$)$_2$), 1.50 (s, 9H, Boc-(CH_3)$_3$), 2.31 (m, 1H, C$^\beta H$), 4.20 (d, 1H, *J* = 3.7 Hz, C$^\alpha H$), 7.74 (s, 1H, N*H*).

(S)-Boc-NH–O–CHMe–CONHiPr (5b). (71% yield). White powder M.p.: 86 °C; *m*/*z* [ES] calcd 246.16, found 247.1 for $[M+H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 1.16 and 1.19 (2d, 6H, *J* = 6.3 Hz, iPr-(CH_3)$_2$), 1.42 (d, 3H, *J* = 7.0 Hz, C$^\beta H_3$), 1.48 (s, 9H, Boc-(CH_3)$_3$), 4.08 (m, 1H, iPr-C*H*), 4.23 (q, 1H, *J* = 7.0 Hz, C$^\alpha H$), 7.33 (s, 1H, N*HO*), 7.71 (b, 1H, N*H*).

The optically pure *N*-protected-α-amino aldehydes are commonly obtained by reduction of the Weinreb *N,O*-dimethylhydroxamate [32,33], or more recently of the UNCA [34] or morpholinamide [35] with $LiAlH_4$. The Weinreb procedure was applied with success to the synthesis of the Boc-aldehydes Boc-Ala-H **10a** and Boc-Pro-H **10c** (Scheme 2), but failed to give the Piv-aldehydes Piv-Val-H **10b** and Piv-Pro-H (**10d**) in good yield. Therefore another procedure was used, compatible with most usual *N*-protections [36]: the mixed anhydride of Piv-Val-OH **7b** or Piv-Pro-OH **7d** with ICF was first reduced with $NaBH_4$ at −15 °C into the Piv-β-amino alcohol



Scheme 1 Synthesis of the α-aminoxy acid derivatives.



Scheme 2 Synthesis of the α-amino aldehydes.

9a,b [37] which was then oxidized with Swern reagent (DMSO/$(COCl)_2$ mixture) in the presence of DIEA [38] to give Piv-Val-H **10b** and Piv-Pro-H **10d** (Scheme 2). Due to easy epimerization, aldehydes **10a–d** were prepared just before use without further purification.

According to the procedure recommended by Rodriguez *et al.* [37], to the Piv-α-amino acid (10 mmol) in DME (10 ml) at −15 °C was successively added dropwise NMM (1.10 ml, 10 mmol) and ICF (1.30 ml, 10 mmol). The NMM salt was filtered off 1 min later and washed with DME (2 ml, 5 times). The organic phases were collected and cooled to 0 °C. $NaBH_4$ (0.57 g, 15 mmol) in water (5 ml) was added in one portion, and additional water (250 ml) was immediately added. The β-amino alcohol was extracted with DCM (20 ml, 5 times), and the organic phase was successively washed with 5% aqueous $KHSO_4$ (15 ml, 3 times), 5% aqueous $KHCO_3$ (15 ml, 3 times), and NaCl saturated water (15 ml, 3 times), and then dried over $MgSO_4$. DCM was evaporated under reduced pressure, and the β-amino alcohol was oxidized without further purification. The β-amino alcohol in DCM (25 ml) was added dropwise under a stream of N_2 to the Swern reagent at −60 °C (DMSO (1.06 ml, 15 mmol) added to oxalyl chloride (0.65 ml, 7.5 mmol) in DCM (12.5 ml) at −60 °C) [36]. The mixture was stirred for 15 min at −60 °C, and DIEA (5.13 ml, 30 mmol) was added to the mixture, which was allowed to reach room temperature. Water (125 ml) was added, and stirring was maintained for 10 min. The Piv-α-amino aldehyde was extracted with DCM (30 ml, 3 times). The organic layer was washed with 5% aqueous $KHSO_4$ (15 ml, 3 times), NaCl saturated water (15 ml, 3 times), and dried over $MgSO_4$. DCM was evaporated under reduced pressure, and the Piv-α-amino aldehyde was used without further purification.

Piv-L-Val-H (10b). (30% yield). Colourless oil. ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 0.99 (2d, 6H, $J = 7$ Hz, $(C^{\gamma}H_3)_2$), 1.25 (s, 9H, Piv-$(CH_3)_3$), 2.29 (m, 1H, $C^{\beta}H$), 4.23 (m, 1H, $C^{\alpha}H$), 6.65 (b, 1H, N*H*), 9.66 (s, 1H, C*H*O).

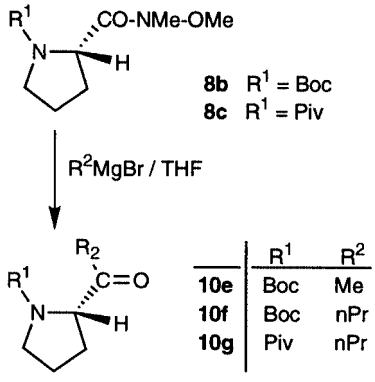
Piv-L-Pro-H (10d). (35% yield). Colourless oil; ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 1.29 (s, 9H, Piv-$(CH_3)_3$), 1.84–1.99 (m, 4H, $C^{\beta}H_2 + C^{\gamma}H_2$), 3.75 (m, 2H, $C^{\delta}H_2$), 4.44 (b, 1H, $C^{\alpha}H$), 9.45 (d, 1H, $J = 2.4$ Hz, C*H*O).

Enantiomerically pure α-amino ketones are starting molecules for obtaining chiral β-amino secondary alcohols with good diastereomeric excess [39]. Most of the procedures are based on the nucleophilic addition to carboxylic derivatives of organometallic reagents, such as organozinc reagents with thiol esters [40], Grignard reagents with Weinreb amides [32] and organolithium reagents with morpholine amides [41]. The Weinreb procedure was applied for introducing the methyl and *n*-propyl groups on proline, so obtaining the α-amino ketones Boc-Pro-Me **10e**, Boc-Pro-nPr **10f** and Piv-Pro-nPr **10g** (Scheme 3). α-Amino ketones are not stable enough to be stored and were prepared just before use.

To the *N*-Boc-Weinreb amide (10 mmol) in THF (50 ml) at −15 °C was added dropwise the Grignard reagent (15 mmol) in THF. Stirring was maintained at −15 °C for 2 h, and a cold 5% $KHSO_4$ aqueous solution (50 ml) was added. The organic phase was concentrated under reduced pressure, the residue taken up with DCM (80 ml), and washed with 5% aqueous $KHSO_4$ (15 ml, 3 times), 5% aqueous $KHCO_3$ (15 ml, 3 times) and NaCl saturated water (15 ml, 3 times). The organic phase was dried over $MgSO_4$, evaporated under reduced pressure, and **10e and 10f** were purified by silica gel chromatography just before use. **10g** is not stable enough to be purified.

Boc-Pro-Me (10e). R_f (EtOAc/hexanes 70/30) = 0.70 (42% yield). Colourless oil; ^{1}H-NMR (200 MHz) in $CDCl_3$, two conformers A/B 2/1, δ ppm: 1.41(A) and 1.45(B) (2s, 9H, Boc-$(CH_3)_3$), 1.85(A + B) (m, 3H, $C^{\beta}H + C^{\gamma}H_2$), 2.12(A) and 2.16(B) (2s, 3H, $COCH_3$), 2.16 (A + B) (m, 1H, $C^{\beta}H$), 3.52(A + B) (m, 2H, $C^{\delta}H_2$), 4.18(A) and 4.32(B) (2m, 1H, $C^{\alpha}H$).

Boc-Pro-nPr (10f). R_f (EtOAc/hexanes 70/30) = 0.83 (19% yield). Colourless oil; *m/z* [ES] calcd 241.17, found 240.1 for $[M - H]^-$. ^{1}H-NMR (200 MHz) in $CDCl_3$, two conformers A/B 2/1, δ



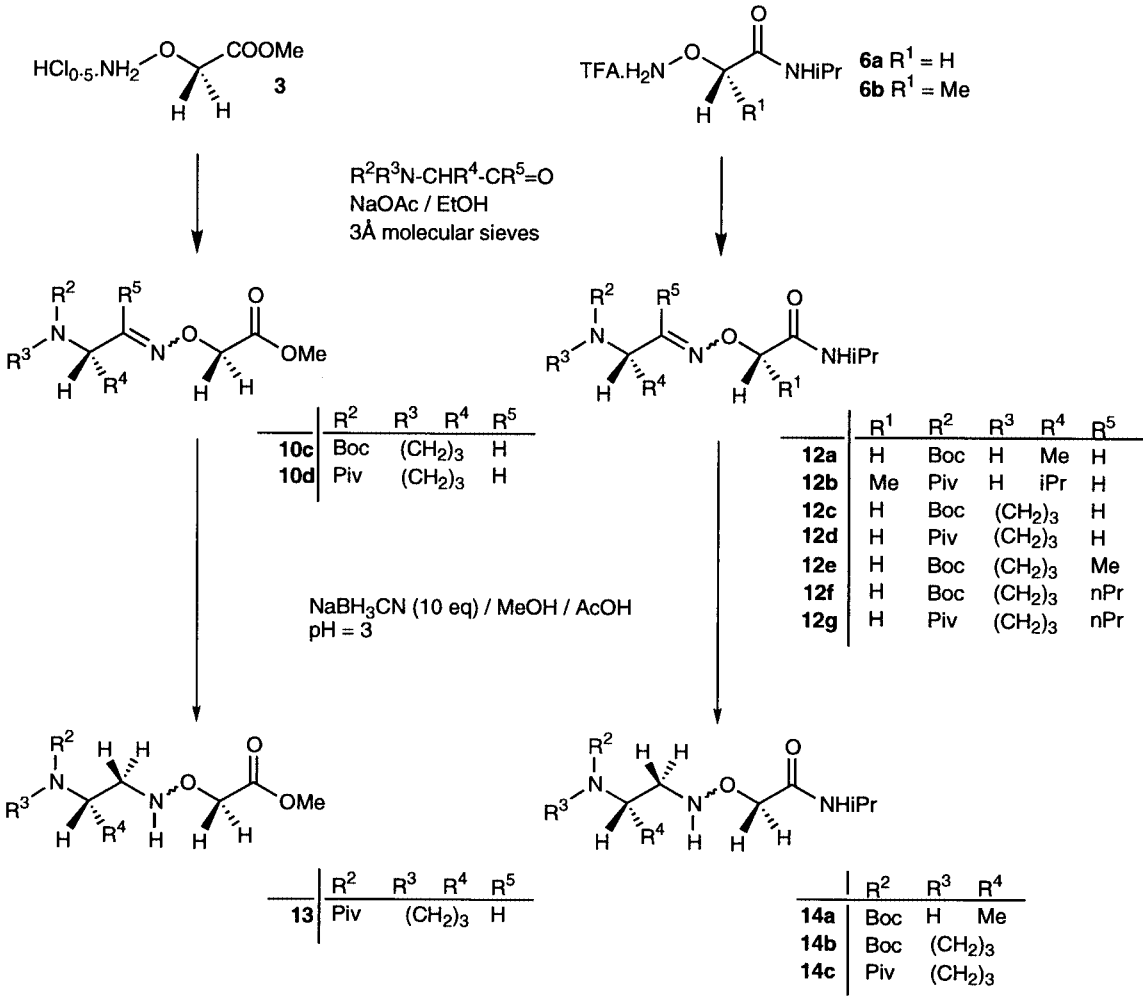
	R^1	R^2
10e	Boc	Me
10f	Boc	nPr
10g	Piv	nPr

Scheme 3 Synthesis of the α-amino ketones.

ppm: 0.92(A + B) (m, 3H, nPr-CH_3), 1.40(A) and 1.45(B) (2s, 9H, Boc-(CH_3)$_3$), 1.61(A + B) (m, 2H, nPr-CH_2), 1.83–2.16(A + B) (m, 4H, C$^{\beta}H_2$ + C$^{\gamma}H_2$), 2.44(A + B) (m, 2H, COCH_2), 3.50(A + B) (m, 2H, C$^{\delta}H_2$), 4.23(A) and 4.35(B) (2m, 1H, C$^{\alpha}H$).

The aminoxy derivatives **3, 6a** and **6b** were coupled to the aldehyde or ketone derivatives **10a–g** (Scheme 4) to give the oxime link ψ[C=N–O] under the same conditions as those already used for obtaining the semicarbazone pseudopeptide fragment C$^{\alpha}$–CH=N–N$^{\alpha}$R–CO–NH–C$^{\alpha}$ [42]. The aminoxy terminus in **3, 6a** and **6b** was regenerated from the HCl and TFA salt with NMM, and added to a twofold excess of the carbonyl derivative in EtOH containing four equivalents of NaOAc. The equilibrium was displaced to a moderate to good overnight yield by trapping water with molecular sieves. Ketones required larger reaction times and resulted in lower yields than aldehydes. The organic solution was washed three times with 5% aqueous $KHSO_4$, 5% aqueous $KHCO_3$ and NaCl saturated water, and then dried over $MgSO_4$. The solvent was evaporated under reduced pressure, and the oxime derivative was purified by silica gel chromatography.

The oxime peptide actually accommodates two different conformations arising from the *Z*/*E* isomerism of the imine bond, and it was noticed that the *Z*/*E* ratio for the Piv-oxime dipeptides in some cases depended on the synthesis procedure, and essentially on the fact that strong acidic conditions had been used or not (see below). For this reason, the Piv-α-amino oxime peptides **11d, 12b, 12d** and **12g** were obtained in two ways: (i) from the Piv-α-amino aldehyde **10b** and **10d** or ketone **10g** using the same procedure as above and (ii) from the corresponding Boc-derivatives **11c, 12c** and **12f** by



	R2	R3	R4	R5
10c	Boc	(CH2)3	H	
10d	Piv	(CH2)3	H	

	R1	R2	R3	R4	R5
12a	H	Boc	H	Me	H
12b	Me	Piv	H	iPr	H
12c	H	Boc	(CH2)3		H
12d	H	Piv	(CH2)3		H
12e	H	Boc	(CH2)3		Me
12f	H	Boc	(CH2)3		nPr
12g	H	Piv	(CH2)3		nPr

	R2	R3	R4	R5
13	Piv	(CH2)3	H	

	R2	R3	R4
14a	Boc	H	Me
14b	Boc	(CH2)3	
14c	Piv	(CH2)3	

Scheme 4 Synthesis of the oxime and hydroxylamine dipeptides.

classical Boc/Piv exchange (Scheme 5) using TFA in DCM (40/60) for Boc elimination and pivaloyl chloride. The Piv-derivatives were purified by silica gel chromatography.

Boc-Proψ(CH=N–O)Gly-OMe 11c. R_f (EtOAc/hexanes 70/30) = 0.77 (53% yield). Colourless oil; *m/z* [ES] calcd 286.15, found 309.14 for $[M + Na]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, major *E*-conformer, δ ppm: 1.44 (s, 9H, Boc-(CH_3)$_3$), 1.86–2.03 (m, 4H, $C^\beta H_2 + C^\gamma H_2$), 3.40 (m, 2H, $C^\delta H_2$), 3.76 (s, 3H, OCH_3), 4.33 (m, 1H, $C^\alpha H$), 4.58 (s, 2H, OCH_2), 7.42 (d, 1H, J = 5.0 Hz, =C*H*).

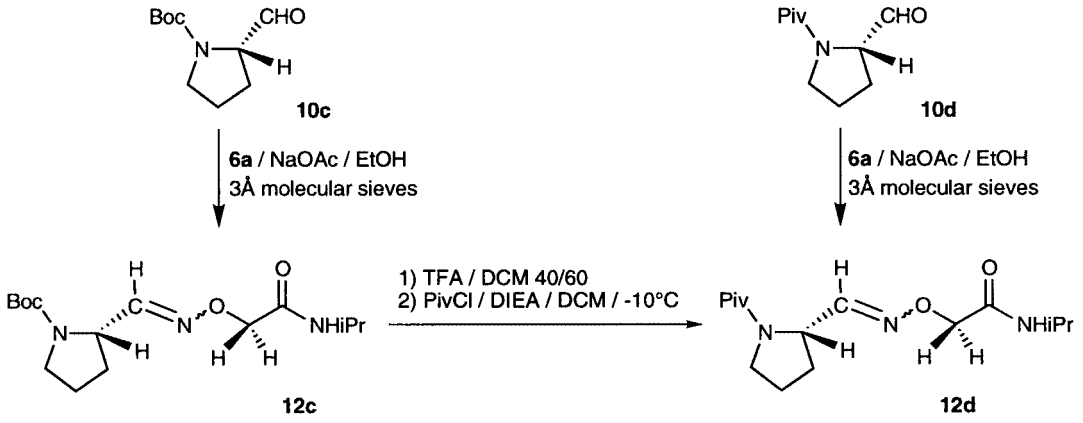
Piv-Proψ(CH=N–O)Gly-OMe 11d. R_f (EtOAc/hexanes 70/30) = 0.63 (81% yield). Yellowish oil; *m/z* [ES] calcd 270.16, found 271.15 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, two isomers *Z/E*, δ ppm: 1.26(*Z* + *E*) (s, 9H, Piv-(CH_3)$_3$), 1.96–2.20(*Z* + *E*) (m, 4H, $C^\beta H_2 + C^\gamma H_2$), 3.65(*Z* + *E*) (m, 2H, $C^\delta H_2$), 3.75(*Z* + *E*) (s, 3H, OCH_3), 4.56(*Z* + *E*) (s, 2H, OCH_2), 4.79(*E*) and 5.06(*Z*) (2m, 1H, $C^\alpha H$), 6.65(*Z*) (d, 1H, J = 5.5 Hz, =CH), 7.42(*E*) (d, 1H, J = 4.6 Hz, =C*H*).

Boc-Alaψ(CH=N–O)Gly-NHiPr 12a. R_f (EtOAc/hexanes 70/30) = 0.55 (35% yield). Colourless oil; *m/z* [ES] calcd 287.18, found 288.15 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, two isomers *Z/E* 1/2, δ ppm: 1.19(*Z* + *E*) (d, 6H, J = 6.6 Hz, iPr-(CH_3)$_2$), 1.24(*Z*) and 1.31(*E*) (2d, 3H, J = 6.6 Hz and J = 6.9 Hz, $C^\beta H_3$), 1.45(*Z* + *E*) (s, 9H, Boc-(CH_3)$_3$), 4.17(*Z* + *E*) (m, 1H, iPr-C*H*), 4.35(*E*) and 4.95(*Z*) (2m, 1H, $C^\alpha H$), 4.48(*E*) (s, 2H, OCH_2), 4.52(*Z*) and 4.63 (*Z*) (AB, 2H, J = 15.9 Hz, OCH_2), 4.68(*Z*) and 4.86(*E*) (2b, 1H, N*H*), 6.02(*E*) and 6.98(*Z*) (2b, 1H, N*H*iPr), 6.54(*Z*) (d, 1H, J = 7.4 Hz, =CH), 7.52(*E*) (d, 1H, J = 4.1 Hz, =C*H*).

Piv-Valψ(CH=N–O)Ala-NHiPr 12b. R_f (EtOAc/hexanes 70/30) = 0.62 (35% yield). Gummy solid; *m/z* [ES] calcd 313.24, found 314.22 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, two isomers *Z/E*, δ ppm: 0.91(*Z* + *E*) (d, 3H, J = 6.7 Hz, $C^\gamma H_3$), 0.98(*Z* + *E*) (d, 3H, J = 6.8 Hz, $C^\gamma H_3$), 1.16(*Z* + *E*) (m, 6H, iPr-(CH_3)$_2$), 1.20(*Z* + *E*) (s, 9H, Piv-(CH_3)$_3$), 1.42(*E*) (d, 3H, J = 7.2 Hz), OCH(CH_3), 1.48(*Z*) (d, 3H, J = 6.6 Hz), OCH(CH_3), 1.90(*Z*) and 2.09(*E*) (2m, 1H, $C^\beta H$), 4.11(*Z* + *E*) (m, 1H, iPr-C*H*), 4.56(*E*) and 4.89(*Z*) (2m, 1H, $C^\alpha H$), 4,64 (*Z* + *E*) (m, 1H, OC*H*, 5.77(*Z*) (d, 1H, J = 7.7 Hz, Piv-N*H*), 5.89(*E*) (d, 1H, J = 7.9 Hz, N*H*iPr), 6.12(*E*) (d, 1H, J = 7.6 Hz, Piv-N*H*), 6.65(*Z*) (d, 1H, J = 6.6 Hz, =CH), 6.83(*Z*) (b, 1H, N*H*iPr), 7.54(*E*) (d, 1H, J = 3.7 Hz, =CH).

Boc-Proψ(CH=N–O)Gly-NHiPr 12c. R_f (EtOAc/hexanes 70/30) = 0.53 (82% yield). Colourless oil; *m/z* [ES] calcd 313.39, found 314.16 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, major *E*-conformer, δ ppm: 1.10 (d, 6H, J = 6.6 Hz, iPr-(CH_3)$_2$), 1.38 (s, 9H, Boc-(CH_3)$_3$), 1.81–1.95 (m, 4H, $C^\beta H_2 + C^\gamma H_2$), 3.32 (m, 2H, $C^\delta H_2$), 4.06 (m, 1H, iPr-C*H*), 4.31 (m, 1H, $C^\alpha H$), 4.39 (s, 2H, OCH_2), 6.03 (b, 1H, N*H*), 7.40 (b, 1H, =C*H*).

Piv-Proψ(CH=N–O)Gly-NHiPr 12d. R_f (EtOH/EtOAc/hexanes 10/60/30) = 0.67 (77% yield). Gummy solid; *m/z* [ES] calcd 297.21, found 298.2 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, two isomers *Z/E*, δ ppm: 1.17(*Z* + *E*) (d, 6H, J = 6.6 Hz, iPr-(CH_3)$_2$), 1.26(*E*) and 1.28(*Z*) (2s, 9H, Piv-(CH_3)$_3$), 1.98–2.11(*Z* + *E*) (m, 4H, $C^\beta H_2 + C^\gamma H_2$), 3.68(*E*) and 3.77(*Z*) (2m, 2H, $C^\delta H_2$), 4,14(*E*) and 4.27(*Z*) (2m, 1H, iPr-C*H*), 4.45(*E*) (s, 2H, OCH_2), 4.61(*Z*) and 4.72 (*Z*) (AB, 2H, J = 16.8 Hz, OCH_2), 4.73(*E*) and 5.28(*Z*) (2m, 1H, $C^\alpha H$), 6.06(*E*) and 7.53(*Z*) (2b, 1H, N*H*iPr),



Scheme 5 Synthesis of the N-Piv-protected oxime dipeptide **12d** by two procedures with or without acidic treatment.

6.45(*Z*) (d, 1H, $J = 7.9$ Hz, =CH), 7.56(*E*) (d, 1H, $J = 4.5$ Hz, =C*H*).

Boc-Proψ(C(Me)=N–O)Gly-NHiPr 12e. R_f (EtOAc/hexanes 70/30) = 0.44 (32% yield). Colourless oil; *m/z* [ES] calcd 327.22, found 327.52 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, major *E*-conformer, δ ppm: 1.17 (d, 6H, $J = 6.5$ Hz, iPr (CH_3)$_2$), 1.42 (s, 9H, Boc-(CH_3)$_3$), 1.80 (s, 3H, (CH_3)C=), 1.81–2.06 (m, 4H, $C^\beta H_2 + C^\gamma H_2$), 3.38–3.55 (m, 2H, $C^\delta H_2$), 4.15 (m, 1H, iPr-C*H*), 4.29 (m, 1H, $C^\alpha H$), 4.46 (s, 2H, OCH_2), 6.00–6.15 (b, 1H, N*H*).

Boc-Proψ(C(Pr)=N–O)Gly-NHiPr 12f. R_f (EtOAc/hexanes 70/30) = 0.71 (43% yield). Colourless oil; *m/z* [ES] calcd 355.47, found 356.26 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, major *E*-conformer, δ ppm: 1.00 (t, 3H, $J = 7.3$ Hz, Pr-(CH_3)), 1.17 (d, 6H, $J = 6.5$ Hz, iPr (CH_3)$_2$), 1.44 (s, 9H, Boc-(CH_3)$_3$), 1.58–2.30 (m, 8H, $C^\beta H_2 + C^\gamma H_2$ + Pr(CH_2)$_2$), 3.41(m, 2H, $C^\delta H_2$), 4.14 (m, 1H, iPr-C*H*), 4.37 (m, 1H, $C^\alpha H$), 4.45 (s, 2H, OCH_2), 6.01–6.13 (b, 1H, N*H*).

Piv-Proψ(C(Pr)=NO)Gly-NHiPr 12g. R_f (EtOAc/hexanes 70/30) = 0.49 (68% yield). Colourless oil; *m/z* [ES] calcd 339.25, found 345.53 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, two isomers *Z*/*E* δ ppm: 0.89(*Z*) and 1.00(*E*) (2t, 3H, $J = 7.3$ Hz, Pr-(CH_3)), 1.16(*E*) and 1.21(*Z*) (2d, 6H, $J = 6.5$ Hz, iPr-(CH_3)$_2$), 1.22(*E*) and 1.27(*Z*) (2s, 9H, Piv-(CH_3)$_3$), 1.54–2.45(*Z* + *E*) (m, 8H, $C^\beta H_2 + C^\gamma H_2$ + Pr-(CH_2)$_2$), 3.53–3.93(*Z* + *E*) (m, 2H, $C^\delta H_2$), 4.09 (*E*) and 4.27(*Z*) (2m, 1H, iPr-C*H*), 4.40(*E*) (s, 2H, OCH_2), 4.55(*Z*) and 4.66(*Z*) (AB, 2H, $J = 16.6$ Hz, OCH_2), 4.66(*E*) and 5.44(*Z*) (2m, 1H, $C^\alpha H$), 6.02(*E*) and 7.74 (*Z*) (2b, 1H, N*H*).

Various catalytic hydrogenation or reductive conditions have been tested for reducing the oxime ψ[CH=N–O] into the hydroxylamine ψ[CH_2–NH–O] link. The standard conditions (2 eq $NaBH_3CN$/MeOH/1% AcOH/12 h) [43] for reductive amination of the peptide aldehydes giving the so-called reduced amide bond ψ[CH_2–NH] was found to be inefficient. Catalytic hydrogenation (H_2/Pd), which is known to give good results for the reduction of semicarbazones into semicarbazides [44–46], cleaved the N–O bond. The same held true with $NaBH_4$, whereas the milder reducing agent $NaBH(OAc)_3$ [47] was inefficient. Satisfactory results were actually obtained using $NaBH_3CN$ under the following conditions. The oxime peptide (2 mmol) in MeOH (10 ml) was treated portionwise under stirring at room temperature for 6 days with $NaBH_3CN$ (1.26 g, 20 mmol) while the pH is continuously adjusted to 3 by the addition of AcOH. A 5% aqueous K_2CO_3 solution (20 ml) was added, and the reduced amidoxy peptide was extracted with DCM (3 × 10 ml). The organic phase was washed with NaCl saturated water (5 ml) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure, and the reduced amidoxy peptide was purified by silica gel chromatography.

Piv-Pro ψ(CH$_2$–NH–O)Gly-OMe 13. R_f (EtOAc/hexanes 70/30) = 0.32 (48% yield). Colourless oil; *m/z* [ES] calcd 272.17, found 273.43 for $[M + H]^+$. ^{1}H–NMR (200 MHz) in $CDCl_3$, δ ppm: 1.24 (s, 9H, Piv-(CH_3)$_3$), 1.88 (m, 4H, $C^\beta H_2 + CH_2$), 2.94 (ABX, 1H, $J = 12.8$ and 6.9 Hz, C*H*N), 3.20 (ABX, 1H, $J = 12.8$ and 4.1 Hz, C*H*N), 3.50 and 3.69 (2m, 2H, $C^\delta H_2$), 3.74 (s, 3H, OCH_3), 4.25 (s, 2H, OCH_2), 4.30 (m, 1H, $C^\alpha H$).

Boc-Ala ψ(CH$_2$–NH–O)Gly-NHiPr 14a. R_f (EtOAc/hexanes 70/30) = 0.22 (50% yield). Colourless oil; *m/z* [ES] calcd 289.2, found 290.43 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 1.16 (m, 9H, iPr-(CH_3)$_2$ + $C^\beta H_3$), 1.43 (s, 9H, Boc-(CH_3)$_3$), 2.83 (ABX, 1H, $J = 13.5$ and 7.6 Hz, C*H*N), 3.00 (ABX, 1H, $J = 13, 5$ and 4.5 Hz, C*H*N), 3.94 (m, 1H, $C^\alpha H$), 4.09 (m, 1H, iPr-C*H*), 4.14 (s, 2H, OCH_2), 4.59 (d, 1H, $J = 8.0$ Hz, N*H*), 6.47 (b, 1H, N*H*iPr).

Boc-Pro ψ(CH$_2$–NH–O)Gly-NHiPr 14b. R_f (EtOH/EtOAc/hexanes 10/60/30) = 0.60 (18% yield). Colourless oil; *m/z* [ES] calcd 315.22, found 315.51 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in DMSO-d_6, δ ppm: 1.20(d, 6H, $J = 6.6$ Hz, iPr-(CH_3)$_2$), 1.41 (s, 9H, Boc-(CH_3)$_3$), 1.79 (m, 4H, $C^\beta H_2 + C^\gamma H_2$), 2.64 (m, 1H, $J = 12.9$, 8.8 and 5.1 Hz, C*H*N), 3.07 (m, 1H, $J = 12.9$, 3.3 and 6.6 Hz, C*H*N), 3.21 (m, 2H, $C^\delta H_2$), 3.88 (m, 2H, $C^\alpha H$ + iPr-C*H*), 3.94 (s, 2H, OCH_2), 6.97 (b, 1H, N*H*O), 7.45 (d, 1H, $J = 7.5$ Hz, N*H*iPr).

Piv-Pro ψ(CH$_2$–NH–O)Gly-NHiPr 14c. R_f (EtOH/EtOAc/hexanes 10/60/30) = 0.45 (22% yield). Gummy solid; *m/z* [ES] calcd 299.22, found 300.4 for $[M + H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 1.20 (d, 6H, $J = 6.6$ Hz, iPr-(CH_3)$_2$), 1.26 (s, 9H, Piv-(CH_3)$_3$), 1.92 (m, 4H, $C^\beta H_2 + C^\gamma H_2$), 2.87 (ABX, 1H, $J = 13.4$ and 6.0 Hz, C*H*N), 3.16 (ABX, 1H, $J = 13.4$ and 4.8 Hz, C*H*N), 3.55–3.70 (m; 2H, $C^\delta H_2$), 4.17 (s, 2H, OCH_2), 4.17 (m, 1H, iPr-C*H*), 4.43 (m, 1H, $C^\alpha H$), 6.90 (b, 1H, N*H*).

In order to determine the eventual interactions between the amide group and the oxime or hydroxylamine link, the two model molecules tBu-CH=N–O–CH_2–CONHiPr **15** and tBu-CH_2–

NHO–CH_2–CO–NHiPr **16** were prepared using the afore-mentioned procedures (Scheme 6). The aminoxy link was also suppressed, but retained an oxygen atom, in MeO–CH_2–CO–NHiPr **17** [46].

tBuCH=N-O-CH₂-CO-NHiPr 15. R_f (EtOAc/hexanes 50/50) = 0.40 (79% yield). Yellowish oil; *m/z* [ES] calcd 200.15, found 201.43 for $[M+H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, major *E*-conformer, δ ppm: 1.07 (s, 9H, tBu-(CH_3)$_3$), 1.13 (d, 6H, $J = 6.6$ Hz, iPr-(CH_3)$_2$), 4.11 (m, 1H, iPr-C*H*), 4.41 (s, 2H, CH_2), 6.05 (b, 1H, N*H*iPr), 7.41 (s, 1H, =C*H*).

tBuCH₂-NH-O-CH₂-CO-NHiPr 16. R_f (EtOAc/hexanes 50/50) = 0.58 (60% yield). Gummy solid; *m/z* [ES] calcd 202.17, found 203.23 for $[M+H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 0.93 (s, 9H, tBu-(CH_3)$_3$), 1.18 (d, 6H, $J = 6.2$ Hz, iPr-(CH_3)$_2$), 2.77 (s, 2H, CH_2N), 4.13 (m, 1H, iPr-C*H*), 5.79 (s, 1H, N*H*O), 7,45 (b, 1H, N*H*iPr).

MeO-CH₂-CO-NHiPr 17. [46]. *m/z* [ES] calcd 131.09, found 132.14 for $[M+H]^+$. ^{1}H-NMR (200 MHz) in $CDCl_3$, δ ppm: 1.19 (d, 6H, $J = 6.5$ Hz, iPr-(CH_3)$_2$), 3.42 (s, 3H, OCH_3), 3.87 (s, 2H. CH_2), 4.14 (m, 1H, iPr-C*H*), 6.33 (b, 1H, N*H*iPr).

Synthesis of the α-Aminoxy Acid Deriving Hexapeptides

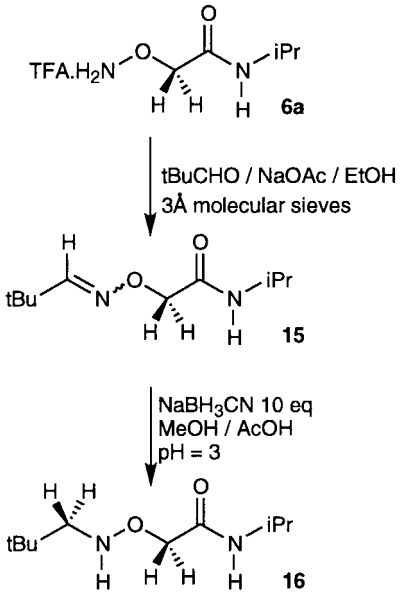
The synthetic procedures for the six amidoxy **23a,b**, oxime **29a,b**, and reduced amidoxy analogues **30a,b** of the HLE substrate Z-Ala^1-Ala^2-Pro^3-Val^4-Ala^5-Ala^6-NHiPr are specified in Schemes 7–10. The pseudohexapeptides have been characterized by electrospray mass spectroscopy and 2D-NMR using COSY, TOCSY and NOESY experiments. The characteristics of the six pseudohexapeptides are listed in Table 1.

Z-Ala-Ala-Proψ[CO–NH–O]Val-Ala-Ala-NHiPr **23a**
Z-Ala-Ala-Pro-Valψ[CO–NH–O]Ala-Ala-NHiPr **23b**
Z-Ala-Ala-Proψ[CH=N–O]Val-Ala-Ala-NHiPr **29a**
Z-Ala-Ala-Pro-Valψ[CH=N–O]Ala-Ala-NHiPr **29b**
Z-Ala-Ala-Proψ[CH_2–NH–O]Val-Ala-Ala-NHiPr **30a**
Z-Ala-Ala-Pro-Valψ[CH_2–NH–O]Ala-Ala-NHiPr **30b**

Taking advantage of the Pro residue that is not epimerized *via* the oxazolidinone intermediate, the amidoxy analogues **23a** and **23b** have been obtained by fragment condensation using Boc protection (Schemes 7 and 8). The peptide coupling steps have been classically carried out with TBTU, and TBTU/HOBt was used for the fragment condensation. The yield in fragment condensation after 3 days was rather low either due to the weak nucleophilicity of the aminoxy nitrogen compared with amines, or rather to the low solubility in organic solvents of **23b** which was recovered by washing with water.

The oxime link in analogues **29a** and **29b** was also created by fragment condensation of the *C*-terminal aminoxy peptide and the *N*-terminal peptide aldehyde (Schemes 9 and 10) which was classically obtained by reduction of the corresponding Weinreb amide with $LiAlH_4$ [33]. The preparation of the two peptide aldehydes **28** and **35** was difficult due to the low solubility of the corresponding Weinreb amides **27** and **34**. Furthermore, in previous works it was observed that the use of $LiAlH_4$ for the reduction of proline-containing peptide aldehydes often resulted in the reduction of the Pro-preceding amide carbonyl. For this reason the coupling yield was low (22% for **29a** and 12% for **29b**), but both oxime hexapeptides **29a** and **29b** were easily purified by silica gel chromatography.

The oxime link in **29a** and **29b** was almost quantitatively reduced with $NaBH_3CN$ into the reduced amidoxy link (Schemes 9 and 10). The reaction was carried out in MeOH with progressive addition over 6 days of $NaBH_3CN$ (10 eq) and AcOH in order to adjust the pH to about 3. Both reduced amidoxy hexapeptides **30a** and **30b** were purified by silica gel chromatography.

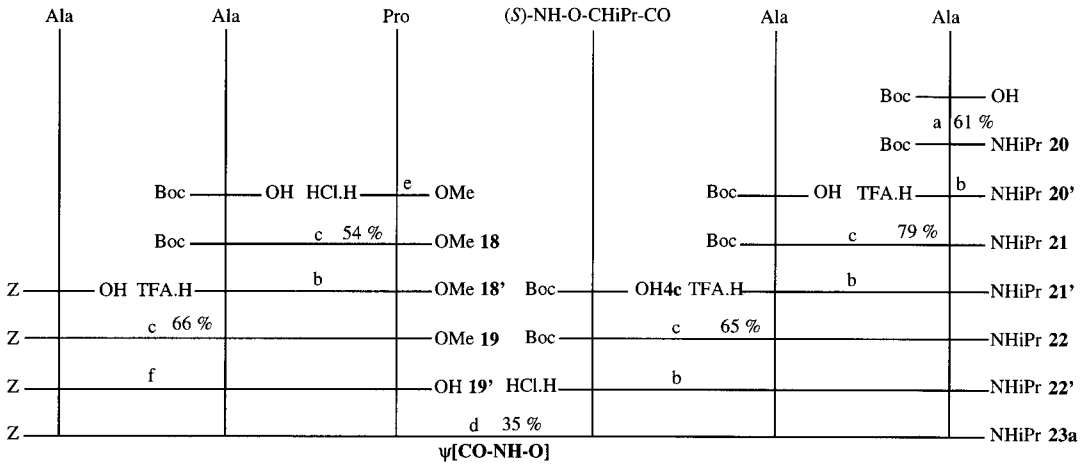


Scheme 6 Synthesis of the oxime **15** and hydroxylamine **16** model molecules.

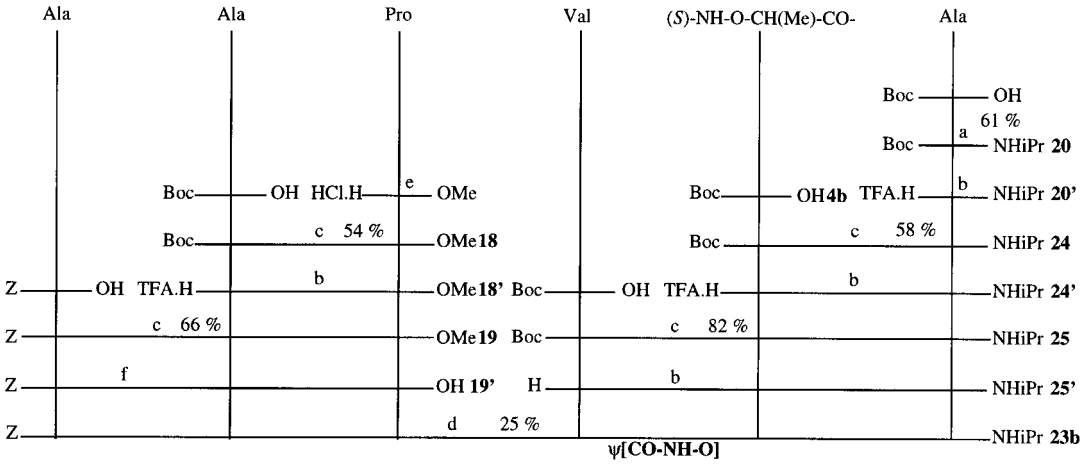
Table 1 Characteristic Parameters for the Amidoxy, Oxime and Reduced Amidoxy Hexapeptides

	Proψ[CO-NH-O]Val hexapeptide **23a**	Valψ[CO-NH-O]Ala hexapeptide **23b**	Proψ[CH=N-O]Val hexapeptide **29a**[a]	Valψ[CH=N-O]Ala hexapeptide **29b**[b]	Proψ[CH_2-NH-O]Val hexapeptide **30a**[c]	Valψ[CH_2-NH-O]Ala hexapeptide **30b**[d]
R_f 0.83[e]	Not soluble	0.54[e]	0.53[f]	0.35[e]	0.34[f]	
Molecular weight						
Theoretical	689.37	689.37	673.38	673.38	675.40	675.40
Experimental[g] $[M+H]^+$	690.34	690.34	674.33	674.33	676.36	676.36
Proton resonances (400 MHz, DMSO-d_6)						
Z CH_2	5.01	5.01	5.00	5.01	5.01	5.00
C_6H_5	7.31–7.36	7.31–7.36	7.31–7.36	7.31–7.37	7.31–7.36	7.31–7.36
Ala[1] NH	7.41	7.43	7.42	7.43	7.44(t)/7.38(c)	7.43
$C^{\alpha}H$	4.05	4.05	4.05	4.05	4.05(t)/4.10(c)	4.03
$C^{\beta}H_3$	1.17	1.19	1.17	1.18	1.18	1.19
Ala[2] NH	8.01	8.01	7.98	8.01	7.96(t)/8.07(c)	8.04
$C^{\alpha}H$	4.48	4.49	4.44	4.49	4.44(t)/4.62(c)	4.51
$C^{\beta}H_3$	1.17	1.19	1.18	1.78	1.18	1.17
Pro[3] $C^{\alpha}H$	4.16	4.43	4.57	4.33	4.13	4.28
$C^{\beta}H_2 + C^{\gamma}H_2$	1.76–1.97	1.82–1.97	1.88–2.02	1.80–1,99	1.75–1.97	1.84–1.98
$C^{\delta}H_2$	3.50, 3.59	3.51, 3.60	3.46, 3.55[c]	3.51, 3.60	3.44, 3.49	3.54, 3.59
Val[4] NH	11.16	7.87	—	7.92	6.74(t)/7.02(c)	7.42
$C^{\alpha}H$	3.91	3.93	4.09	4.13	3.65(t)/3.69(c)	3.72
$C^{\beta}H$	1.96	1.86	2.00	1.86	1.88(t)/1.80(c)	1.67
$C^{\gamma}H_3$	0.91, 0.94	0.80, 0.84	0.88, 0.89	0.84, 0.86[d]	0.87, 0.88(t) 0.84, 0.86(c)	0.81, 0.83
Ala[5] NH	8.28	11.33	7.69	—	7.85(t)/7.84(c)	6.46
$C^{\alpha}H$	4.28	4.31	4.31	4.51	4.31	4.00
$C^{\beta}H_3$	1.23	1.26	1.21	1.27	1.23	1.14
Ala[6] NH	7.83	8.17	7.88	7.61	7.78(t)/7.81(c)	7.59
$C^{\alpha}H$	4.18	4.20	4.17	4.21	4.19	4.22
$C^{\beta}H_3$	1.17	1.17	1.16	1.18	1.17	1.20
NH 7.63	7.76	7.66	7.76	7.65	7.65(t)/7.71(c)	
iPr CH	3.78	3.80	3.79	3.79	3.78	3.80
CH_3	1.02, 1.03	1.03, 1.05	1.02, 1.04	1.03, 1.04	1.02, 1.04	1.02, 1.04

[a] CH=, 7.49 ppm.
[b] CH=, 7.43 ppm.
[c] CH_2N, 2.61(t), 2.72(c), 3.10(t) and 3.23(c) ppm (t and c refer to the *trans* or *cis* conformation of the Ala-Pro amide bond, respectively).
[d] CH_2N, 2.69 and 2.96 ppm.
[e] Eluent, EtOH/EtOAc/hexanes 20/50/30.
[f] Eluent, EtOH/EtOAc/hexanes 16/54/30.
[g] Electrospray.



Scheme 7 Synthesis of the amidoxy hexapeptide **23a**. a: NMM, ICF, $iPrNH_2$, THF, −18 °C; b: TFA/DCM 40/60 v/v; c: DIEA, TBTU, DCM; d: DIEA, TBTU, HOBt, DMF; e: $SOCl_2$, MeOH, 45 °C; f: NaOH, H_2O/MeOH 50/50.

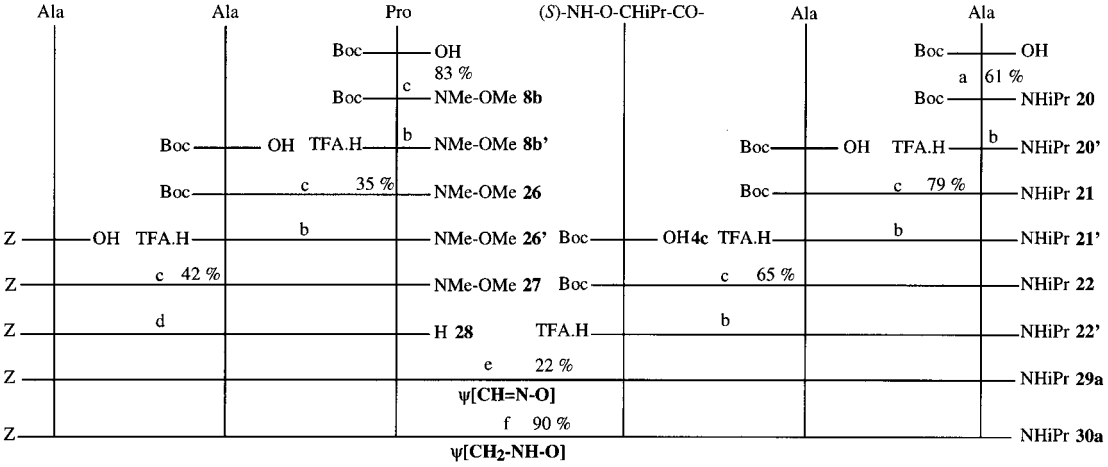


Scheme 8 Synthesis of the amidoxy hexapeptide **23b**. a: NMM, ICF, $iPrNH_2$, THF, −18 °C; b: TFA/DCM 40/60 v/v; c: DIEA, TBTU, DCM; d: DIEA, TBTU, HOBt, DMF; e: $SOCl_2$, MeOH, 45 °C; f: NaOH, H_2O/MeOH 50/50.

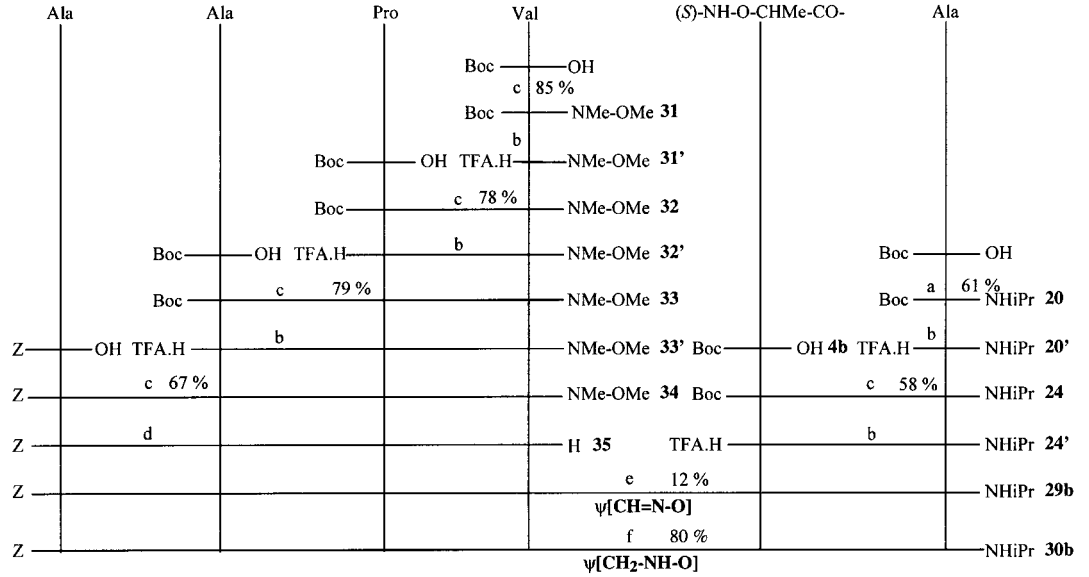
Spectroscopic Studies

^{1}H-NMR spectra were run on AC-200P (pseudodipeptides) and DRX-400 (pseudohexapeptides) Bruker apparatus with Me_4Si as internal reference, and the spin systems were solved by COSY and TOCSY experiments. The solvent accessibility, and therefore the extent of free or hydrogen-bonded character for the N*H* protons, was investigated in the pseudodipeptides by considering the N*H* proton resonance shift in $CDCl_3$/DMSO-d_6 mixtures with increasing DMSO-d_6 content [46,50]. The signal of a hydrogen-bonded, solvent-shielded N*H* is only slightly sensitive to DMSO content whereas the signal of a free, solvent-exposed N*H* is downfield shifted due to DMSO-d_6 solvation. In the case of a rapid equilibrium between an extended (solvent-exposed N*H*) and a folded (solvent-protected N*H*) conformer, the average solvent sensitivity of the N*H* resonance translates the relative percentages of both conformers.

IR spectra were scanned on a Bruker IFS-25 apparatus using a cell path length of 0.5 mm in order to investigate the amide N–H (3200–3500 cm^{-1}) and C=O (1580–1720 cm^{-1}) stretching frequencies. The peptide concentration was 0.005 M in DCM and in DMSO, and further dilution confirmed the absence of molecular aggregation. The N–H and C=O stretching frequencies were assigned on the basis of previous studies on related pseudopeptides [42,46] and of the spectroscopic data for model molecules **15** and **16**. In DCM, the free amide N–H



Scheme 9 Synthesis of the oxime **29a** and reduced amidoxy hexapeptide **30a**. a: NMM, ICF, $iPrNH_2$, THF, −18 °C; b: TFA/DCM 40/60 v/v; c: DIEA, TBTU, DCM; d: (1) $LiAlH_4$, THF, −15 °C, (2) 5% $KHSO_4$; e: AcONa, molecular sieves, MeOH; f: $NaBH_3CN$ 10 eq portionwise, 6 days, MeOH, AcOH, pH 3.



Scheme 10 Synthesis of the oxime **29b** and reduced amidoxy hexapeptide **30b**. a: NMM, ICF, $iPrNH_2$, THF, −18 °C; b: TFA/DCM 40/60 v/v; c: DIEA, TBTU, DCM; d: (1) $LiAlH_4$, THF, −10 °C, (2) 5% $KHSO_4$; e: AcONa, molecular sieves, MeOH; f: $NaBH_3CN$ 10 eq portionwise, 6 days, MeOH, AcOH, pH 3.

gives a sharp absorption in the 3400–3450 cm^{-1} domain, and the free Piv-*CO* a strong contribution at 1610–1625 cm^{-1}.

The molecular masses were determined by electrospray using a Platform II Micromass in negative or positive mode.

Enzymological Tests

Human elastase leukocyte enzyme (HLE) was purchased from Elastin Products Co. The chromogenic substrate MeO-Suc-Ala-Ala-Pro-Val-*p*NA was obtained from Sigma and acetonitrile (HPLC quality) from SDS. Spectrophotometric measurements were performed using a Uvikon 941 Kontron UV-vis thermostat-controlled spectrophotometer. A Waters model 600 MS system using a Lichrosorb C_{18}, 5 µM reverse-phase column (Interchim; 250 × 4.6 mm) was used to perform reverse-phase HPLC.

Solutions of enzyme were conserved at −20 °C in 0.01 M sodium acetate, pH 5.0 containing 0.5 M

NaCl and 0.03% $Brij_{35}$. The enzymatic activities were measured at pH 8.0 and 25 °C in 0.1 M Hepes, 0.5 M NaCl, 0.1% Tween 80, 10% v/v DMSO by following at 405 nm the continuous release of *p*-nitroaniline from MeO-Suc-Ala-Ala-Pro-Val-*p*NA (40 μM) in the presence of 20 nM HLE.

Compounds **23a,b, 29a,b** and **30a,b** (225 μM and 450 μM) were incubated at 25 °C and pH 8.0 in 500 μl buffer (0.1 M Hepes, 0.5 M NaCl, 0.1% Tween 80, 10% v/v DMSO) in the presence of 20 nM of HLE. After 1 or 10 min, the enzymatic assay was initiated by addition of substrate MeO-Suc-Ala-Ala-Pro-Val-*p*NA to the reaction mixture (final concentration: 40 μM). The constant K_i for **23b** was determined using a Dixon plot. The initial rates were measured using six concentrations of **23b** ranging from 70 to 450 μM and four concentrations of chromogenic substrate (20–500 μM).

The putative enzymatic hydrolysis was detected spectrophotometrically at pH 8.0 and 25 °C by scanning between 250 and 400 nm a reaction mixture containing 222 nM HLE and 450 μM of **23a,b, 29a,b** and **30a,b** over a period of 1 h. Alternatively, the reaction mixture was analysed to detect hydrolysis products. Compounds **23a,b, 29a,b** and **30a,b** (450 μM) were incubated at 25 °C for 1, 2 or 4 h in the presence of enzyme (24 or 240 nM). At the end of the incubation period, the pH was dropped to 3.5 to quench enzyme activity. Then the reaction products were separated from the enzyme by filtration/centrifugation on a Centricon 10 instrument (Amicon). The filtrates (25 μl) were analysed by reverse-phase HPLC using a 60 min (0–60%) linear gradient (Milli-Q water 0.1% TFA/acetonitrile 0.07% TFA) at a flow rate of 0.75 ml/min. The absorbance of the effluents was monitored at 220 nm. The eventual spontaneous hydrolysis of **23a,b, 29a,b** and **30a,b** was checked by following an identical procedure in the absence of enzyme. Peptide Z-Ala-Ala-Pro-Val-OH was used to calibrate the column.

RESULTS

Most spectroscopic experiments were carried out on the oxime dipeptides and the reduced amidoxy dipeptide containing the Piv group which is known to favour the *trans* conformer of the Piv-Pro amide bond [48,49]. In contrast to the reduced amide ψ[CH_2–NH] link [46], neither the oxime ψ[C=N–O] nor the reduced amidoxy ψ[CH_2–NH–O] groups are protonated in the pH range 4–12 in water as their proton resonances are not significantly affected in this domain. From this point of view, they have the same properties as the imino aza C^{α}–CH=N–N^{α} and reduced aza peptide C^{α}–CH_2–NH–N^{α} fragments [46,50].

Oxime Dipeptides

Most of the investigated oxime dipeptides exhibit split proton NMR resonances, and the quantitative ratio for each resonance not only depends on the compound, but also varies with the time in some cases (Table 2). The presence or absence of *NOE* correlations between the oxime C*H* and the OCH_2 protons for **12d** indicates that the equilibrium between the *E* and *Z*-stereomers of the oxime group is responsible for signal splitting. The existence of *Z* and *E*-oxime is well documented, and it is known that the transition from one to the other stereomer is catalysed by acids [51].

In a fresh $CDCl_3$ solution of **12d**, obtained from **12c** by Piv for Boc exchange after TFA treatment (Scheme 5), a single set of proton resonances is observed, and the existence of a *NOE* correlation between the oxime C*H* and the OCH_2 protons is in favour of the *E*-oxime. A second set of proton resonances progressively appears and increases over 10 days up to a 70/30 ratio. The absence of *NOE* correlation between the afore-mentioned protons supports the occurrence of the *Z*-oxime in the second stereomer. In contrast, the Boc-protected homologue **12c** only exhibits the *E*-oxime, and there is no evolution of the spectrum with the time. Therefore **12d** was prepared using a second procedure in which the Piv group is introduced in a first step so that the oxime group has no contact with a strong acidic medium (Scheme 5). Under these conditions, the ^{1}H-NMR spectrum of **12d** does not change with the time, and is typical of the *E*-oxime. However, the addition of TFA or gaseous HCl traces restores the progressive *E*/*Z* evolution with time.

The solvent accessibility of the N*H* protons has been followed in $CDCl_3$/DMSO-d_6 mixtures, in which DMSO-solvation of the N*H*s induces a shift to lower fields of the solvent-exposed N*H* signals [46,49]. The *E* and *Z*-conformers of the aldoxime and ketoxime dipeptides **12a–g** exhibit quite different variations of the N*H* resonance (Figure 1). The highest N*H*-accessibility is observed for the *E*-oxime, and is quite similar to that for model derivative **15**, and for MeO-CH_2-CONHiPr **17** in which the N*H* is *H*-bonded to the ether oxygen [46]. Therefore it is concluded that the N*H* bond in the *E*-stereomer of

Table 2 Percentage of the Z Conformer for Various Oxime Dipeptides in $CDCl_3$ Prepared by Different Strategies

Compound	Strategy	Crude material	After TFA treatment
11d Piv-Proψ[CH=NO]Gly-OMe	[a]	[b]	37
12a Boc-Alaψ[CH=NO]Gly-NHiPr	[c]	30	[d]
12b Piv-Valψ[CH=NO]Ala-NHiPr	[e]	30	20
12c Boc-Proψ[CH=NO]Gly-NHiPr	[c]	<2	[d]
12d Piv-Proψ[CH=NO]Gly-NHiPr	[e]	<2	70
12d Piv-Proψ[CH=NO]Gly-NHiPr	[a]	[b]	70
12g Piv-Proψ[CnPr=NO]Gly-NHiPr	[a]	[b]	80

[a] Coupling of the N-Boc-α-amino aldehyde with the aminoxy peptide, followed by Piv for Boc exchange with TFA treatment.
[b] Slow evolution with the time toward the same Z percentage as after TFA treatment.
[c] Coupling of the N-Boc-α-amino aldehyde with the aminoxy peptide.
[d] Boc cleavage by TFA.
[e] Coupling of the N-Piv-α-amino aldehyde with the aminoxy peptide.

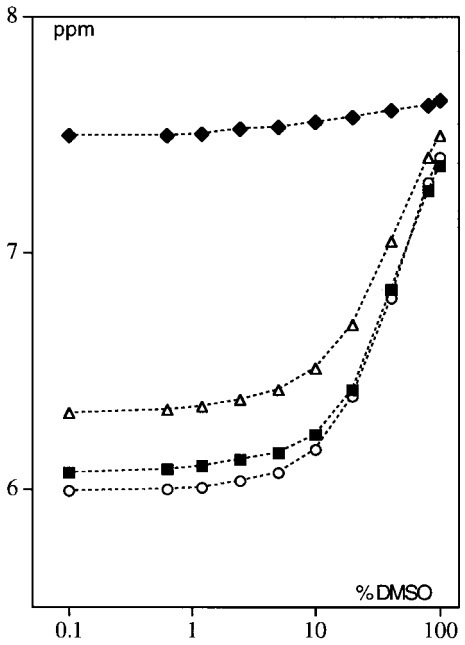


Figure 1 Influence of DMSO-d_6 addition on the N*H*iPr proton resonance for the *Z* (♦) and *E* (■) stereomers for Piv-Proψ[CH=N–O]Gly-NHiPr **12d** in $CDCl_3$. Comparison with tBuCH=N–O–CH_2–CONHiPr **15** (○) and MeO–CH_2–CO–NHiPr **17** (Δ).

12a–g probably interacts with the oxime oxygen in a 5-membered chelation ring. The vicinal coupling constant of 4.4 Hz in the $C^{\alpha}H$–C*H*= fragment and the magnetically equivalent $C^{\alpha}H_2$ protons for **12a** and **12c–g** indicate a rather flexible structure. In contrast, the weak solvent sensitivity of the N*H* resonance for the *Z*-isomers of **12a–g** is typical of an N*H*-bonded proton. It is concluded that the *Z*-oxime is folded by an N-H···O=C *H*-bond closing an 11-membered cycle. The non-equivalent $C^{\alpha}H_2$ protons for **12a** and **12c–g**, and the higher vicinal coupling constant of 8.0 Hz for the $C^{\alpha}H$–C*H*= fragment in **12d**, corresponding to the transoid disposition of the vicinal protons, are in favour of the rigid structure depicted in Figure 2.

IR spectroscopy confirms the above conclusions. For example, the *E*-conformer of **12c** and **12d** in DCM exhibits the same high NH stretching frequency at 3422 cm^{-1}, exactly as the model compound **15** lacking the Pro residue. This frequency is typical of a free, non H-bonded vibrator. After evolution with time in favour of the *Z*-conformer in DCM, a second NH absorption progressively grows for **12d** at 3343 cm^{-1} and denotes the occurrence of an intramolecular NH to Piv-CO *H*-bond closing an 11-membered ring (Figure 2). In DMSO, the higher absorption shifts down to about 3270 cm^{-1} that corresponds to a DMSO-solvated NH, and therefore to an open structure containing a trans-oxime link, while the retention of an absorption at 3335 cm^{-1} illustrates the stability of the folded structure containing the *cis*-oxime link. It is surprising to note that the Piv-CO absorption for **12d** in DCM (1616 cm^{-1}) is quite similar to that for ester

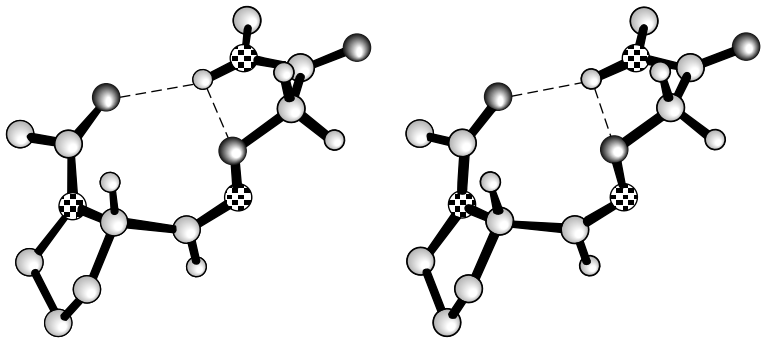


Figure 2 Stereoviews of the folded structure, with a bifurcate H-bond, adopted by the *Z*-stereomer of **12d**. Only the NH, =C*H* and C$^{\alpha}$H hydrogens have been indicated for clarity.

11d, and is not very sensitive to the *cis*-oxime percentage and therefore to the existence of the NH to Piv-CO H-bond. In contrast, the Piv-CO absorption for ester **11d** in DMSO (1617 cm^{-1}) actually exceeds by 4 cm^{-1} that for **12d** in DMSO (1613 cm^{-1}).

The percentage of the *Z*-oxime stereomer largely depends on the existence of the aforementioned *H*-bond, which stabilizes the folded structure in Figure 2. It decreases for **11c** where the *C*-terminal ester is substituted for the amide bond in **12d**. The same is observed for **12a** and **12b** where Ala or Val is substituted for Pro which is known to be a better turn promoting residue in the peptides than Ala or Val [52].

Reduced Amidoxy Dipeptides

The solvent sensitivity of the N*H* proton resonances in the reduced amidoxy dipeptide **14c**, compared with that for the model molecule tBuCH$_2$–NH–CH$_2$–CO–NHiPr **16** and for MeO–CH$_2$–CO–NHiPr **17**, is represented in Figure 3. The variations for the amide N*H*iPr proton for **16** and **17** are quite similar, and denote the occurrence of a weak *H*-bond between the amide N*H* and the sp^3 oxygen [46]. This weak *H*-bond, closing a 5-membered cycle, is actually present in the crystal molecular structure of **14c** (Figure 4a) [53]. The lower N*H* variation for **14c** probably denotes an additional, weak interaction with the Piv-carbonyl (Figure 4b) which effectively presents a lower C=O stretching frequency (1608 cm^{-1}) than for the homologous ester **13** (1613 cm^{-1}). The corresponding NH frequency at 3417 cm^{-1} in DCM denotes a weak interaction, and shifts in DMSO to about 3270 cm^{-1}, a value typical of a DMSO-solvated NH.

The hydroxylamine N*H* proton is also less solvent-accessible in **14c** than in **16**, likely indicating that it is, at least partially, also engaged in a weak

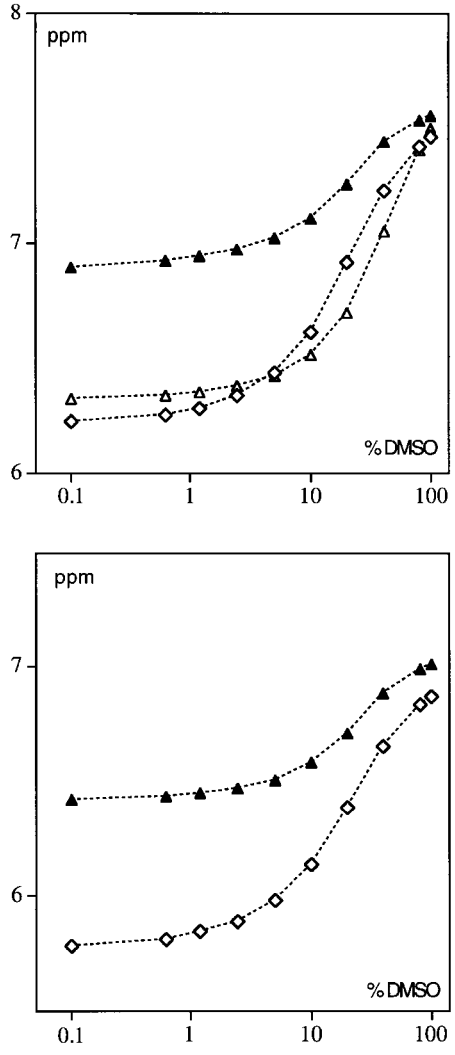


Figure 3 Influence of DMSO-d_6 addition on the N*H*iPr (up) and N*H*O (down) proton resonances for Piv-Proψ[CH$_2$–NH–O]Gly-NHiPr **14c** (▲) in CDCl$_3$. Comparison with tBuCH$_2$-NH–O–CH$_2$–CO–NHiPr **16** (Δ) and MeO–CH$_2$–CO–NHiPr **17** (◊).

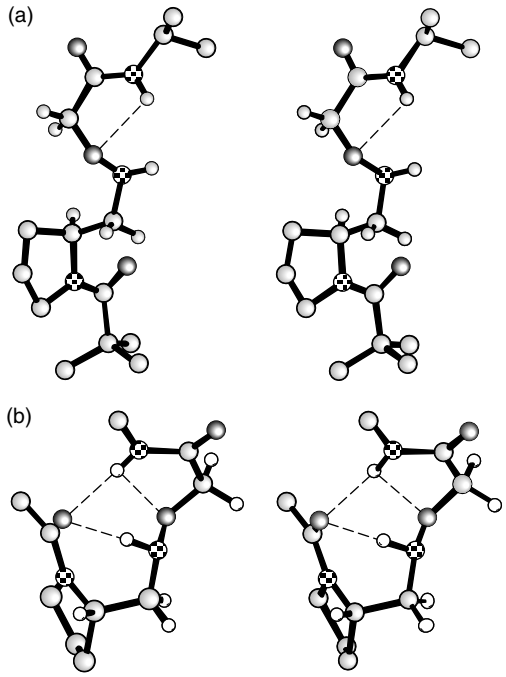


Figure 4 Stereoviews of the extended (a) and folded structure (b) adopted by **14c** in the solid state and in $CHCl_3$ solution, respectively. Only the NH, $C^{\alpha}CH_2$ and $C^{\alpha}H$ hydrogens have been indicated for clarity.

interaction with the Piv-carbonyl. The resulting folded conformation (Figure 4b) is probably in rapid equilibrium with an open conformation similar to that observed in the solid state (Figure 4a). The open and folded structures essentially differ by a rotation of 120° for the torsion angles about the $C^{\alpha}H-CH_2$ and CH_2-N bonds.

HLE Inhibition

The potential inhibitory properties of the pseudopeptides **23a,b, 29a,b** and **30a,b** were studied using the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-*p*NA. A crucial point was to determine whether pseudopeptides **23a,b, 29a,b** and **30a,b** acted as substrates or inhibitors of HLE. No spectral evolution with time was observed when these compounds were incubated for 1 h in the presence of enzyme at pH 8.0 and 25 °C. However, the hydrolysis products might have absorbance spectra which did not differ significantly from that of the uncleaved pseudopeptides. So the reaction mixtures were analysed by HPLC with UV detection at 220 nm. The peak corresponding to pseudopeptide **23b** (retention time of 43.3 min) disappeared when incubated with HLE 240 nM for 2 h while a new peak (retention time of 40.3 min) appeared, with no detectable secondary peaks. The new peak had the same retention time as Z-Ala-Ala-Pro-Val-OH demonstrating that the amidoxy link in **23b** is cleaved in the presence (but not in its absence) of HLE. Under identical experimental conditions, no cleavage was observed for compounds **23a, 29a,b** and **30a,b** (retention times of 44.9, 50.0, 48.2, 46.9 and 47.5 min, respectively) in the presence or absence of enzyme.

It has already been reported that both the Z-Ala-Ala-Pro-Val-Ala-Ala-NHiPr peptide and the Z-Ala-Ala-Pro-Valψ[CO–NH–NH]Ala-Ala-NHiPr hydrazine analogue act as HLE substrates, and are cleaved at the Val-Ala position [6]. Similarly to ψ[CO–NH–NH], the amidoxy ψ[CO–NH–O] link (**23b**) does not prevent HLE-catalysed hydrolysis. Compound **23b** has a better apparent affinity ($K_m = 382$ μM) than the analogous hydrazine peptide ($K_m = 712$ μM), while the peptide itself displays the best apparent affinity ($K_m = 131$ μM). As might be expected due to the suppression of the carbonyl electrophilic centre, the ψ[CH=N–O] (**29b**) or ψ[CH_2–NH–O] (**30b**) link prevents hydrolysis. Similar to the hydrazide analogue Z-Ala-Ala-Proψ[CO–NH–NH]Val-Ala-Ala-NHiPr [6], the ψ[CO-NH-O] (**23a**) or ψ[CH=N–O] (**29a**) bond between Pro and Val prevented the enzymatic hydrolysis of the Val-Ala peptide bond.

As a matter of fact, only compounds **23a** and **29b** acted as weak inhibitors (17% and 32% inhibition at 450 μM, respectively). The inhibition is dose, but not time-dependent. The inhibitory effect of **29b** on HLE hydrolysis of the chromogenic substrate was used to evaluate the Michaelis constant K_m for the enzymatic hydrolysis of **23b** [54]. The Dixon plot is consistent with a competitive inhibition characterized by the dissociation constant K_i of 382 μM, a value which is identical to K_m (Figure 5).

CONCLUSION

The α-hydrazino and α-aminoxy acids are isosteric molecules, which can be prepared from the corresponding α-amino acids. However, the α-aminoxy acids are much more easily obtained, and present no problem of regioselectivity during *N*-coupling. The α-aminoxy acids can be *N*-coupled to a peptide aldehyde to give the oxime link ψ[CH=N–O] which in turn can be reduced into the reduced amidoxy link ψ[CH_2–NH–O]. The *E*-oxime group is generally obtained, but may progressively change into the *Z*-form when submitted to strong acidic conditions.

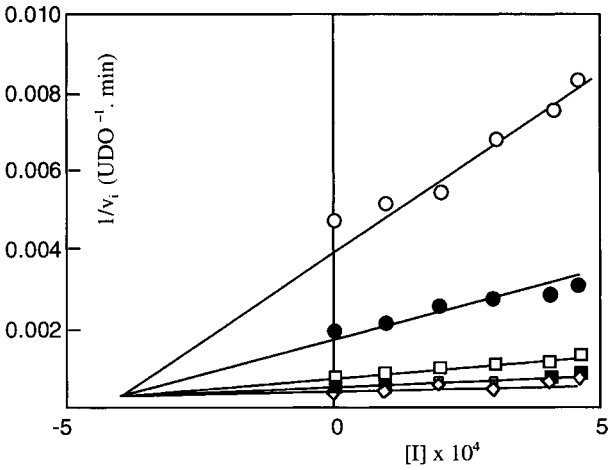


Figure 5 Inhibition of HLE by compound **29b** at 25 °C and pH 8.0 ($[HLE]_o = 20$ nM; $[S]_o = 2 \times 10^{-5}$ M (○), 5×10^{-5} M (●), 10^{-4} M (□), 2×10^{-4} M (■), 5×10^{-4} M (◊). (inhibitor I, **29b**; substrate S, MeO-Suc-Ala-Ala-Pro-Val-*p*NA).

The *E*-stereomer adopts an extended conformation in solution while the *Z*-stereomer is folded in a β-like conformation by a strong bifurcate hydrogen bond. The reduced amidoxy group induces more flexibility resulting in an equilibrium between extended and folded conformations in solution. An extended conformation has been characterized in the solid state of the Piv-Proψ[CH_2–NH–O]Gly-NHiPr **14c**. In all cases, the backbone sp^3 oxygen is H-bonded to the following amide NH in a 5-membered pseudocycle.

The oxime and reduced amidoxy link, together with the amidoxy surrogate, have been introduced in the Pro-Val or Val-Ala position of the HLE hexapeptide substrate Z-Ala-Ala-Pro-Val-Ala-Ala-NHiPr and tested for an eventual inhibitory activity.

The amidoxy Proψ[CO–NH–O]Val (**23a**) and Valψ[CO–NH–O]Ala (**23b**) hexapeptides have the same behaviour as the analogous hydrazide Proψ[CO–NH–NH]Val and Valψ[CO–NH–NH]Ala hexapeptides, respectively, thus confirming the close structural and electronic similarity for both amide surrogates. The Proψ[CO–NH–O]Val (**23a**) and Proψ[CO–NH–NH]Val analogues are true competitive inhibitors while the amidoxy and hydrazide bonds in the Valψ[CO–NH–O]Ala (**23b**) and Valψ[CO–NH–NH] Ala analogues are slowly cleaved. The oxime Valψ[CH=N–O]Ala hexapeptide (**29b**) is also a competitive inhibitor while the other three Proψ[CH=N–O]Val (**29a**), Proψ[CH_2–NH–O]Val (**30a**) and Valψ[CO–NH–O]Ala (**30b**) analogues have no affinity to HLE.

The α-aminoxy acids appear to be interesting precursors capable of giving three different pseudopeptide links depending on the carboxylic, aldehydic or ketonic nature of the coupling partner. They are good candidates to chemical diversity in the design of bioactive pseudopeptide analogues.

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