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Triphosgene: An Efficient Carbonylating Agent for Liquid and Solid-phase Aza-peptide Synthesis. Application to the Synthesis of Two Aza-analogues of the AChR MIR Decapeptide

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Abstract: The $N^{\alpha}/C^{\alpha}H$ exchange in aza-peptides has the advantage of preserving the side chain. Bis(trichloromethyl)carbonate or triphosgene is a solid, stable phosgene substitute which retains its high reactivity. Temperature and coupling times are greatly reduced with reference to other usually recommended carbonylating agents, while purity and yield are increased. It has been used, in both liquid- and solid-phase procedures, for the synthesis of various aza-analogues of dipeptides, tripeptides and decapeptides containing the alanine, aspartic acid and asparagine aza-residue. ©1997 European Peptide Society and John Wiley & Sons, Ltd.

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Aza-peptides, in which nitrogen has been substituted for at least one of the CH^{α} groups, constitute a pseudopeptide series characterized by an α-modification allowing easy retention of the side chains. Initially introduced by Goldschmidt and Wick [1], then by Niedrich and Oehme in eledoisin analogues [2], aza-peptides have been actively developed by Gante [3, 4] and by Dutta and Morley [5] for the design of hormone analogues and various protein inhibitors. More recently, their synthesis has been particularly investigated by Gante [6] and by Boussard and co-workers [7, 8] in liquid-phase synthesis, and by Gray *et al.* [9] and Quibell *et al.* [10] in solid-phase synthesis.

Incorporation of an aza-residue in a peptide chain is a combination of hydrazine and peptide chemistry. The most frequent method consists in adding an adequately protected hydrazine to an isocyanate obtained by action of phosgene or activated aryl chloroformates or carbonates on the peptide N-terminus. [4, 5, 11]. This route is not applicable when proline occupies the N-terminal position of a growing peptide chain. Another method is based on activated aryl esters either of the peptide N-terminus [11, 12] or of the aza-residue (carbazic residue) [5,

Abbreviations: P, P′ N-protective groups, MA mixed anhydride method; NMM, *N*-methylmorpholine; IBCF, isobutylchloroformate; PE, Petroleum ether (Eb: 35–65°C); b, broad band (NMR spectra).

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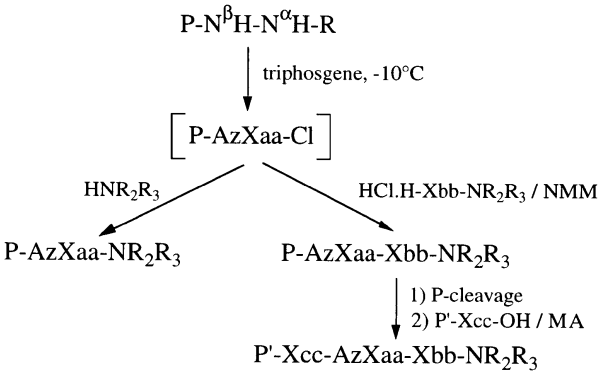
11, 13]. However, coupling requires in both cases a high temperature and long reaction times, and often results in low yields with numerous side products such as phenolic derivatives, hydantoins [9, 10] and oxadiazolones [4, 12, 14].

We have been engaged both in structural investigations and in unnatural antigen synthesis of aza-peptides, and we propose here triphosgene (bis(trichloromethyl) carbonate) as a mild, easy-to-handle and efficient carbonylating agent for aza-peptide synthesis in both liquid- and solid-phase procedures. In the present paper, we report the synthesis of several aza-alanine, aza-asparagine or aza-aspartic acid-containing derivatives (the aza-analogue of the amino acid residue Xaa is denoted by AzXaa in the following).

Different Pro-AzAsx and AzAsx-Pro model compounds, designed for structural analysis purposes (Xaa = Ala and Asx = Asp or Asn) were synthesized in the liquid-phase. In order to evaluate the impact of the AzXaa/Xaa substitution in a longer peptide chain, we have also prepared in the liquid-phase Boc-Trp-AzAsn-Pro-NH^{i}Pr, an analogue of the N-terminal tripeptide sequence in the main immunogenic region (MIR = Trp-Asn-Pro-Ala-Asp-Tyr-Gly-Gly-Ile-Lys) of the *Torpedo californica* electric organ acetylcholine receptor (AChR) which is the target in an experimental autoimmune response related to myasthenia gravis [15, 16]. Triphosgene was also used for the solid-phase synthesis of two aza-analogues of the MIR decapeptide containing either AzAsn[2] or AzAla[4].

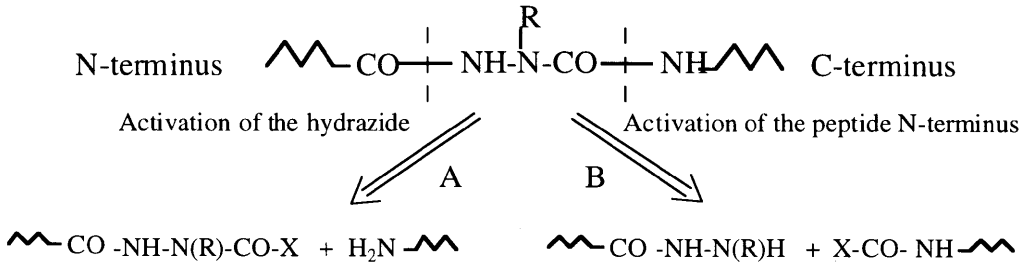
If the method based on the activation of the hydrazide (Path A; Scheme 1) is chosen and if the spontaneous decarboxylation of carbazic acids (aza-analogues of the α-amino acids) is kept in mind, the aza-residue has to be generated by carbonylation of the corresponding hydrazine before its incorporation into the peptide chain (Scheme 1). Thus the crucial steps in that aza-peptide synthesis are: (i) regioselective N^{β}-protection of an N^{α}-substituted hydrazine, (ii) carbonylation-activation and coupling of the latter to the peptide N-terminus, and (iii) elongation of the hydrazino terminus. Here, the N^{β}-protection must be a carbamate in order to prevent the formation of 1,3,4-oxadiazol-2(3H)-ones which are known far less reactive than the corresponding azlactones in classical peptide chemistry [4]. Triphosgene is proposed as a mild chlorocarbonylating agent of any substituted hydrazine, and to react the adequately protected resulting carbazic acid chloride further under mild conditions (low temperature and short reaction times) with the peptide amino terminus (Scheme 2) without formation of quasi non-separable product mixtures.



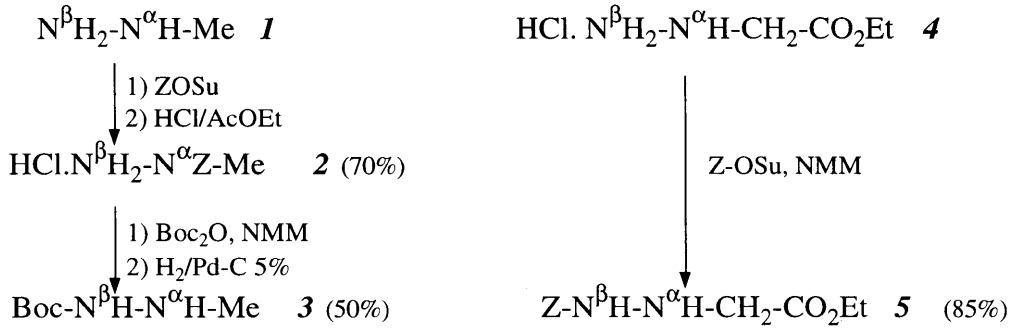
Scheme 2 Triphosgene employment in the synthesis of oligo aza-peptides.

N^{β}-protected-N^{α}-substituted Hydrazines

The hydrazine precursors of the AzAla and AzAsx residues were the commercially available methylhydrazine ($N^{\beta}H_2$–N^{α}H–Me **1**) and ethyl hydrazino acetate hydrochloride (HCl.$N^{\beta}H_2$–N^{α}H–CH_2–CO_2Et **4**), respectively. Previous studies in the laboratory have shown that their nitrogen atoms practically display opposite regioselectivities [8]. For example, Z–OSu acylates preferentially the α-nitrogen of methylhydrazine **(2)**, and the β-nitrogen of ethyl



Scheme 1 Basic retrosynthetic pathways for the incorporation of an aza-residue in a peptide chain.



Scheme 3 Preparation of N^{β}-protected,N^{α}-substituted hydrazides.

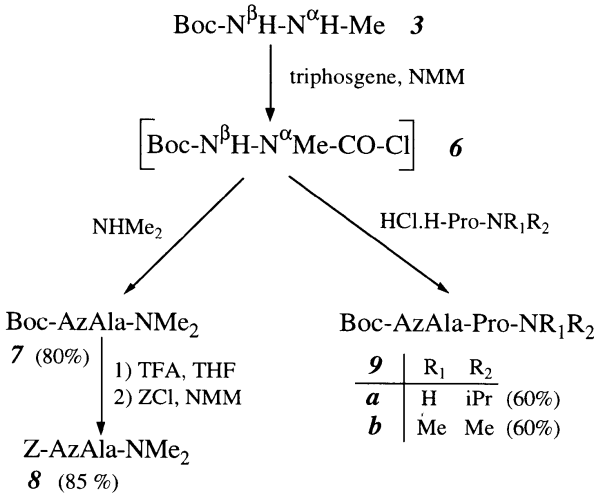
hydrazino acetate **(5)** (Scheme 3). Boc-N^{β}H–N^{α}HMe **3** was obtained by action of Boc_2O on **2** followed by catalytic hydrogenolysis of the N^{α}–Z group. No reagent was found to introduce the Boc group with such a regioselectivity in either α or β position in **1**.

Liquid-phase Aza-peptide Synthesis

Bis(trichloromethyl) carbonate (Aldrich), a stable solid derivative, which is known for its typical reactions, e.g. chloroformylation, carbonylation, chlorination, dehydration, chemically behaves as three phosgene molecules, hence its name of triphosgene [17]. It readily converts primary amines into isocyanate [17]. With the N^{α}-substituted-N^{β}-protected hydrazide precursors **3** and **5** of the AzAla and AzAsx residues, triphosgene reacts mildly at −10 °C to give likely the corresponding carbazic acid chlorides which are not isolable and must be used *in situ* (Scheme 4). They react rapidly at low temperature (−10 °C) with an amine to afford a N^{β}-protected carbazamide, or with the amino terminus of a C-protected amino acid to give an aza-dipeptide (Scheme 2). Then the β-nitrogen can be deprotected and coupled to a N-protected amino acid.

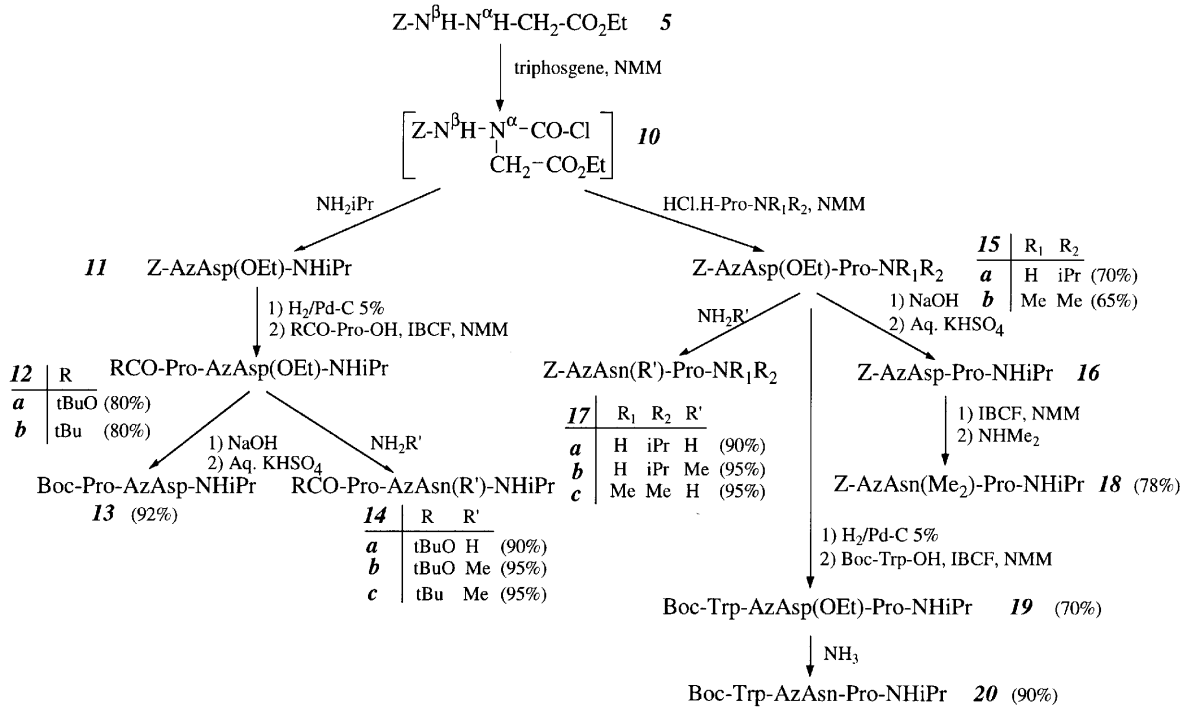
Triphosgene was employed for the synthesis of various aza-peptides containing either AzAla or AzAsp or AzAsn residue. The AzAla-containing derivatives were prepared according to Scheme 4. The carbazic acid chloride **6** reacted at −10 °C with a secondary amine (dimethylamine) to give **7**. Acidolysis of the Boc group allowed the introduction of the N^{β}-Z protection in **7**. **6** also coupled with the amino terminus of a proline derivative to obtain the aza-dipeptides **9a,b** with the AzAla-Pro sequence.

The Z–N^{β}H–N^{α}H–CH_2–CO_2Et protected hydrazine **5** presented the same possibilities as **3**



Scheme 4 Preparation of aza-alanine derivatives.

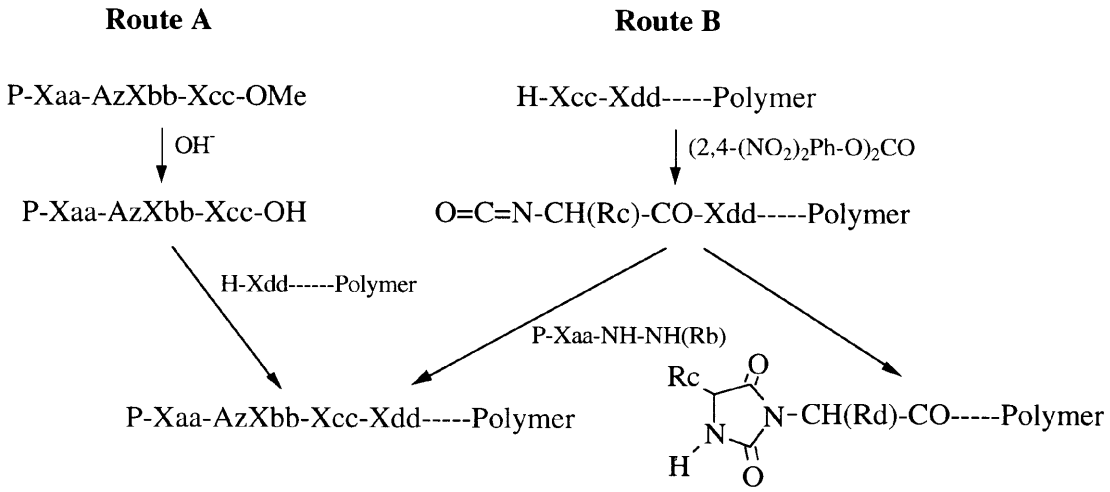
(Scheme 5). Moreover, after amidification of the carbazic acid chloride **10**, the ester function of **11**, **15** and **19** can be saponified into a carboxylic group **(13**, **16)** or converted into a carboxamide group by aminolysis **(14a–c**, **17a–c** and **20)**, so giving access to the AzAsp or AzAsn residue. The carboxylic group in **16** also reacted with a secondary amine (dimethylamine) to give a N^{δ},N^{δ}-disubstituted AzAsn residue **(18)**. Using these procedures, the aza-dipeptides with the Pro-AzAsx **(13** and **14a–c)** or AzAsx-Pro **(16**, **17a–c** and **18)** sequences were obtained (Asx = Asp or Asn), and the aza-analogue **20** of the MIR N-terminal tripeptide. It is to be noted that the mixed anhydride method needed shorter reaction times and lower temperature than the activated ester pathway, and gave much better yields than the procedures based on DCC/HOBt or BOP reagents, for N^{β}-acylation of the AzAsx residue.



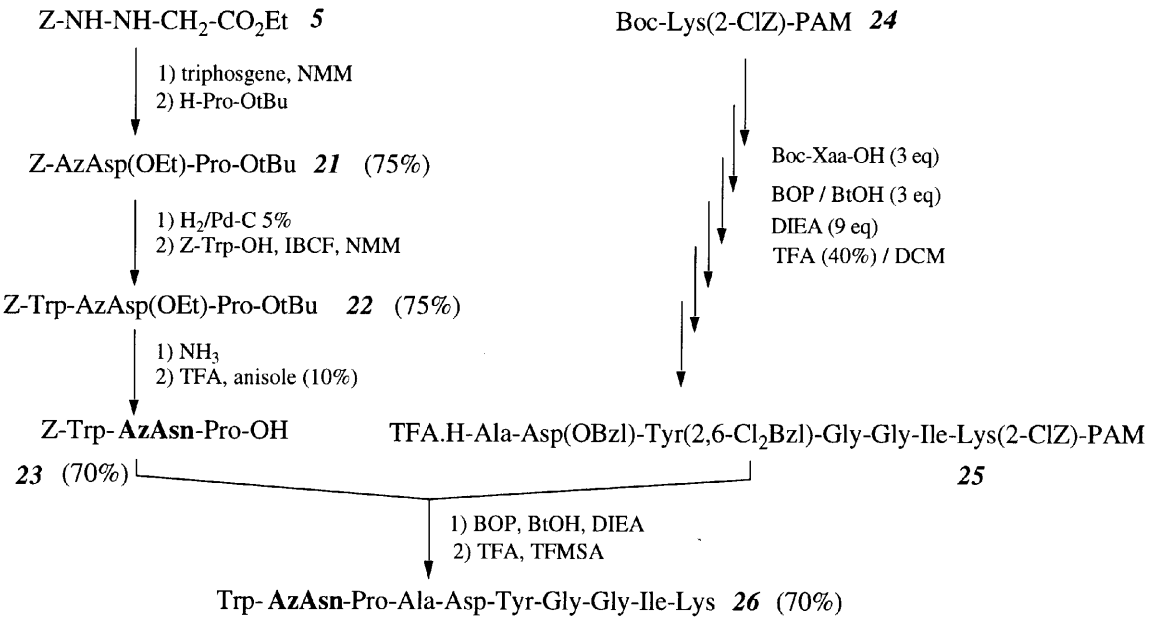
Scheme 5 Synthesis of several AzAsx-containing oligo aza-peptides.

Solid-phase Synthesis of Two Aza-decapeptides

The synthesis of longer aza-peptides of biological interest requires preferentially solid-phase procedures. Two strategies for solid-phase aza-peptide synthesis have been reported in the literature (Scheme 6) [9]. Route A consists of coupling a preformed aza-tripeptide synthon to the N-terminus of a resin-bonded peptide. However, the authors point out the partial epimerization of the Xaa residue during saponification of the C-terminal ester in the synthon [9], and of the Xcc residue (except for glycine or proline) during coupling of the synthon to the resin. Route B is singularized by the condensation of a peptide hydrazide on the isocyanate N-terminus of a resin-bonded peptide, but the N-terminal isocyanate is more or less partially converted into a hydantoin moiety whatever the Boc or



Scheme 6 Introduction of an aza-residue in a peptide chain built according to a solid phase procedure [9].



Scheme 7 Preparation of the [AzAsn^2]MIR aza-decapeptide.

Fmoc strategy [9, 10]. Therefore, both procedures suffer some significant limitations resulting in low yields and difficulties in the purification of the crude material which contains epimerized aza-peptides and side products.

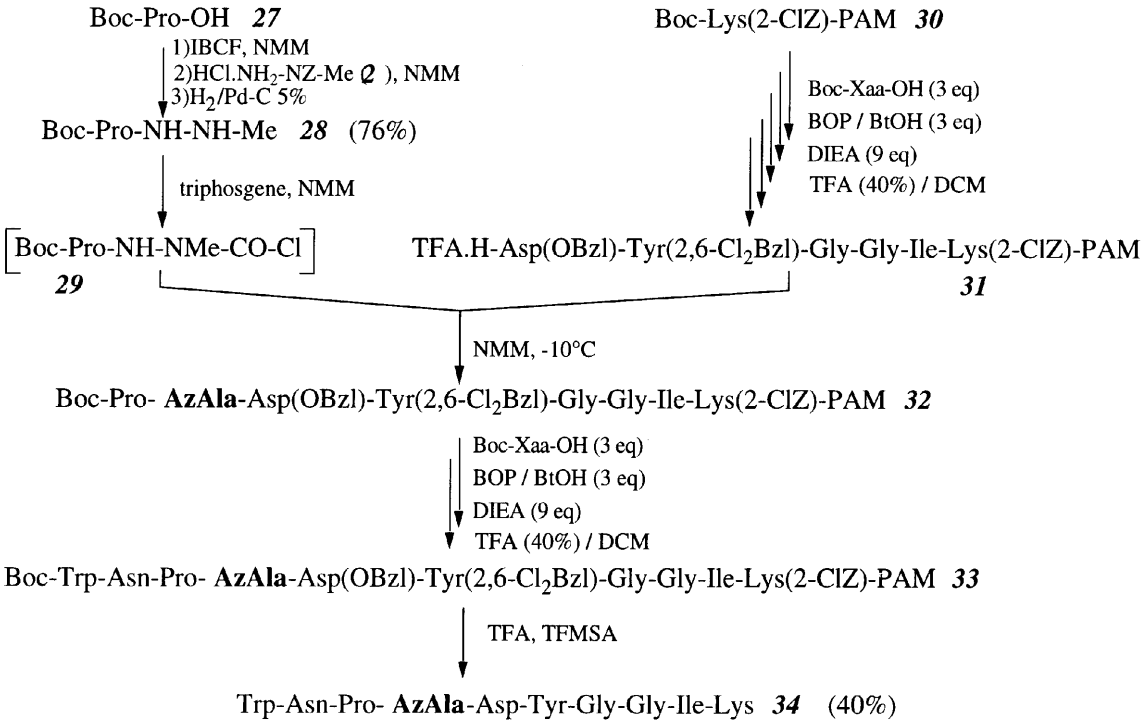
We reinvestigated the above two methods, aiming at better yields and reduced side-product formation. The [AzAsn^2]MIR **26** and [AzAla^4]MIR **34** aza-analogues of the main immunogenic region (Trp-Asn-Pro-Ala-Asp-Tyr-Gly-Gly-Ile-Lys) in the *Torpedo californica* electric organ acetylcholine receptor were prepared according to Schemes 7 and 8. In both cases, we used the Boc strategy with a PAM (phenyl-*para*-acetamido-methyl) polystyrene resin, BOP as coupling agent and TFA for Boc cleavage. The aza-peptides were detached from the resin by a TFA/TFMSA mixture [18].

The aza-tripeptide synthon strategy was used for construction of **26** (Scheme 7). Potential epimerization of Trp in **23** was minimized by using the tBu ester **22** cleavable in acidic TFA medium. Moreover, the OtBu ester protection was suitable to prevent cyclization of **21** into the 3,6-dioxohexahydro-1,2,4 triazine [19] from occurring during the urethane deprotection step. Owing to the presence of proline in the C-terminal position in **23**, epimerization of this residue when coupling to the resin-bonded peptide was expected to be quite small if present at all. **34** was prepared by direct coupling of the Boc-Pro-NH-NH-Me **28** peptide hydrazide to the amino terminus of the resin-bonded peptide **31** by using triphosgene (Scheme 8). The resulting aza-peptide **32** is classically elongated by using BOP as coupling agent. After cleavage and deprotection, the aza-decapeptides were obtained in 70% **(26)** and 40% **(34)** yield as crude desalted material. HPLC purification (Figure 1) afforded the pure aza-decapeptides whose MS molecular peaks confirm the expected molecular weights.

26 was found to be totally inactive with reference to the mAb6 monoclonal antibody raised against *Torpedo californica* AChR receptor whereas **34** was observed to retain about 30% of the MIR mAb6-binding affinity. Surprisingly, **34** was as active as MIR with reference to the anti-human AChR monoclonal antibody mAbl98. This confirms that N^{α} substitution for $C^{\alpha}H$ induces conformational perturbations as already highlighted in the case of short aza-peptide models [7, 8, 20]. From this point of view, the AzAsn^2/Asn^2 substitution is more significant than the AzAla^4/Ala^4 one. Moreover, the mAb6 and mAbl98 antibodies do not probably recognize the same part of the MIR epitope. Investigations are in progress in this direction.

EXPERIMENTAL PART

Solid-phase syntheses were carried out using a Dupont Coupler 250. Semi-preparative HPLC was



Scheme 8 Preparation of the [AzAla^4]MIR aza-decapeptide.

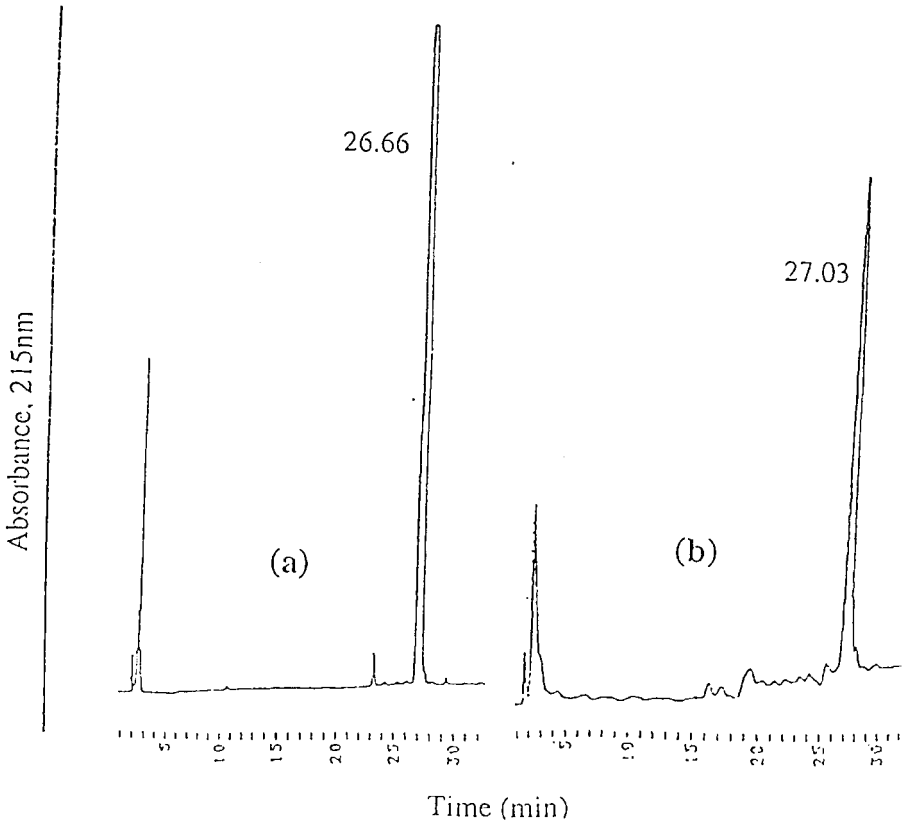


Figure 1 Analytical HPLC profiles of purified [AzAsn^2]MIR (a) and [AzAla^4]MIR (b).

performed using a Merck Lichrosorb RP 18, 7 μm column, 14–28% CH_3CN/H_2O/0.1% TFA gradient (over 28 min) elution, and analytical HPLC was carried out on a Merck Lichrospher RP18, 5 μm column using 5–30% CH_3CN/H_20/0.1% TFA gradient (over 30 min) elution, with 215 nm UV detection. IR spectra were run in the Fourier transform mode on a Bruker IFS-25 apparatus, and ^{1}H-NMR spectra on a Bruker AC-200P spectrometer with Me_4Si as internal reference. Mass spectra were obtained on an Brucker Protein TOF spectrometer. Specific rotations were measured with a Perkin Elmer automatic model 141 ($e = 10$ cm; $c = 10$ g/l in MeOH; 20 °C]. The melting points (m.p.) were uncorrected. R_F values were calculated from thin liquid layer chromatography (Merck, Silicagel 60 F_{254} plates No. 5735).

Anti-MIR antibody binding to the aza-peptides was measured by a solid-phase radioimmunoassay [21]. Some 50 μl of peptide dilutions (100 μg/ml in 50 mM carbonate buffer pH 9.6) were placed in each well of a 96 well Immuno plate (MaxiSorp F96, Nunc) and allowed to be absorbed overnight at 4 °C. Wells were washed three times with phosphate-buffered saline (PBS)–0.05% Tween-20 (PBS-Tween). Wells were then saturated with 200 μl of 2.5% bovine serum albumin (BSA) in PBS–Tween for 30 min at room temperature. After one washing, the wells were incubated with 50 μl of the test rat monoclonal antibody (dilution 1 : 500) in PBS–Tween supplemented with 0.2% BSA (PBS–Tween–BSA) for 2 h at room temperature. Subsequently the wells were washed three times and 50 μl of the second antibody (rabbit anti-rat) was added (dilution 1 : 100) in PBS–Tween–BSA for 45 min at room temperature. After three washes, the wells were incubated with 50 μl of ^{125}I-protein A in PBS–Tween–BSA (150.000 c.p.m./well, 3 nM) for 30 min. Finally, the wells were washed six times and 200 μl of 1% SDS was added for 20 min, in order to release the bound radioactivity from the plate. The content of the wells was transferred in Eppendorf tubes and radioactivity was measured in a γ-counter. All determinations were performed in triplicate.

Three monoclonal antibodies (mAbs) were tested: mAb 6 was derived from rats immunized with *Torpedo* AChR. It binds strongly to the Torpedo AChR and weakly to the human AChR; mAb 198 was derived from rats immunized with human AChR and binds to both *Torpedo* and human AChR; mAb 25 does not bind to the *Torpedo* or human AChR nor to the MIR peptides and it is used as a negative control.

Liquid-phase Syntheses

General Coupling Procedure Using Triphosgene. NMM (0.44 ml, 4 mM) in THF (1 ml) was added dropwise, under stirring, to a cold solution (−10 °C) of triphosgene (0.39 g, 1.3 mM, Aldrich 33,075-2) and the N^{β}-protected hydrazide P-N^{β}H-N^{α}H-R in THF (4 ml); stirring at −10 °C was maintained for 45 min. Then, the amino compound (4 mM) and NMM (4 mM) were added progressively under stirring at −10 °C for 1 h, and then back to room temperature for 2 h. After filtration, THF was evaporated under reduced pressure and the crude material, after extraction into DCM, was washed twice by aqueous $KHSO_4$ 0.5 M, and twice by a saturated NaCl aqueous solution, and then dried over Na_2SO_4.

***Boc-NH-NHMe* (3)**. Methylhydrazine **1** (2 ml, 37.6 mM) was progressively added to ZOSu (9.35 g, 37.6 mM) dissolved in THF (20 ml) at 0 °C, and stirring continued for 4 h at room temperature. THF was removed under reduced pressure. The hydrochloride **2** was precipitated by addition of an HCl/AcOEt 3 M solution and crystallized in absolute ethanol (70% yield). A suspension of **2** (3 g, 13.8 mM) in THF (10 ml) at 0 °C was successively treated by Boc_2O (3.32 g, 15.2 mM), 10% DMAP as a catalyst and NMM (1.6 ml, 13.8 mM). Stirring at room temperature was maintained for 12 h. HCl.NMM was filtered off, THF was evaporated and the residue was taken up by DCM (20 ml). The organic phase was washed with brine. Boc-NH-NZ-Me was crystallized from an AcOEt/PE solution. The Z groups was further hydrogenized in MeOH (10 ml) in the presence of Pd/C 5% as a catalyst under vigorous stirring at room temperature and near atmospheric pressure. After filtration and evaporation of MeOH, **3** was recovered as thin white needles in an overall yield of 50% based on methylhydrazine **1**; m.p. = 52–54 °C; $R_F = 0.37$ (AcOEt). ^{1}H-NMR ($CDCl_3$): 1.45 (s, 9H, Boc-(CH_3)$_3$); 2.60 (s, 3H, N−CH_3); 3.82 (s, 1H, N*H*−Me); 6.16 (s, 1H, N*H*−CO).

***Boc-AzAla-NMe$_2$* (7)** and Z-AzAla-NMe_2 **(8)**. **3** was treated by the triphosgene procedure with dimethylamine as the amino compound. **7** was purified by silica gel chromatography with an AcOEt : PE (80/20) mixture as eluent (80% yield); m.p. = 77 °C; $R_F = 0.38$ (*i*PrOH/DCM: 7/93); IR(KBr), 3310 cm^{-1} (NH); 1720 cm^{-1} (urethane CO); 1652 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.43 (s, 9H, Boc-(CH_3)$_3$); 2.85 (b, 6H, N−(CH_3)$_2$); 3.01 (s, 3H, AzAla-C$^{\beta}H_3$); 6.58 (s, 1H, AzAla-N*H*). **7** (0.44 g, 2 mM) was treated under

stirring with a solution of HCl/AcOEt 3 M (10 ml) for 1 h at room temperature. After evaporation of the solvent, the resulting hydrochloride HCl. H-AzAla-NMe_2 was dispersed in anhydrous THF (4 ml) and NMM (0.44 ml, 4 mM) and ZCl (0.30 ml, 2.1 mM) were successively added. After evaporation under reduced pressure, DCM (10 ml) was substituted for THF. HCl.NMM was then eliminated by filtration, before the organic phase was washed with an aqueous $KHSO_4$ 0.5 M solution, followed by an NaCl saturated aqueous solution, and finally dried over Na_2SO_4. **8** was crystallized from AcOEt (85% yield); m.p. = 105 °C; $R_F = 0.37$ (iPrOH/DCM: 7/93); IR(KBr), 3315 cm^{-1} (NH); 1730 cm^{-1} (urethane CO); 1640 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 2.88 (b, 6H, N–(CH_3)$_2$); 3.05 (s, 3H, AzAla-C$^\beta H_3$); 5.17 (s, 2H, Z-CH_2); 6.86 (s, 1H, AzAla-N*H*); 7.37 (b, 5H, Z-C_6H_5).

Boc-AzAla-Pro-NR$_1$R$_2$ **(9a,b)**. **3** was treated by the triphosgene procedure with respectively H-Pro-NHiPr or H-Pro-NMe_2 as the amino compound. **9a** was first purified by gravity silica gel chromatography with an iPrOH : DCM (8/92) mixture as eluent, and further crystallized from an AcOEt/DCM mixture (60% yield); m.p. = 156 °C; $R_F = 0.29$ (iPrOH/DCM: 8/92); $[\alpha]_D = 6.9°$; IR(KBr), 3347 and 3269 cm^{-1} (NH); 1713 cm^{-1} (urethane CO); 1669 and 1633 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.17 (dd, $^3J = 6.6$ Hz, 6H, iPr-(CH_3)$_2$); 1.50 (s, 9H, Boc-(CH_3)$_3$); 1.88 (m, 3H, Pro-C$^\beta H$ + C$^\gamma H_2$); 2.24 (m, 1H, Pro-C$^\beta H$); 3.08 (s, 3H, AzAla-C$^\beta H_3$); 3.45 (m, 2H, Pro-C$^\delta H_2$); 4.05 (m, 1H, *i*Pr-C*H*); 4.49 (m, 1H, Pro-C$^\alpha H$); 6.52 (s, 1H, AzAla-N*H*); 6.76 (b, 1 H, N*H*-*i*Pr). **9b** (60 % yield) was purified by gravity silica gel chromatography with an iPrOH: DCM (7/93) mixture as eluent; m.p. = 154 °C; $R_F = 0.31$ (iPrOH/DCM: 7/93); $[\alpha]_D = 20.5°$; IR(KBr), 3338 and 3257 cm^{-1} (NH); 1710 cm^{-1} (urethane CO); 1664 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.47 (s, 9H, Boc-(CH_3)$_3$); 1.96 (m, 4H, Pro-C$^\beta H_2$ + C$^\gamma H_2$); 2.92 and 3.06 (2s, 6H, N(CH_3)$_2$); 3.16 (s, 3H, AzAla-C$^\beta H_3$); 3.58 (m, 2H, Pro-C$^\delta H_2$); 4.82 (m, 1H, Pro-C$^\alpha H$); 6.47 (s, 1H, AzAla-N*H*).

Z-NH-NH-CH$_2$-CO$_2$Et **(5)**. NMM (2.1 ml) in THF (5 ml) was added dropwise to a stirred cold suspension (0 °C) of ethyl hydrazinoacetate hydrochloride **4** (3.0 g, 19.4 mM) and ZOSu (5.8 g, 23.3 mM, Aldrich 22,779-1) in THF (20 ml). Stirring was maintained for 4 h with progressive return to room temperature. After filtration, THF was evaporated, the crude material was dissolved in DCM and washed three times with brine. The organic phase was finally dried over Na_2SO_4. **5** was crystallized from AcOEt (85% yield); m.p. = 98–100 °C; $R_F = 0.40$ (iPrOH/DCM: 3/97). ^{1}H-NMR ($CDCl_3$): 1.27 (t, $^3J = 7.1$ Hz, 3H, Et-CH_3); 3.68 (b, 2H, N-CH_2); 4.20 (q, $^3J = 7.1$ Hz, 2H, Et-CH_2); 5.14 (s, 2H, Z-CH_2); 6.80 (s, 1H, N*H*-CO); 7.35 (b, 5H, C_6H_5).

Z-AzAsp(OEt)-NHiPr **(11)**. **5** was treated by triphosgene and $iPrNH_2$ in ethanol. **11** was crystallized from an AcOEt/PE mixture (95% yield); m.p. = 142 °C; $R_F = 0.37$ (AcOEt/PE: 50/50); IR (KBr), 3378 and 3300 cm^{-1} (NH); 1748 cm^{-1} (ester CO); 1710 cm^{-1} (urethane CO); 1654 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.09 (d, $^3J = 6.6$ Hz, 6H, iPr-(CH_3)$_2$); 1.23 (t, $^3J = 7.1$ Hz, 3H, Et-CH_3); 3.90 (m, 1H, iPr-C*H*); 4.17 (q, $^3J = 7.1$ Hz, 2H, Et-CH_2); 4.25 (b, 2H, AzAsp-C$^\beta H_2$); 5.45 (d, $^3J = 7.8$ Hz, 1H, N*H*-iPr); 7.38 (b, 5H, Z-C_6H_5); 7.42 (s, 1H, AzAsp-N*H*).

RCO-Pro-AzAsp(OEt)-NHiPr **(12a,b)**. The Z group in **11** was hydrogenolized in MeOH in the presence of Pd/C 5% as a catalyst, under vigorous stirring, at room temperature and nearly atmospheric pressure to regenerate the aza-derivative. Boc-Pro-OH **(a)** or Piv-Pro-OH **(b)** (2 mM) was dissolved in THF containing NMM (2 mM) before being treated at −18 °C by IBCF (2 mM). The aza-derivative (2 mM) was further added after stirring for 20 min at −18 °C. Stirring was kept for 4 h with progressive return to room temperature. After filtration, THF was evaporated and **12a,b** was taken up into DCM (20 ml). The organic phase was washed with brine and dried over Na_2SO_4. **12a** (80% yield) was purified by gravity silica gel chromatography (eluent, iPrOH/DCM 6/94). Oil, $R_F = 0.31$ (iPrOH/DCM: 6/94); IR (KBr), 3361 and 3258 cm^{-1} (NH); 1750 cm^{-1} (ester and urethane CO); 1679 (amide CO). ^{1}H-NMR ($CDCl_3$): 1.18 (dd, $^3J = 6.6$ Hz, 6H, iPr-(CH_3)$_2$); 1.29 (t, $^3J = 7.2$ Hz, 3H, Et-CH_3); 1.47 (s, 9H, Boc-(CH_3)$_3$) ; 2.01 (m, 4H, Pro-C$^\beta H_2$ + C$^\gamma H_2$); 3.48 (m, 2H, Pro-C$^\delta H_2$); 3.94 (m, 1H, iPr-C*H*); 3.99 (m, 1H, Pro-C$^\alpha H$); 4.20 (q, $^3J = 7.2$ Hz, 2H, Et-CH_2); 4.32 (b, 2H, AzAsp-C$^\beta H_2$); 6.08 (d, $^3J = 6.9$ Hz, 1H, N*H*-iPr); 8.10 (s, 1H, AzAsp-N*H*). **12b** (80% yield) was crystallized from an AcOEt/PE mixture; m.p. = 150–152 °C; $R_F = 0.56$ (iPrOH/DCM: 9/91); IR (KBr), 3380 and 3261 cm^{-1} (NH); 1749 cm^{-1} (ester CO); 1689 cm^{-1} (amide CO); 1605 cm^{-1} (pivalyl CO). ^{1}H-NMR ($CDCl_3$): 1.16 (dd, $^3J = 6.6$ Hz, 6H, iPr(CH_3)$_2$); 1.23 (t, $^3J = 7.2$ Hz, 3H, Et-CH_3); 1.26 (s, 9H, Piv-(CH_3)$_3$); 2.04 (m, 4H, Pro-C$^\beta H_2$ + C$^\gamma H_2$); 3.74 (m, 2H, Pro-C$^\delta H_2$); 3.91 (m, 1H, iPr-C*H*); 4.17 (q, $^3J = 7.2$ Hz, 2H, Et-CH_2); 4.17 (m, 1H, Pro-C$^\alpha H$); 4.62 (b, 2H, AzAsp-C$^\beta H_2$); 6.12 (b, 1H, N*H*-iPr); 8.05 (s, 1H, AzAsp-N*H*).

***Boc-Pro-AzAsp-NHiPr* (13)**. The ester function of **12a** (1.8 mM) dissolved in acetone (10 ml) was saponified by NaOH 1 M (2 ml) under stirring for 1 h at room temperature. Acetone was removed, water (10 ml) was added and pH was lowered to 2 with aqueous $KHSO_4$ 0.5 M. After extraction by DCM (4 × 10 ml) **13** was obtained after elimination of the solvent. **13** was recovered as a solid (90% yield); m.p. = 201–203 °C; $[\alpha]_D = -16.8°$; IR(KBr), 3351 and 3210 cm^{-1} (NH); 1750 cm^{-1} (acid CO); 1676 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.17 (dd, $^3J = 6.4$ Hz, 6H, iPr-(CH_3)$_2$); 1.48 (s, 9H, Boc(CH_3)$_3$); 2.03 (m, 4H, Pro-$C^\beta H_2 + C^\gamma H_2$); 3.47 (m, 2H, Pro-$C^\delta H_2$); 3.94 (m, 1H, iPr-$CH$); 4.12 (b, 2H, AzAsp-$C^\beta H_2$); 4.21 (m, 1H, Pro-$C^\alpha H$); 6.21 (b, 1H, N*H*-iPr); 8.65 (s, 1H, AzAsp-N*H*).

***RCO-Pro-AzAsn(R')-NHiPr* (14a–c)**. **12a,b** (1.5 mM) was stirred overnight in an NH_3 **(14a)** or $MeNH_2$ **(14b,c)** (8 M) ethanolic solution (10 ml). Ethanol was evaporated under reduced pressure, and the AzAsx derivative **14a–c** was crystallized. **14a** (90% yield): m.p. = 207 °C; $R_F = 0.46$ (MeOH/$CHCl_3$: 10/90) $[\alpha]_D = -10.3°$; crystallized from AcOEt/EtOH; IR(KBr) 3480 and 3340 cm^{-1} (NH); 1696 cm^{-1} (carboxamide CO); 1680 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.16 (dd, $^3J = 6.6$ Hz, 6H, iPr-(CH_3)$_2$); 1.50 (s, 9H, Boc-(CH_3)$_3$); 2.04 (m, 4H, Pro-$C^\beta H_2 + C^\gamma H_2$); 3.47 (m, 2H, Pro-$C^\delta H_2$); 3.92 (m, 1H, iPr-$CH$); 4.06 and 4.20 (AB, $^2J = 17.6$ Hz, 2H, AzAsn-$C^\beta H_2$); 4.13 (m, 1H, Pro-$C^\alpha H$); 5.47 and 7.64 (2s, 2H, AzAsn-$N^\delta H_2$); 6.16 (d, $^3J = 8.0$ Hz, 1H, N*H*-iPr); 8.46 (s, 1H, AzAsn-N*H*). **14b** (95% yield): m.p. = 223 °C; $R_F = 0.42$ (MeOH/$CHCl_3$: 7/93); $[\alpha]_D = -8.9°$; crystallized from AcOEt/PE; IR(KBr), 3349 cm^{-1} (NH); 1676 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.17 (dd, $^3J = 6.6$ Hz, 6H, iPr-(CH_3)$_2$); 1.46 (s, 9H, Boc-(CH_3)$_3$); 2.16 (m, 4H, Pro-$C^\beta H_2 + C^\gamma H_2$); 2.78 (d, $^3J = 4.4$ Hz, 3H, N^δ-CH_3); 3.78 (m, 2H, Pro-$C^\delta H_2$); 3.91 (m, 1H, iPr-CH); 4.17 (m, 1H, Pro-$C^\alpha H$); 3.83 and 4.44 (AB, $^2J = 16.8$ Hz, 2H, AzAsn-$C^\beta H_2$); 6.56 (d, $^3J = 7.3$ Hz, 1H, N*H*-iPr); 7.67 (b, 1H, AzAsn-$N^\delta H$); 8.14 (s, 1H, AzAsn-N*H*). **14c** (95 % yield): m.p. = 196 °C; $R_F = 0.44$ (iPrOH/DCM: 12/88); $[\alpha]_D = 44.4°$; crystallized from iPr_2O/THF; IR(KBr), 3360 and 3281 cm^{-1} (NH); 1689 and 1666 cm^{-1} (amide CO); 1607 cm^{-1} (pivalyl CO). ^{1}H-NMR ($CDCl_3$): 1.17 (dd, $^3J = 6.5$ Hz, 6H, iPr-(CH_3)$_2$); 1.28 (s, 9H, Boc-(CH_3)$_3$); 2.05 (m, 4H, Pro-$C^\beta H_2 + C^\gamma H_2$); 2.78 (d, $^3J = 4.8$ Hz, 3H, N^δ-CH_3); 3.78 (m, 2H, Pro-$C^\delta H_2$); 3.92 (m, 1H, iPr-CH); 4.17 (m, 1H, Pro-$C^\alpha H$); 3.82 and 4.44 (AB, $^2J = 17.3$ Hz, 2H, AzAsn-$C^\beta H_2$); 6.57 (d, $^3J = 7.6$ Hz, 1H, N*H*-iPr); 7.66 (b, 1H, AzAsn-$N^\delta H$); 8.15 (s, 1H, AzAsn-N*H*).

***Z-AzAsp(OEt)-Pro-NR$_1$R$_2$* (15a,b)**. **5** was treated by triphosgene, and the amino compound used was either H-Pro-NHiPr for **15a** or H-Pro-NMe_2 for **15b**. **15a** (70% yield) was crystallized from the crude product in an AcOEt/DCM mixture; m.p. = 175–176 °C; $R_F = 0.57$ (iPrOH/DCM: 10/90); IR(KBr): 3203 cm^{-1} (NH); 1758 cm^{-1} (ester CO); 1723 cm^{-1} (urethane CO); 1641 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.12 (dd, $^3J = 6.6$ Hz, 6H, iPr-(CH_3)$_2$); 1.28 (t, $^3J = 7.2$ Hz, 3H, Et-CH_3); 1.96 (m, 4H, Pro-$C^\beta H_2 + C^\gamma H_2$); 3.43 (m, 2H, Pro-$C^\delta H_2$); 4.03 (m, 1H, iPr-$CH$); 4.03 and 4.44 (AB, $^2J = 16.8$ Hz, 2H, AzAsp-$C^\beta H_2$); 4.20 (q, $^3J = 7.2$ Hz, 2H, Et-CH_2); 4.44 (m, 1H, Pro-$C^\alpha H$); 5.18 (s, 2H, Z-CH_2); 6.41 (b, 1H, N*H*-iPr); 7.37 (b, 6H, Z-C_6H_5 + AzAsn-N*H*). **15b** (65% yield) was crystallized from the crude product in an AcOEt/PE mixture; m.p. = 118–119 °C; $R_F = 0.35$ (iPrOH/DCM: 5/95); IR(KBr), 3263 cm^{-1} (NH); 1752 cm^{-1} (ester CO); 1717 cm^{-1} (urethane CO); 1656 (amide CO). ^{1}H-NMR ($CDCl_3$): 1.25 (t, $^3J = 7.2$ Hz, 3H, Et-CH_3); 1.88 (m, 4H, Pro-$C^\beta H_2 + C^\gamma H_2$); 2.89 and 3.02 (2s, 6H, N-(CH_3)$_2$); 3.53 (m, 2H, Pro-$C^\delta H_2$); 4.09 (b, 2H, AzAsn-$C^\beta H_2$); 4.18 (q, $^3J = 7.2$ Hz, 2H, Et-CH_2); 4.76 (m, 1H, Pro-$C^\alpha H$); 5.18 (s, 2H, Z-CH_2); 7.36 (b, 6H, Z-C_6H_5 + AzAsn-N*H*).

***Z-AzAsn(R')-Pro-NR$_1$R$_2$* (17a–c)**. **15a,b** (1.5 mM) was stirred overnight in an NH_3 or $MeNH_2$ (8 M) ethanolic solution (10 ml). Ethanol was removed under reduced pressure and the AzAsn product **17a–c** was crystallized from the crude material. **17a** (90% yield): m.p. = 166–167 °C; $R_F = 0.31$ (iPrOH/DCM: 10/90); $[\alpha]_D = 56.6°$; crystallized from AcOEt/iPrOH; IR(KBr), 3446, 3338 and 3313 cm^{-1} (NH); 1726 cm^{-1} (urethane CO); 1687 cm^{-1} (carboxamide CO); 1643 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.13 (dd, $^3J = 6.6$ Hz, 6H, iPr-(CH_3)$_2$); 1.91 (m, 4H, Pro-$C^\beta H_2 + C^\gamma H_2$); 3.39 (m, 2H, Pro-$C^\delta H_2$); 4.01 (m, 1H, iPr-$CH$); 4.05 and 4.24 (AB, $^2J = 14.7$ Hz, 2H, AzAsn-$C^\beta H_2$); 4.39 (m, 1H, Pro-$C^\alpha H$); 5.14 and 5.22 (AB, $^2J = 12.2$ Hz, 2H, Z-CH_2); 5.42 and 6.55 (2s, AzAsn-$N^\delta H_2$); 6.38 (b, 1H, N*H*-iPr); 7.36 (b, 5H, Z-C_6H_5); 7.55 (s, 1H, AzAsn-N*H*). **17b** (95% yield): m.p. = 180–181 °C; $R_F = 0.37$ (iPrOH/DCM: 10/90); $[\alpha]_D = 58.2°$; crystallized from AcOEt/MeOH; IR(KBr), 3419, 3353 and 3212 cm^{-1} (NH); 1726 cm^{-1} (urethane CO); 1674 and 1631 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.12 (dd, $^3J = 6.5$ Hz, 6H, iPr-(CH_3)$_2$); 1.76 (m, 3H, Pro-$C^\beta H$ + $C^\gamma H_2$); 2.20 (m, 1H, Pro-$C^\beta H$); 2.76 (d, $^3J = 4.8$ Hz, 3H, N^δ-CH_3); 3.37 (m, 2H, Pro-$C^\delta H_2$); 3.98 and 4.21 (AB, $^2J = 16.6$ Hz, 2H, AzAsn-$C^\beta H_2$); 4.03 (m, 1H, iPr-

C*H*); 4.36 (m, 1H, Pro-C$^{\alpha}$*H*); 5.13 and 5.22 (AB, $^{2}J = 12.1$ Hz, 2H, Z-CH_2); 6.45 (d, $^{3}J = 6.9$ Hz, 1H, N*H*-iPr); 6.56 (b, 1H, AzAsn-N$^{\delta}$*H*); 7.35 (b, 5H, Z-C$_6H_5$); 7.53 (s, 1H, AzAsn-N*H*). **17c** (95% yield): m.p. = 108 °C; $R_F = 0.30$ (iPrOH/DCM: 13/87); $[\alpha]_D = -3.1$ °; crystallized from AcOEt/MeOH; IR(KBr): 3461 and 3290 cm^{-1} (NH); 1721 cm^{-1} (urethane CO); 1696 cm^{-1} (carboxamide CO); 1651 (amide CO). ^{1}H-NMR ($CDCl_3$): 1.87 (m, 4H, Pro-C$^{\beta}H_2$ + C$^{\gamma}H_2$); 2.82 and 2.95 (2s, 6H, N(CH_3)$_2$); 3.48 (m, 2H, Pro-C$^{\delta}H_2$); 4.02 (b, 2H, AzAsn-C$^{\beta}H_2$); 4.69 (m, 1H, Pro-C$^{\alpha}$*H*); 5.06 and 5.13 (AB, $^{2}J = 11.7$ Hz, 2H, Z-CH_2); 5.52 and 7.54 (2s, 2H, AzAsn-N$^{\delta}H_2$); 7.29 (b, 5H, Z-C$_6H_5$); 7.44 (s, 1H, AzAsn-N*H*).

***Z-AzAsp-Pro-NHiPr* (16)**. **16** was obtained as a solid (90% yield) by the same procedure as **13**; m.p. = 185–186 °C; $R_F = 0.19$ (MeOH/$CHCl_3$: 10/90); $[\alpha]_D = 41.6$ °; IR(KBr), 3351 cm^{-1} (NH); 1750 cm^{-1} (acid CO); 1715 cm^{-1} (urethane CO); 1676 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.10 (d, $^{3}J = 6.4$ Hz, 6H, iPr-(CH_3)$_2$); 1.80 (m, 3H, Pro-C$^{\beta}H$ + C$^{\gamma}H_2$); 2.11 (m, 1H, Pro-C$^{\beta}$*H*); 3.41 (m, 2H, Pro-C$^{\delta}H_2$); 3.97 (m; 1H, iPr-C*H*); 4.21 (b, 2H, AzAsp-C$^{\beta}H_2$); 4.37 (m, 1H, Pro-C$^{\alpha}$*H*); 5.16 (s, 2H, Z-CH_2); 6.46 (b, 1H, N*H*-iPr); 7.35 (b, 5H, Z-C$_6H_5$); 7.83 (s, 1H, AzAsp-N*H*).

***Z-AzAsn(Me$_2$)-Pro-NHiPr* (18)**. **16** (1.5 mM) was dissolved in a THF/DMF (v/v) mixture (3 ml) containing NMM (1.5 mM) before treatment at −18 °C by IBCF (1.5 mM). Me_2NH (0.5 ml) was further added after stirring for 20 min at −18 °C. Stirring was maintained for 4 h with a gradual return to room temperature. After filtration, THF and DMF were removed under reduced pressure, and **18** was taken up into DCM (20 ml). The organic phase was washed with brine and dried over Na_2SO_4. **18** (78% yield) was purified by gravity silica gel chromatography (eluent: iPrOH/DCM: 9/91); m.p. = 196 °C; $R_F = 0.41$ (iPrOH/DCM: 9/91); $[\alpha]_D$, 45.2 °. IR(KBr), 3360 and 3220 cm^{-1} (NH); 1728 cm^{-1} (urethane CO); 1660 cm^{-1} (amide CO). ^{1}H-NMR ($CDCl_3$): 1.13 (dd, $^{3}J = 6.6$ Hz, 6H, iPr-(CH_3)$_2$); 2.02 (m, 4H, Pro-C$^{\beta}H_2$ + C$^{\gamma}H_2$); 2.93 (b, 6H, N$^{\delta}$(CH_3)$_2$); 3.43 (m, 2H, Pro-C$^{\delta}H_2$); 4.07 (m; 1H, iPr-C*H*); 4.12 (b, 1H, AzAsn-C$^{\beta}$*H*); 4.43 (m, 1H, Pro-C$^{\alpha}$*H*); 4.54 (b, 1H, AzAsn-C$^{\beta}$*H*); 5.13 (s, 2H, Z-CH_2); 6.44 (b, 1H, N*H*-iPr); 7.36 (m, 5H, Z-C$_6H_5$); 7.71 (s, 1H, AzAsn-N*H*).

***Boc-Trp-AzAsp(OEt)-Pro-NHiPr* (19)**. The Z-group of **15a** was hydrogenolyzed in the presence of Pd/C 5% with regeneration of the aza-peptide derivative. Boc-Trp-OH (2 mM) was dissolved in THF containing NMM (2 mM) before being treated at −18 °C by IBCF (2 mM). The aza-peptide derivative (2 mM) was further added by portion after stirring for 20 min. Stirring was maintained for 4 h with a gradual return to room temperature. After filtration, THF was evaporated and crude **19** was purified by gravity silica gel chromatography (eluent: iPrOH/DCM 5/95). **19** (70% yield) was obtained as an oil; $R_F = 0.57$ (iPrOH/DCM: 9/91). ^{1}H-NMR ($CDCl_3$): 1.17 (m, 9H, Et-CH_3 + iPr-(CH_3)$_2$); 1.44 (s, 9H, Boc-(CH_3)$_3$); 1.62 (m, 3H, Pro-C$^{\beta}H$ + C$^{\gamma}H_2$); 2.18 (m, 1H, Pro-C$^{\beta}$*H*); 2.71 (m, 1H, Pro-C$^{\delta}$*H*); 3.14 (m, 1H, Pro-C$^{\delta}$*H*); 3.25 (b, 2H, Trp-C$^{\beta}H_2$); 3.70 and 4.05 (AB, $^{2}J = 17.7$ Hz, 2H, AzAsp-C$^{\beta}H_2$); 4.09 (m, 3H, Et-CH_2 + iPr-C*H*); 4.30 (m, 1H, Pro-C$^{\alpha}$*H*); 4.46 (m, 1H, Trp-C$^{\alpha}$*H*); 5.06 (d, $^{3}J = 7.6$ Hz, 1H, Trp-N*H*); 6.69 (d, $^{3}J = 8.0$ Hz, 1H, N*H*-iPr); 7.16 (m, 3H, Trp-*arom.*); 7.33 (m, 1H, Trp-*arom.*); 7.59 (m, 1H, Trp-C$^{\delta}$*H*); 8.25 (s, 1H, AzAsp-N*H*); 8.57 (s, 1H, Trp-N$^{\varepsilon}$*H*).

***Boc-Trp-AzAsn-Pro-NHiPr* (20)**. **19** (1 mM) was stirred overnight in a NH_3 ethanolic solution (8 M). Ethanol was removed under reduced pressure and the AzAsn derivative was first purified by gravity silica gel chromatography (eluent: iPrOH/DCM 18/82) and then crystallized from an AcOEt/EtOH solution (90% yield); m.p. = 165–166 °C; $[\alpha]_D$: 27.9 °; $R_F = 0.40$ (iPrOH/DCM: 18/82). ^{1}H-NMR (DMSO-d$_6$): 1.10 (dd, $^{3}J = 6.5$ Hz, 6H, iPr-(CH_3)$_2$); 1.35 (s, 9H, Boc-(CH_3)$_3$); 1.47 (m, 3H, Pro-C$^{\beta}H$ + C$^{\gamma}H_2$); 2.02 (m, 1H, Pro-C$^{\beta}$*H*); 2.51 (m, 1H, Pro-C$^{\delta}$*H*); 2.94 and 3.13 (ABX, ^{3}*J*AX = 6.5 Hz, ^{3}*J*BX = 7.3 Hz, $^{2}J = 14.3$ Hz, 2H, Trp-C$^{\beta}H_2$); 3.18 (m, 1H, Pro-C$^{\delta}$*H*); 3.42 and 4.14 (AB, $^{2}J = 18.4$ Hz, 2H, AzAsp-C$^{\beta}H_2$); 3.85 (m; 1H, iPr-C*H*); 4.05 (m, 1H, Pro-C$^{\alpha}$*H*); 4.33 (m, 1H, Trp-C$^{\alpha}$*H*); 7.04 (m, 5H, N*H*-iPr + AzAsn-N$^{\delta}$*H* + Trp-*arom.*); 7.31 (m, 3H, AzAsn-N$^{\delta}$*H* + Trp-N*H* + Trp-*arom.*); 7.61 (m, 1H, Trp-C$^{\delta}$*H*); 10.52 (s, 1H, AzAsp-N*H*); 10.88 (s, 1H, Trp-N$^{\varepsilon}$*H*).

***Z-AzAsp(OEt)-Pro-OtBu* (21)**. **5** was treated by triphosgene and H-Pro-OtBu as the amino compound. **21** (75% yield) was purified by gravity silica gel chromatography (eluent: AcOEt/EP: 35/65). Oil; $R_F = 0.32$ (AcOEt/PE: 35/65). ^{1}H-NMR ($CDCl_3$): 1.17 (t, $^{3}J = 7.1$ Hz, 3H, Et-CH_3); 1.38 (s, 9H, tBu-(CH_3)$_3$); 1.96 (m, 4H, Pro-C$^{\beta}H_2$ + C$^{\gamma}H_2$); 3.40 (m, 2H, Pro-C$^{\delta}H_2$); 4.05 (b, 2H, AzAsp-C$^{\beta}H_2$); 4.10 (q, $^{3}J = 7.1$ Hz, 2H, Et-CH_2); 4.23 (m, 1H, Pro-C$^{\alpha}$*H*); 5.07 (s, 2H, Z-CH_2); 7.35 (b, 5H, Z-C$_6H_5$); 7.45 (s, 1H, AzAsp-N*H*).

***Z-Trp-AzAsp(OEt)-Pro-OtBu* (22)**. The Z group in **21** was hydrogenolyzed in the presence of Pd/C 5% to regenerate the aza-peptide derivative. Z-Trp-OH (2 mM) was dissolved in THF containing NMM (2 mM) before being treated at −18 °C by IBCF (2 mM). The aza-peptide derivative (2 mM) was further added after stirring for 20 min. Stirring was maintained for 4 h with progressive return to room temperature. After filtration, THF was evaporated and **22** was taken up into DCM (20 ml). The organic phase was washed with brine and finally dried over Na_2SO_4. **22** (75% yield) was crystallized from AcOEt/DCM; m.p. = 167–168 °C; $R_F = 0.32$ (AcOEt/PE: 60/40). ^{1}H-NMR ($CDCl_3$): 1.11 (t, $^3J = 7.2$ Hz, 3H, Et-CH_3); 1.40 (s, 9H, tBu-(CH_3)$_3$); 1.68 (m, 3H, Pro-C$^\beta H$ + C$^\gamma H_2$); 2.03 (m, 1H, Pro-C$^\beta H$); 3.22 (m, 4H, Trp-C$^\beta H_2$ + Pro-C$^\delta H_2$) 3.42 and 3.92 (AB, 2H, $^2J = 19.5$ Hz, AzAsp-C$^\beta H_2$); 3.96 (q, $^3J = 7.2$ Hz, 2H, Et-CH_2); 4.21 (m, 1H, Pro-C$^\alpha H$); 4.54 (m, 1H, Trp-C$^\alpha H$); 5.10 (s, 2H, Z-CH_2); 5.44 (d, $^3J = 7.8$ Hz, 1H, Trp-NH); 7.19 (m, 4H, Trp-*arom.*); 7.35 (b, 5H, Z-C$_6H_5$); 7.60 (m, 1H, Trp-C$^\delta H$); 8.20 (s, 1H, AzAsp-NH); 8.27 (s, 1H, Trp-N$^\varepsilon H$).

***Z-Trp-AzAsn-Pro-OH* (23)**. **22** (2 mM) was stirred overnight in a NH_3 ethanolic solution (8 M). Ethanol was removed under reduced pressure and the AzAsn derivative (75% yield) was purified by gravity silica gel chromatography (eluent: iPrOH/DCM: 8/92). One gram (1.65 mM) of this latter compound was treated upon stirring with a solution (11 ml) of TFA/DCM/anisole (5/5/1) for 2 h at 0 °C. The solvents were removed under reduced pressure. After lyophilization, **23** was used directly in the SPPS procedure. ^{1}H-NMR (DMSO-d$_6$): 1.68 (m, 3H, Pro-C$^\beta H$ + C$^\gamma H_2$); 1.98 (m, 1H, Pro-C$^\beta H$); 3.05 (m, 2H, Trp-C$^\beta H_2$); 3.28 (m, 2H, Pro-C$^\delta H_2$); 3.77 (b, 2H, AzAsp-C$^\beta H_2$); 4.09 (m, 1H, Pro-C$^\alpha H$); 4.22 (m, 1H, Trp-C$^\alpha H$); 4.92 and 5.01 (AB, $^2J = 11.76$ Hz, 2H, Z-CH_2); 7.21 (m, 11H, Z-C$_6H_5$ + AzAsn-N$^\delta H_2$ + Trp-*arom.*); 7.64 (m, 2H, Trp-NH + Trp-C$^\delta H$); 10.44 (s, 1H, AzAsp-NH); 10.82 (s, 1H, Trp-N$^\varepsilon H$).

***Boc-Pro-NH-NH-CH$_3$* (28)**. Boc-Pro-OH (4 mM) was dissolved in THF containing NMM (2 mM) before being treated at −18 °C by IBCF (4 mM). **2** (4 mM) and NMM (4 mM) were further added after stirring for 20 min at −18 °C. Stirring was maintained for 4 h with a gradual return to room temperature. After filtration, THF was evaporated. The Z group in the resulting product was hydrogenolyzed on Pd/C 5%. **28** was obtained as a white solid crystallized from an AcOEt/PE solution (76% yield); m.p. = 95 °C; $R_F = 0.37$ (AcOEt). ^{1}H-NMR ($CDCl_3$): 1.30 (s, 9H, Boc-(CH_3)$_3$); 1.85 (m, 3H, Pro-C$^\beta H$ + C$^\gamma H_2$); 2.05 (m, 1H, Pro-C$^\beta H$); 2.50 (b, 3H, N-CH_3); 3.30 (m, 2H, Pro-C$^\delta H_2$); 4.05 (m, 1H, Pro-C$^\alpha H$); 5.00 (b, 1H, NH-Me); 8.30 (b, 1H, NH-CO).

Solid-phase Syntheses

The aza-peptides [AzAsn^2]MIR **26** and [AzAla^4]MIR **34** were prepared from N$^\alpha$-Boc-Lys(2-ClZ)-PAM (Neosystem, 0.68 mM/g substitution). The peptide chain was assembled by sequential coupling of the N$^\alpha$-Boc-amino acids (3 equiv.) in the presence of BOP (3 equiv.), BtOH (3 equiv.) and diisopropylethylamine (DIEA, 9 equiv.) in DMF for 5 h [18]. Completion of each coupling was monitored by the Kaiser ninhydrin test. Deprotection of the Boc group was achieved by 40% trifluoroacetic acid (TFA) in DCM.

Incorporation of the AzAsn residue into the MIR decapeptide. The 3 equivalent of **23** (1.0 mM) was added to the resin-bonded heptapeptide H-Ala-Asp(OBzl)-Tyr(2,6-Cl$_2$-Bzl)-Gly-Gly-Ile-Lys(2-ClZ)-PAM (0.34 mM), which was prepared by the standard Boc/BOP SPPS protocol using DMF (6 ml). Then, BOP (1.0 mM), BtOH (1 mM) and DIEA (3.0 mM) were successively added. Reaction proceeded for 5 h and the resin was then washed according to a normal washing cycle [18] (3 × 10 ml DCM, 3 × 10 ml MeOH and 3 × 10 ml DCM). A standard cleavage using a mixture (0.38 ml) of thioanisole/ethanedithiol (2/1) as scavengers, 2.5 ml TFA and 0.25 ml of TFMSA afforded the crude deprotected aza-decapeptide. This latter was desalted on Sephadex G-25 (eluent: nBuOH/pyridine/AcOH/H_2O 15/10/3/12) and purified by semipreparative HPLC. The aza-decapeptide yield, estimated by HPLC, was about 70%. Fractions determined to be pure by analytical reversed-phase HPLC were pooled, concentrated under reduced pressure and lyophilized to obtain the aza-peptide as a white solid (> 98% pure, Figure 1(a)). The aza-peptide was characterized by time-of-flight mass spectoscopy (calculated) 1121.4 ($M+1$); found, 1122.2) and by 2D-NMR spectroscopy. The AzAsn residue is identified by the singlet at 9.15 p.p.m. (AzAsn-NH) and by the two peaks at 3.35 ppm and 4.10 ppm (AzAsn-C$^\beta H_2$).

Incorporation of the AzAla Residue into the MIR Decapeptide. The synthon **28** (2.7 mM) was treated by triphosgene according to the general procedure, and in this case, the amino compound is the N-terminus of the resin-bonded hexapeptide

H-Asp(OBzl)-Tyr(2-BrZ)-Gly-Gly-Ile-Lys(2-ClZ)-PAM (0.34 mM). After reaction, the PAM-bonded aza-octapeptide **32** is washed three times by DCM. Incorporation of Asp and Trp was classically achieved by successive cycles of coupling (BOP/BtOH)/deprotection (TFA/DCM, 40/60). The aza-decapeptide **34** was purified as indicated above and recovered as a white powder (>95% pure, Figure 1(b)). It was characterized by time-of-flight mass spectoscopy (calculated, 1121.4 ($M+1$); found, 1122.9) and by 2D-NMR. Incorporation of the AzAla residue is confirmed by the two singlets at 9.20 p.p.m. (AzAla-N*H*) and 3.2 p.p.m. (AzAla-C$^{\beta}H_3$).

General Remarks

In aza-peptide chemistry, the reagents usually used for the carbonylation-activation step are either nitrophenylchloroformates or bis(2,4-dinitrophenyl)carbonate. The resulting nitrophenyl carbazates are not very reactive and require long coupling times and mostly high temperature, thus giving rise to poor yields and numerous side products. Gaseous phosgene or toluene solution has been also recommended, but its use suffers from toxicity and difficulties in measuring easily defined and precise quantities. Bis(trichloromethyl)carbonate (triphosgene) is a phosgene substitute that retains the high reactivity of phosgene but that can be handled with the safety of a stable crystalline solid. Moreover, temperature and condensation time are greatly reduced while purity and yield are increased. It has been used with success, in both liquid- and solid-phase procedures, for the synthesis of various aza-peptides containing the aza-analogues of alanine, aspartic acid and asparagine. Last but not least, the N$^{\alpha}$/CH$^{\alpha}$ apparently tiny substitution is able to perturb locally and largely the tridimensional structure and therefore the behaviour of a biologically active peptide, thus highlighting all its interest in pseudopeptide chemistry.

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REFERENCES

1. S. Goldschmidt and M. Wick (1952). Uber peptid-synthesen I. *Liebigs Ann. Chem. 575*, 217–231.
2. H. Niedrich and C. Oehme (1972). Synthese von eledoisin-octapeptiden mit den carbazylresten azaglycin und α-azaasparagin statt glycin und asparagin. *J. Prakt. Chem. 314*, 759–768.
3. J. Gante (1965). Uber neuartige azapeptide. *Chem. Ber. 98*, 540–547.
4. J. Gante (1989). Azapeptides. *Synthesis*, 405–413.
5. A. S. Dutta and J. S. Morley (1975). Polypeptides. Part XIII. Preparation of α-aza-amino-acid (carbazic acid) derivatives and intermediates for the preparation of α-aza-peptides. *J. Chem. Soc. Perkin Trans. I*, 1712–1720.
6. J. Gante (1994). Peptidomimetics-tailored enzyme inhibitors. *Angew. Chem. Int. Ed. Engl. 33*, 1699–1720.
7. Z. Benatalah, A. Aubry, G. Boussard and M. Marraud (1991). Evidence for a β-turn in an azadipeptide sequence. Synthesis and crystal structure of Bu^{t}CO-Pro-AzaAla NHPr^{i}. *Int. J. Peptide Protein Res. 38*, 603–605.
8. A. Lecoq, G. Boussard, M. Marraud and A. Aubry (1992). The couple Pro/AzaPro: A means of β-turn formation control. Synthesis and conformation of two AzaPro-containing dipeptides. *Tetrahedron Lett. 33*, 5209–5212.
9. C. J. Gray, M. Quibell, N. Baggett and T. Hammerle (1992). Incorporation of azaglutamine residues into peptides synthesized by the ultra-high load solid (gel)-phase technique. *Int. J. Peptide Protein Res. 40*, 351–362.
10. M. Quibell, W. G. Turnell and T. Johnson (1993). Synthesis of azapeptides by the Fmoc/tert-butyl/-polyamide technique. *J. Chem. ,Soc. Perkin Trans. I*, 2843–2849.
11. C. J. Gray, M. Quibell, K.-L. Jiang and N. Baggett (1991). Synthesis and spectroscopic properties of azaglutamine amino acid and peptide derivatives. *Synthesis 3*, 141–146.
12. C. J. Gray, J. C. Ireson and R. C. Parker (1977). Preparation and properties of some aza-amino-acid derivatives; their possible use in peptide synthesis. *Tetrahedron 33*, 739–743.
13. J. Gante, M. Krug, G. Lauterbach, R. Weitzel and W. Hiller (1995). Synthesis and properties of the first all-aza analogue of a biologically active peptide. *J. Peptide Sci. 2*, 201–206.
14. A. N. Kurtz and C. Niemann (1961). Synthesis and properties of 1-acetyl-2-benzyl carbazate, an analog of acetyl-D- and L-phenylalanine ethyl ester. *J. Org. Chem. 26*, 1843–1846.
15. M. T. Cung, M. Marraud, I. Hadjidakis, E. Bairaktari, C. Sakarellos, A. Kokla and S. J. Tzartos (1989). Two-dimensional ^{1}H-NMR study of synthetic peptides containing the main immunogenic region of the *Torpedo* acetylcholine receptor. *Biopolymers 28*, 465–478.

16. I. Papadouli, S. Potamianos, I. Hadjidakis, E. Bairaktari, V. Tsikaris, C. Sakarellos, M. T. Cung, M. Marraud and S. J. Tzartos (1990). Antigenic role of single residues within the main immunogenic region of the nicotinic acetylcholine receptor. *Biochem. J. 269*, 239–245.
17. H. Eckert and B. Forster (1987). Triphosgene, a crystalline phosgene substitute. *Angew. Chem. Int. Ed. Engl. 26*, 894–895.
18. E. Atherton and R. C. Sheppard in: *Solid phase synthesis, a practical approach*, D. Rickwood and B. Hames, Eds., Oxford University Press, Oxford 1989.
19. F. Pinnen, G. Luisi, G. Lucente, E. Gavuzzo and S. Cerrini (1993). Approaches to pseudopeptidic ergopeptines. *J. Chem. Soc. Perkin Trans. I*, 819–824.
20. M. Marraud and A. Aubry (1996). Crystal structures of peptides and modified peptides. *Biopolym (Peptide Sci.) 40*, 45–83.
21. S. J. Tzartos, A. Kokla, S. Walgrave and B. Conti-tronconi (1988). Localization of the main immunogenic region of human muscle acetylcholine receptor to residues 67–76 of the α-subunit. *Proc. Natl. Acad. Sci. USA 85*, 2899–2903.

