p-Nitrobenzyloxycarbonyl (pNZ) as Temporary N^{α}-Protecting Group for Mild Solid-Phase Peptide Synthesis. Avoiding Diketopiperazine and Aspartimide Formation

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Abstract. *p*-Nitrobenzyloxycarbonyl was used as temporary protecting group for the α-amino function in solid-phase peptide synthesis. The corresponding derivatives are solids, easy to be synthesized, and perform well in the solid-phase mode. pNZ is removed in practical neutral conditions in the presence of catalytic amounts of acid. They are orthogonal with the most common protecting groups used in peptide chemistry. They are specially useful in combination with Fmoc chemistry to overcome those side reactions associated with the used of the piperidine such DKP and aspartiimide formation. The flexibility of pNZ can be very useful for the preparation of libraries of small organic molecules.

Keywords. Orthogonal protecting group, Nitro reduction, Side-reactions, combinatorial chemistry.

Introduction

The development of the solid-phase methodology for the preparation of peptides carried by Bruce R. Merrifield[1] in the late fifties and early sixties has been crucial for positionating peptides as important biochemical tools and, more important, to play an important role in several therapeutic areas.[2] Nowdays, more than 40 peptides are into the market, 4 more in registration, 200 in clinical phases, and more of 400 in advanced preclinical.[3]

Solid-phase peptide synthesis (SPPS) strategies come dictated by the lability of both the *temporary* (for the N^{α}-amino function) and the *permanent* (for side-chain functions and for the anchoring of the *C*-terminal to the solid support trough the handle) protecting groups.[4] The seminal SPPS strategy proposed by Merrifield and fine tuned over the years is based on a graduated acid lability of the *tert*-butyolxycarbonyl (Boc) as *temporary* group, which is removed by trifluoroacetic acid (TFA) usually 25-50% in CH_2Cl_2; and benzyl type *permanent* protecting groups, which are removed by strong acids, such as anhydrous HF or trifluoromethanesulfonic acid.[4] Although the Boc based method has been showed to be successful for the preparation of large number of peptides, the long exposure of the peptide chain to TFA in the removal of the Boc group can also cause premature removal of the benzyl protecting group. Furthermore some fragile residue containing peptides do not survive the relatively harsh acid

conditions. Finally, HF can be considered a dangerous gas that requires to be treated in an special Teflon reactor. All these have fuelled the development of milder strategies avoiding the use of the harsh acid conditions. Thus, nowdays most of the peptides are prepared using the Fmoc–*t*-butyl strategy.[4,5] This has the additional advantage of being an orthogonal scheme[6] as the Fmoc (*temporary group*) is removed by piperidine usually 20% in *N,N-dimethylformamide* (DMF) and the *permanent* protecting groups are removed by high percentages of TFA in the presence of scavengers. Thus, the selective deprotection is governed by alternative cleavage mechanism rather than by reaction rates. Although this has become the strategy of choice for the preparation of peptides, the preparation of complex molecules such as cyclic or branched systems could require the use of other protecting groups.[7] Furthermore, the conditions used to remove the Fmoc group are still, in some cases, too harsh and can be incompatible with several sequences. The main drawbacks associated with the use of the Fmoc are that both the piperidine, which is a base and a rather good nucleophile, and the free amine that is formed can provoke some side reactions. Thus, an optimal group for the protection of the N^{α}-amino function as alternative to the Fmoc group should be removed in practical neutral conditions and the deprotection step should left the amine function masked to prevent side reactions. In addition, the protecting group should be orthogonal with *t*-butyl based protecting groups and others such as the allyl based and the same Fmoc if they should be used for the preparation of complex peptides or other organic molecules.

Herein, the use of the *p*-nitrobenzyloxycarbonyl (pNZ) (Figure 1) group for the *temporary* protection of the α-amino function in a SPPS strategy is reported.

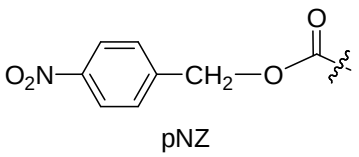

Figure 1. Structure of pNZ protecting group

The pNZ group, which is a carbamate type protecting group, was first described by Carpenter and Gish as an alternative to the benzyloxycarbonyl (Z).[8] Furthermore, it has been used for the protection of

the ε-amino function of Lys.[9] The related *p*-nitrobenzyl (pNB) group has been used in peptide synthesis for the protection of the *C*-terminal carboxylic function in side-chain anchoring strategies for the SPS of head-to-tail cyclic peptides,[10] and for another function in classical organic chemistry.[11]

Results and Discussion

Preparation of *p*-nitrobenzyloxycarbonyl(pNZ)-amino acids

The classical method for the preparation of N^{α}-carbamate protected amino acids is through the corresponding and reactive chloroformate reagents under Schotten-Baumann conditions, which can lead to the formation of protected dipeptides as side products.[12] This high incidence of protected dipeptide can lead to the insertion of an extra amino acid in the final peptide synthesis, which cannot be tolerated for use in therapeutic applications. Several approaches based on the use of different activated formates have been proposed to minimize this problematic side reaction.[13] An efficient, competitive and inexpensive procedure is the azide method.[12,14,15] In this case, an optimized *one-pot* protocol based on the azide, which was recently described, was used.[13] pNZ-Cl reacts with NaN_3 and without isolating the pNZ-N_3, the reaction with the free amino acids leads to the pNZ-amino acids. These derivatives, which were obtained in relatively high yields (71-94%) and purity, were characterized by HPLC, IR, $^{1}H/^{13}C$-NMR, and HRMS.

Solubility of pNZ – and Fmoc-amino acids

A good solubility of the protected amino acids is important for running reactions at high concentration and for facilitating the use of these derivatives in an automatic mode, which is a technique very used for SPPS. In this regards, pNZ derivatives have shown to present superior solubility in DMF to the corresponding Fmoc-derivatives (Table 1).

Table 1. Solubility comparison of pNZ-aa-OH *versus* to Fmoc derivatives

Amino Acid	Solubility in DMF[a] (g/mL)
pNZ-L-Phe-OH	0.80
Fmoc-L-Phe-OH	0.31
pNZ-L-Gly-OH	1.33
Fmoc-L-Gly-OH	0.80
pNZ-L-Asp(O^{t}Bu)-OH	1.00
Fmoc-L-Asp(O^{t}Bu)-OH	0.67

[a]All derivatives except pNZ-L-Asp(O^{t}Bu)-OH show negligible solubility in CH_2Cl_2.

Removal of the pNZ group

The pNZ can be removed by catalytic hydrogenation and other reducing agents of the nitro group. In this case, the first step is the reduction of the nitro group to give the *p*-aminobenzylozycarbonyl derivative, which suffers a spontaneous collapse *via* 1,6-electron pair shift to render the quinonimine methide and the carbamic acid. Finally, this decomposes to give the free amine (Figure 2).[16] If this process is carried out in the presence of catalytic amounts of an acid, the ammonium salt is obtained.

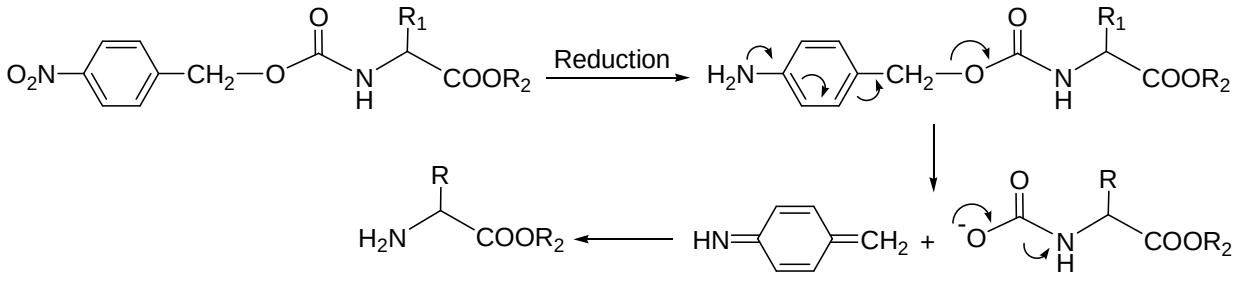

Figure 2. Mechanism for the removal of the pNZ group

As it is shown in Figure 2, the key step in the pNZ removal process is the reduction of the nitro group to the corresponding amino. Although the most common method for this functional group interconversion is catalytic hidrogention,[17] it is not useful for SPPS. Other common methods are the use of metals such as Zn,[11b] Fe,[18] or Sn[18] in acidic solutions, but the use of this chemistry for the present

purposes has two main problems: (i) the difficulty in working with metals on solid phase and (ii) the strong acidic conditions required. Finally, two reagents, $Na_2S_2O_4$[19] and $SnCl_2$,[8b,20,21] which in principle could be compatible with solid phase synthesis were essayed.

$Na_2S_2O_4$

Although $Na_2S_2O_4$ is a good reductor of nitro groups in basic medium,[22] there are also described several applications of its use in neutral or nearly neutral medium.[19]

The main problem for the use of $Na_2S_2O_4$ in solid-phase in its solubility. Thus, $Na_2S_2O_4$ is very insoluble in DMF and other solvents that swells well the resin. Furthermore, $Na_2S_2O_4$ for reducing the nitro groups needs H_2O, which is not a good solvent for the most of solid supports. To try to overcome that, 15-Crown-5 was used to solubilize the $Na_2S_2O_4$. First of all, several attempts of removing the pNZ group of pNZ-Orn(Boc)-OH in solution were carried out varying the amounts of water and 15-Crown-5. It was found by TLC (CONDITIONS) that the rate of cleavage increases with the the concentration of H_2O and 15-Crown-5

$SnCl_2$ is a good reductor of nitro group in the presence of catalytic amounts of acid. The first acid tested was HOAc, but for preventing acetilation of the amino fucntion, other non carboxylic acids were tested directly in solid-phase.

pNZ-Phe-Gly-Gly-Ley-NH-Rink-polystyrene resin was used as a model. Removal of the pNZ group were carried out in different conditions using both $Na_2S_2O_4$ and $SnCl_2$ as reducing agents and the peptide crude was analyzed by HPLC (Table 2).

Table 2. Removal of pNZ group from pNZ-Phe-Gly-Gly-Ley-NH-Rink-polystyrene resin

Entry	Removal Conditions[a]	T(ºC)	Time (min)	%[b]
1	1M $Na_2S_2O_4$ in H_2O-AcCN-EtOH (1:1:1)	rt	360	0
2	1M $Na_2S_2O_4$ and 15-Crown-5 in DMF–H_2O (9:1)	rt	60	11
3	1M $Na_2S_2O_4$ and 15-Crown-5 in DMF–H_2O (9:1)	rt	420	35
4	1M $Na_2S_2O_4$, 15-Crown-5, and DIEA in DMF–H_2O (9:1)	rt	60	12
5	1M $Na_2S_2O_4$, 15-Crown-5, and DIEA in DMF–H_2O (9:1)	rt	420	13
6	8 M $SnCl_2$, 1.6 mM AcOH, 0.04 M phenol in DMF	rt	60	91
7	8 M $SnCl_2$, 1.6 mM AcOH, 0.04 M phenol in DMF	rt	300	100
8	6 M $SnCl_2$, , 0.04 M phenol in DMF–TFE (19:1)	rt	60	68
9	6 M $SnCl_2$, 0.04 M phenol in DMF–TFE (19:1)	rt	300	100
10	6 M $SnCl_2$, 0.04 M phenol in DMF–HFIP (19:1)	rt	60	76
11	6 M $SnCl_2$, 0.04 M phenol in DMF–HFIP (19:1)	rt	300	100
12	6 M $SnCl_2$,1.6 mM TosOH, 0.04 M phenol in DMF	rt	60	86
13	6 M $SnCl_2$,1.6 mM HCl/dioxane, 0.04 M phenol in DMF	rt	60	93
14	6 M $SnCl_2$,1.6 mM HCl/dioxane, 0.04 M phenol in DMF	rt	2 x 30	98
15	6 M $SnCl_2$, 64 mM HCl/dioxane in DMF	rt	2 x 10	58
16	6 M $SnCl_2$, 64 mM HCl/dioxane in DMF	rt	2 x 20	85
17	6 M $SnCl_2$, 64 mM HCl/dioxane in DMF	rt	2 x 30	97
18	6 M $SnCl_2$,1.6 mM HCl/dioxane, 0.04 M of phenol in DMF	50 ºC	2 x 10	97
19	6 M $SnCl_2$,1.6 mM HCl/dioxane, 0.04 M of phenol in DMF	50 ºC	2 x 20	100

[a]Experiments were carried out with 10 mg of resin and 0.5 mL of solvent; [b]Yield was calculated by comparison between the areas of the HPLC peaks corresponding to the protected and the deprotected peptides, as the ε of the unprotected peptide should be lower than the one of the protected, these yields are really higher than expressed.

The data outlined in Table 2 show that $SnCl_2$ based method is superior to the one based on $Na_2S_2O_4$. 6 M $SnCl_2$ solution is more convenient than 8 M, because in this case the solution is supersaturated and some solid $SnCl_2$ can come off (GREG ES CORRECTO? QUEREMOS DECIR QUE PUEDE

APARECER UN SOLIDO). HCl (entry 13) is slightly superior to Tos-OH (entry 12) and AcOH (entry 6), while the performance of acid alcohols [HFIP (entry 10), TFE(entry 8)] are inferior.

As HCl is the best and can not produce capping on the free amine, this is the acid recommended. The increase of the concentration of the HCl from 1.6 mM (entry 16) to 64 mM (entries 18 and 19) does not improve the rate of deprotection. The presence of phenol does not increase neither the rate of deprotection nor the purity of the final product. As it can be expected, a higher temperature, 50 ºC (entry 21) increases the deprotection rate when compared with rt.

Orthogonality of pNZ-amino acids in front of Fmoc, Boc, and Alloc groups

The preparation of complex peptides such as cyclic, branched, or those containing chemical fragile motives require very often the presence of orthogonal protecting groups.[6,7] To show the orthogonality of pNZ-amino acids in front of the most common protecting groups, samples of pNZ-Xx-OH were dissolved in piperidine-DMF (1:4) and TFA (9:1) and after 2 h, the TLC showed that the pNZ-amino acids are totally stable in the presence of those reagents. Likewise, pNZ-Xx-OH showed to be also stable in the presence of Pd(0) (CONDICIONES DE ELIMINAR ALLOC). Furtermore, Boc-Xx-OH, Fmoc-Xx-OH, and Alloc-Xx-OH resulted to be stable to 6 M $SnCl_2$,1.6 mM HCl(dioxane), in DMF for 2 h. These data confirms that the pNZ group is orthogonal in front of Boc, Fmoc, and Alloc protecting groups.

Solid-Phase Peptide Synthesis Using a pNZ/*t*Bu strategy

Using pNZ-amino acids with *t*-butyl side chain protecting groups, Leu-enkephalinamide and human phospholipase A2 (18-23) were synthesized on a Rink-resin.

Two parallel syntheses of H-Tyr-Phe-Gly-Gly-Leu-NH_2 (Leu-enkephalinamide) were carried out. Removal of the pNZ group was carried out with 6 M $SnCl_2$,1.6 mM HCl/dioxane in DMF (2 x 30 min) at room temperature and at 50 ºC, followed by extensive washings with DMF (3 x 30 s), DMF–H_2O (3 x 30 s), THF–H_2O (3 x 30 s), DMF (3 x 30 s), and DCM (3 x 30 s) to remove the excess of $SnCl_2$ as well as the side products coming from the protecting group. Before coupling the following pNZ-amino acid,

the resin was neutralizated with diisopropylethylamine (DIEA)–CH_2Cl_2 (1:9).[23] Couplings were performed using a *N*,*N*-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt) mediated method. After removal of the last pNZ group, free peptides were released from the resin by acidolytic cleavage [TFA–H_2O–DCM (90:5:5)] and worked-up. Both crude products give a good purity by HPLC (Figure 3a) and a correct HPLC-MS. **ALGUNA DIFERENCIA ENTRE LAS DOS SINTESIS.**

Figure 3. HPLCs of peptides

Synthesis H-Ala-Leu-Ser-Tyr-Gly-Phe-NH_2 [human Phospholipase A2 (18-23)] was carried out similarly as outlined above. The pNZ was removed at room temperature, and the couplings were carried out using a 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide (HATU)/DIEA mediated method. In this case the neutralization step was omitted. After final acidolytic cleavage and work-up the crude peptide shown a good purity by HPLC (Figure 3b) and a correct HPLC-MS.

Use of the pNZ group to Circumvent Problems Associated with Fmoc Chemistry

As mentioned above, the main drawback associated with the Fmoc strategy is related with the use of piperidine, which is an excellent nucleophile and a medium strength base. Furthermore, after removal of the Fmoc group, the amino function remains as a free amine. Both, the piperidine and the free amine can cause side reactions such as the formation of diketopiperazines (DKP) and aspartiimides in the case of Asp containing peptides.

DKP Formation

The solid-phase synthesis of peptide *C*-terminal acids sometimes requires special protocols for the incorporation of the second and third amino acids. In the absence of these, the free amino group of the resin-bound dipeptide can attack the peptide-resin anchorage intramolecularly giving rise to cyclic dipeptides or DKPs.[4a,24]

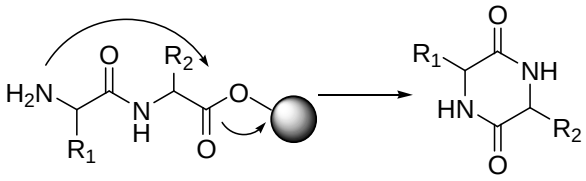

Figure 4. DKP formation mechanism

Although DKP formation is governed by different factors,[25] this side-reaction is mores severe in a Fmoc strategy than in a Boc one. First of all, piperidine catalyzes the side reaction,[26] and, secondly, the removal of the Boc group is carried out in acid medium, so the amine function remains protonated and the incorporation of the third Boc-amino acid can be done with neutralization *in situ*, which minimizes the DKP formation.[27] In Fmoc chemistry, DKP formation can be minimized using resins with large steric hindrance such as those based on trityl (Trt) or *t*-Bu groups[28] or by incorporation of the second residue with Trt protection and then after removal of the Trt group in acid medium, to carry out the incorporation of the third residue with neutralization *in situ.*[29] In mild conditions, DKP can be also minimized by incorporation of the second amino acid protected with the Alloc group in conjunction with a tandem deprotection-coupling reaction, which it is carried out with the corresponding fluoride derivative and it is more facile than DKP formation.[30]

The pNZ as is removed in practical neutral conditions with catalytic amounts of an acid is a good candidate to be used for the protection of the second amino acid in order to prevent or minimize the DKP formation. To demonstrate this methodology, the tripeptide H-Phe-D-Val-Pro-OH was chosen as a model. Thus, pNZ-D-Val-Pro-AB-Leu-aminomethylresin and Fmoc-D-Val-Pro-AB-Leu-aminomethylresin were synthesized with Fmoc-Pro-OH and pNZ/Fmoc-D-Val-OH, using a Leu residue as internal reference amino acid (IRAA) to calculate the yield.[31] While 100% of DKP formation was detected after removal of the Fmoc group of the D-Val residue [piperidine–DMF (2:8)] and incorporation of the Fmoc-Phe-OH, no DKP was observed in the case of the pNZ peptide resin. In this case, the pNZ was removed with 6 M $SnCl_2$,1.6 mM HCl(dioxane) in DMF (2 x 30 min) at room temperature and after extensive washings, the Fmoc-Phe-OH (5 equiv) was incorporated in the presence

of 7-azabenzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) (5 equiv) and DIEA (10 equiv) with neutralization *in situ.*[32]

Aspartimides formation

The cyclization of Asp residues to form aspartimides is a common side reaction in peptide synthesis.[4a,33] Although, this side reaction is favoured in different conditions (strong acids, excess of coupling reagents and basic medium) and depends of the sequence, it is more important when a Fmoc strategy is used due to the repetitive treatment with piperidine. In this case, at the end of the synthetic process besides of the aspartimide containing peptide, it can be found α- and β-peptides (coming from the hydrolysis of the α-imide) and the α- and β-piperidides coming from the attack of piperidine on the aspartiimide.

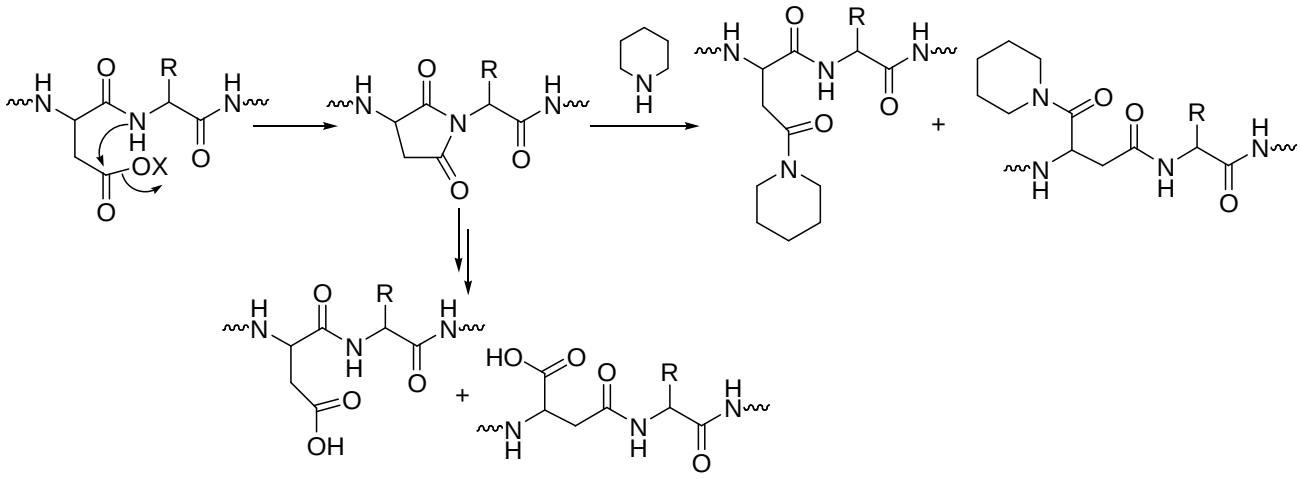

Figure 5. Aspartiimide formation mechanism and side products that can be obtained from the aspartiimide

The side reaction is more severe when the β-carboxyl group of the Asp is protected with the allyl group, due to be a better leaving group than *t*-Bu.[34] Although, the side reaction can try to be minimized when hindered protecting groups[35] for the Asp are used and when the previous amide is protected,[36] a more convenient and general method is needed.

A combination of Fmoc, until the incorporation of the Asp residue, and pNZ for the Asp and the following amino acids can solve this problem, because the removal of the pNZ group does not require

the repetitive presence of a base and at the end of the synthetic process the final cleavage and deprotection is carried out with TFA, which is less prone to give aspartimide than HF used in a Boc/Bzl chemistry strategy.[4a]

The peptide H-Ala-Orn-Asp-Gly-Tyr-Ile-NH_2 was chosen as a model, because it contains the sequence Asp-Gly-Tyr-Ile, which is considered to give a large amount of aspartiimides.[33b] Two parallel syntheses were carried out on a Rink-resin. The first one, Fmoc protection was used for Ile, Gly, and Gly, and pNZ for Orn(Boc) and Ala. Fmoc-amino acids were only used for the second. Furthermore, the peptide H-Ala-Orn-Asp(Gly-Tyr-Ile-NH_2)-OH[37] was synthesized, using Fmoc chemistry, because it can be one of the side products if aspartimide is formed. Fmoc and pNZ peptides were treated 4h with piperidine 20% in DMF and 6M $SnCl_2$ in DMF respectively. While that both piperidides were detected in the synthesis carried out with only Fmoc-amino acids, no aspartimide neither β-peptide were observed in the synthesis carried out with a combination of pNZ and Fmoc.

Conclusions

This work has demonstrated the benefits of using pNZ as temporary protecting group for the α-amino function in SPPS. The corresponding derivatives are easy to be synthesized and in contrast as other α-amino protecting groups such as Trt and Alloc, they are solids. pNZ is removed in practical neutral conditions in the presence of catalytic amounts of acid. pNZ is orthogonal to the most common protecting groups such as *t*-Bu/Boc, Fmoc, and Alloc. Besides to be used for the total elongation of the peptide chain, it can be used in combination with Fmoc chemistry to overcome those side reactions associated with the used of the piperidine such DKP and aspartiimide formation. It is also predictable (GREG en castellano, predecible) that the use of pNZ will avoid the formation of *N*-piperidylalanine residue during the preparation of *C*-terminal Cys peptides as occurs in the Fmoc strategy.[38] The flexibility of pNZ can be very useful for the preparation of libraries of small organic molecules.

Experimental section

General procedures: Commercial-grade reagents and solvents were used without further purification. Resins, linkers and amino acid derivatives, HOBt, DIPCDI, PyAOP, HATU, p-nitrobenzyl

chloroformate and sodium azide were obtained from NovaBiochem (Laüfelfingen, Switzerland), Bachem (Bubendorf, Switzerland), Iris Biotech (Marktredwitz, Germany), Aldrich (Milwaukee, WI), Acros (Geel, Belgium), Neosystem (Strasbourg, France), and Luxembourg Industries (Tel Aviv, Israel).

Analytical HPLC was carried out on a Waters instrument comprising two solvent delivery pumps and automatic injector (Waters Separations Module 2695) and a variable wavelength detector (model Waters 996 Photodiode Array). UV detection was performed at 220 nm, and linear gradients of CH_3CN (0.036% TFA) into H_2O (0.045% TFA) were run at 1.0 mL flow rate. MS-HPLC was carried out on a Waters instrument comprising two solvent delivery pumps and automatic injector (Waters Separations Module 2795), a dual wavelength detector (Waters 2487, Dual λ Absorbance Detector) and an electrospray detector (Waters micromass ZQ).

Amino Acid Analyses were performed using a Beckman System 6300 High Performance Analyzer. Resins were treated with a mixture of HCl and propionic acid (1:1) for 1h at 160ºC, and after evaporating the acid under reduced pressure, they were suspended in amino acid analysis buffer and filtered.

pNZ-Amino Acid Synthesis

Method 1:

p-Nitrobenzyl chloride (1.73g, 8 mmol) was dissolved in 1,4-dioxane (3.5 mL) and a solution of sodium azide (0.624g, 9.6 mmol) in H_2O (2.5 mL) was added. The resulting emulsion was stirred for 2 h. and the formation of the azide can be followed by TLC (DCM). Then, Gly (0.600 g, 8 mmol) dissolved in 1,4-dioxane–2% aqueous Na_2CO_3 (1:1) (10 mL) was added dropwise and the resulting white suspension was stirred for 24 keeping the pH between 9-10 by adding 10% aquous Na_2CO_3. After this time, TLC (CH_2Cl_2) showed that there was not azide left, then H_2O (75 mL) was added and the suspension was washed with *tert*-butyl methyl ether (BME) (3 x 40 mL). The aqueous portion was acidified to pH 2-3 with 12 N HCl–H_2O (3:1), when a white precipitate appeared, which was filtered off and dried to yield 1.44g (71 % of yield) of the title compound as white solid.

Method 2:

The synthesis was performed as in Method 1 but the acidic suspension was extracted with AcOEt. The organic layers were dried on $MgSO_4$, filtered, and the solvent was removed under reduced pressure to yield an oil, which solidified as with solid by washing with diethyl ether or suspended in acetonitrile (AcCN)–H_2O and liofilized (H-D-Val-OH and H-L-Ala-OH). The pNZ-amino acids were recrystalized in H_2O or in diethylether–hexane.

All protected amino acids were characterized by HPLC (Gradient A: from H_2O to AcCN; Gradient B: from AcCN–H_2O (3:7) to AcCN, where H_2O contains 0.045 % of TFA and AcCN contains 0.036% of TFA), HPLC-MS, ^{1}H and ^{13}C NMR, HR-MS, and IR.

Amino Acid	Leu	Ile	D-Val	Phe	Gly	Ser(*t*Bu)	Orn(Boc)	Asp(O*t*Bu)	Ala	Tyr(*t*Bu)
Method	2	2	2	1	1	2	2	2	2	1
Yield (%)	77	94	83	84	71	85	83	89	81	82

pNZ-Ser(tBu)-OH: mp= 92.8-93.8ºC. HPLC rt = 11.28 min.(Gradient A) IR (KBr): ῡ(cm^{-1}) 3364, 3170, 2980, 1741, 1697. ^{1}H NMR (400 MHz, DMSO) δ 8.21 (2H, d, *J*= 8.6), 7.61 (2H, d, *J* = 8.6 Hz), 7.45 (NH, d, *J* = 8.3 Hz), 5.18 (2H, s), 4.10(1H, m), 3,55 (2H, m), 1.09 (9H, s). ^{13}C NMR (100 MHz, DMSO) δ 172.44 (C), 156.42 (C), 147.60 (C), 128.78 (CH Ar), 124.15 (CH Ar), 73.50 (C), 64.93 (CH_2), 62.03 (CH_2), 55.61 (CH), 27.84 (CH_3). HRMS (CI) m/z for $C_{15}H_{21}N_2O_7$ ($M+H^+$) calcd 341.1350, found 341.1359.

pNZ-D-Val-OH: mp= 125.5-127.5 ºC. [12] HPLC rt = 10.5 min. (Gradient A). IR (KBr): ῡ(cm^{-1}) 3322, 2970, 1693, 1540 ^{1}H NMR (400 MHz, DMSO): δ 8.22 (2H, d, *J* = 8.6), 7.63 (NH, d, *J* = unresolved), 7.61 (2H, d, *J* = 8.6 Hz), 5.17 (2H, s), 3.86 (1H, dd, *J*= 8.5 Hz, *J*= 5.9 Hz), 2.48 (1H, m), 0.89 (3H, d, *J* unresolved), 0,87 (3H, d, *J* unresolved). ^{13}C NMR (100 MHz, DMSO) δ 173.82 (C), 156.81 (C), 147.61 (C), 145,76 (C), 128.77 (CH Ar), 124.18 (CH Ar), 64.91 (CH_2), 60.30 (CH), 30.23 (CH), 19.83 (CH_3), 18.68 (CH_3). HRMS (MALDI-TOF) m/z for $C_{13}H_{16}N_2O_6Na$ ($M+Na^+$) calcd 319.0906, found 319.0889.

pNZ-Gly-OH: mp= 121.5-122.5 ºC. HPLC rt = 4.50 min. (Gradient B). IR (KBr): ŭ(cm^{-1}): 3383, 3117, 2941, 1751, 1705, 1522. ^{1}H NMR (400 MHz, DMSO) δ 8.21 (2H, d, *J* =8.7 Hz), 7.70 (NH, t, *J* = 6.1 Hz), 7.64 (2H, d, *J* = 8.7 Hz), 5.18 (2H, s), 3.67 (2H, d, *J* =6.1 Hz). ^{13}C NMR (100 MHz, DMSO) δ 172.12 (C), 156.9 (C), 147.62 (C), 145.7 (C), 128.77 (CH Ar), 124.20 (CH Ar), 64.96 (CH_2), 42.84 (CH_2). HRMS (CI) m/z for $C_{10}H_{11}N_2O_6$ ($M+H^+$) calcd 255.0618, found 255.0611.

pNZ-Leu-OH: mp= 81.0-85.5 ºC. HPLC rt = 7.90 min. (Gradient B) IR (KBr): ŭ(cm^{-1}): 3421.3, 2961.2,1693, 1524. ^{1}H NMR (400 MHz, DMSO) δ 8.21 (2H, d, *J* = 8.8 Hz), 7.70 (NH, d, *J*= 8.2 Hz), 7.58 (2H, d, *J* = 8.8 Hz), 5.15 (2H, s), 3.95 (1H, m), 1.63 (1H, m), 1.50 (2H, m), 0.86 (3H, d, *J* = 6.6 Hz), 0.82 (3H, d, *J* = 6.5 Hz). ^{13}C NMR (100 MHz, DMSO) δ 174.93 (C), 156.55 (C), 147.61 (C), 145.73 (C), 128.75 (CH), 128.47 (CH), 64.88 (CH_2), 52.94 (CH), 40.27 (CH_2), 25.00 (CH), 23.54 (CH_3), 21.80 (CH_3). HRMS (CI) m/z for $C_{14}H_{19}N_2O_6$ ($M+H^+$) calcd 311.1244, found 311.1252.

pNZ-Ile-OH: mp= 79-83ºC. HPLC rt = 11.21 min. (Gradient A) IR (KBr): ŭ(cm^{-1}): 3321, 2967, 1727, 1659, 1538. ^{1}H NMR (400 MHz, DMSO) δ 8.22 (2H, d, *J* = 8.7 Hz), 7.64 (NH, d, *J* = 8.5 Hz), 7.61 (2H, d, *J* = 8.7 Hz), 5.17 (2H, s), 3.90 (1H, dd, *J* = 8.4 Hz, *J'*= 6.1 Hz), 2.48 (1H, m), 1.77 (1H, m), 1.38 (1H, m), 1.20 (1H, m), 0.84 (3H,d, *J* = 6.8 Hz), 0.81 (3H, t, *J* = 7.4 Hz). ^{13}C NMR (100 MHz, DMSO) δ 173.84 (C), 156.72 (C), 147.62 (C), 145.76 (C), 128.78 (CH), 124.19 (CH), 64.92 (CH_2), 59.32 (CH), 36.76 (CH), 25.35 (CH_2), 16.29 (CH_3), 11.95 (CH_3). HRMS (MALDI-TOF) m/z for $C_{14}H_{18}N_2O_6Na$ ($M+Na^+$) calcd 333.1063 found 333.1063.

pNZ-Tyr(*t*Bu)-OH: mp= 130-136.5ºC. HPLC rt = 9.61 min. (Gradient B) IR (KBr): ŭ(cm^{-1}): 3351, 2980, 1703, 1522. ^{1}H NMR (400 MHz, DMSO) δ 8.18 (2H,d, *J* = 8.7 Hz), 7.69 (NH, d, *J* = 8.6 Hz), 7.45 (2H, d, *J* = 8.7 Hz), 7.13 (2H, d, *J* = 8.4 Hz), 6.83 (2H, *J* = 8.4 Hz), 5.09 (2H, m), 4.15 (1H, m), 3.04 (1H, dd, *J*gem= 13.8 Hz, *J* = 4.2 Hz), 2.77 (1H, dd, *J*gem= 13.8 Hz, *J* =10.7 Hz), 1.24 (9, JH, s). ^{13}C NMR (100 MHz, DMSO) δ 173.99 (C), 156.28 (C), 154.15 (C), 147.51 (C), 145.86 (C), 133.22 (C), 130.41 (CH), 128.46 (CH), 124.11 (CH), 123.95 (CH), 78.27 (C), 64.62 (CH_2), 56.47 (CH), 36.70 (CH_2), 29.21 (CH_3). HRMS (CI) m/z for $C_{21}H_{25}N_2O_7$ ($M+H^+$) calcd 417.1663, found 417.1665.

pNZ-Phe-OH: mp= 129.0-135.1 ºC. HPLC rt = 7.90 min. (Gradient B) IR (KBr): ῡ(cm^{-1}): 3329, 1702, 1516. ^{1}H NMR (400 MHz, DMSO) δ 8.17 (2H, d, *J* = 8.7 Hz), 7.76 (NH, d, *J* = 8.5 Hz), 7.48 (2H, d, *J* = 8.7 Hz), 7.25 (5H, m), 5.09 (2H, s), 4.16 (1H, m), 3.08 (1H, dd, *J*gem= 13.8 Hz, *J* = 4.3 Hz), 2.82 (1H, dd, *J*gem= 13.8 Hz, *J* = 10.6 Hz). ^{13}C NMR (100 MHz, DMSO) δ 173.89 (C), 156.34 (C), 147.53 (C), 147.76 (C), 145.76 (C), 138.61 (C), 129.80 (CH), 128.83 (CH), 128.57 (CH), 124.12 (CH), 64.74 (CH_2), 56.33 (CH), 37.20 (CH). HRMS (CI) m/z for $C_{17}H_{17}N_2O_6$ ($M+H^+$) calcd 345.1087, found 345.1079.

pNZ-Ala-OH: amorfous solid. HPLC rt = 9.28 min. (Gradient A) IR (KBr): ῡ(cm^{-1}): 3348, 1697, 1530. ^{1}H NMR (400 MHz, DMSO) δ 8.21 (2H, d, *J* = 8.7 Hz), 7.74 (NH, d, *J* = 7.6 Hz), 7.59 (2H, d, *J* = 8.7 Hz), 5.16 (2H, s), 3.99 (1H, m), 1.26 (3H, d, *J* = 7.4 Hz). ^{13}C NMR (100 MHz, DMSO) δ 174.94 (C), 156.23 (C), 147.61 (C), 145.71 (C), 128.79 (CH), 124.18 (CH), 64.84 (CH_2), 49.97 (CH), 17.71 (CH3). HRMS (MALDI-TOF) m/z for $C_{11}H_{12}N_2O_6Na$ ($M+Na^+$) calcd 291.0593 found 291.0599.

pNZ-Orn(Boc)-OH: amorfous solid. HPLC rt = 7.51 min. (Gradient B) IR (KBr): ῡ(cm^{-1}): ^{1}H NMR (400 MHz, DMSO) δ 8.22 (2H, d, J= 8.6 Hz), 7.68 (NH, d, *J* = 8.0 Hz), 7.60(2H, d, *J* = 8.6 Hz), 6.77 (NH, t, *J* = 4.8 Hz), 5.17 (2H, s), 3.90 (1H, m), 2.91 (2H, m), 1.35 (9H, s). ^{13}C NMR (100 MHz, DMSO) δ 174.40 (C), 156.50 (C), 147.60 (C), 145.77 (C), 128.73 (CH), 124.19 (CH), 78.07 (C), 64.84 (CH_2), 54.45 (CH), 40.09 (CH_2), 28.94 (CH_3), 28.88 (CH_2), 26.87 (CH). HRMS (CI) m/z for $C_{18}H_{26}N_3O_8$ ($M+H^+$) calcd 412.1721, found 412.1719.

pNZ-Asp(O*t*Bu)-OH: mp= 79.5-82.6ºC. HPLC rt = 11.37 min. (Gradient A) IR (KBr): ῡ(cm^{-1}): 3342, 3157, 1723, 1699, 1521. ^{1}H NMR (400 MHz, DMSO) δ 8.21 (2H, d, *J* = 8.7 Hz), 7.76 (NH, d, *J* = 8.6 Hz), 7.59 (2H, d, *J* = 8.7 Hz), 5.17 (2H, s), 4.33 (1H, m), 2.68 (1H, dd, *J*gem= 16.0 Hz, *J* = 5.7 Hz), 2.52 (1H, dd, *J*gem= 16.0 Hz, *J* = 8.4 Hz), 1.35 (9H, s). ^{13}C NMR (100 MHz, DMSO) δ 173.03 (C), 169.87 (C), 156.21 (C), 147.62 (C), 145.62 (C), 128.92 (CH), 124.30 (CH), 80.98 (C), 64.97 (CH_2), 51.32 (CH), 37.88 (CH_2), 28.44 (CH_3). HRMS (CI) m/z for $C_{16}H_{21}N_2O_8$ ($M+H^+$) calcd 369.1299, found 369.1309.

Solid-Phase Synthesis

Solid-phase syntheses were carried out in polypropylene syringues (2-10 mL) fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction.

Fmoc: Removal of the group was carried out with piperidine–DMF (2:8) (2 x 3 min, 1 x 4 min, and 1 x 5 min). Washings between deprotection, coupling, and final deprotecion steps were carried out in DMF (5 x 1 min) and DCM (5 x 1min) using 10 mL solvent/g resin for each wash.

pNZ: Removal of the group was carried out unless indicated otherwise with 6 M $SnCl_2$,1.6 mM HCl(dioxane), in DMF (2 x 30 min). After that the resin was washed with DMF (3 x 30 s), DMF–H_2O(3 x 30 s), THF–H_2O (3 x 30 s), DMF (3 x 30 s), and DCM (3 x 30 s).

Coupling of Fmoc-amino acids and handles were performed by adding the carboxylic acid reagent (4 eq), and the coupling reagent [DIPCDI (4 eq) and HOBt (4 eq), or HATU (3.8 eq) and DIEA (12 eq)] in DMF (0.5 mL) to the resin and the mixture left to stir for 60-90 min.

In all cases, the course of the reactions was followed by ninhydrin test[39] and the coupling repeated if the test gave positive.

Peptide synthesis transformations and washes were performed at 25 ºC unless indicated otherwise.

Final Cleavage and Deprotection: The acidolytic cleavage was carried out with TFA–H_2O–DCM (90:5:5) (10 mL/g of resin). The TFA was removed by evaporation and the residue was taken with AcOH–H_2O (7:3) and washed three times with $CHCl_3$, and the aqueous phase lyophilized.

Synthesis of H-Tyr-Phe-Gly-Gly-Leu-NH_2

Synthesis 1: Rink amide resin (50 mg, 0.66 mmol/g). CouplingsDIPCDI–HOBt method.. Then, the pNZ was removed and the resin was neutralized with DIEA–CH_2Cl_2 (1:9), washed with DCM (5 x 1 min.), and DMF (5 x 1 min.) before performing the next coupling. After final acydolitic cleavage and work up, the crude product was analyzed by HPLC (rt= 6.37 min. Gradient: from H_2O (0.045% of TFA) to AcCN (0.036% of TFA), and HPLC-MS.

Synthesis 2: The same synthetic process as outline above was repeated but carrying out the removal of the pNZ at 50 ºC.

Synthesis of H-Ala-Leu-Ser-Tyr-Gly- Phe-NH_2

Rink amide resin (50 mg, 0.66 mmol/g). Couplings: pNZ-amino acids (4 eq), HATU (3.8 eq) and DIEA (12 eq) in DMF (0.5 mL) were added to the resin and the reaction mixture was left to stir for 1 hour. Then, the pNZ group was removed and the resin was washed before performing the next coupling without previous neutralization. After final acydolitic cleavage and work up, the crude product was analyzed by HPLC (rt= 6.05 min. Gradient: from H_2O:AcCN (95:5) to H_2O:AcCN (5:95) where H_2O contains 0.045% of TFA and AcCN contains 0.036% of TFA) and HPLC-MS.

Avoiding DKPs Formation

Aminomethyl resin (100 mg, f = 1.2 mmol/g) was used as base resin. Fmoc-L-Pro-OH (4 eq), DMAP (0.4 eq) and DIPCDI (4 eq) in DMF (1 mL) were added to the HO-AB-Leu-aminomethyl resin, which is a Wang type resin,[40] and the reaction mixture was left to stir for 1 h. After washing, the TosCl-PNBP test[41] confirmed the correct coupling of the amino acid. Then Fmoc group was removed, the resin was divided in two parts and the peptides were synthesized.

Fmoc synthesis: The Fmoc-D-Val-OH was incorporated using DIPCDI/HOBt mediated coupling, the Fmoc was removed, after washings the Fmoc-Phe-OH was incorporated using the same protocol, the Fmoc was removed and the amino acid analysis of an acid hydrolyzated indicated that the extension of side reaction has been of 100%.

pNZ synthesis: pNZ-D-Val-OH was incorporated using DIPCDI/HOBt mediated coupling, the pNZ was removed, after washings the Fmoc-Phe-OH (4 eq), PyAOP (4 eq),[42] and DIEA (8 eq) in DMF (0.5 mL) were added and the reaction mixture was stirred for 1 h. After washing as indicated above, the ninhydrin test was negative. Then, the resin was washed and the amino acid analysis of an acid hydrolyzated indicated that the side reaction did not occur.

Avoiding Aspartimide Formation

Rink amide resin (200 mg, 0.66 mmol/g) was used and the synthesis were carried out as outlined above using DIPCDI/HOBt mediated couplings. After removal of the Fmoc group of the Gly, the resin was divided in two parts for the Fmoc and the pNZ synthesis. After acidolytic cleavage and work up, the crude were analyzed by HPLC (rt= 9.04 min. Gradient: from H_2O to H_2O-AcCN (7:3), where H_2O contains 0.045 % of TFA and AcCN contains 0.036 % of TFA) and HPLC-MS.

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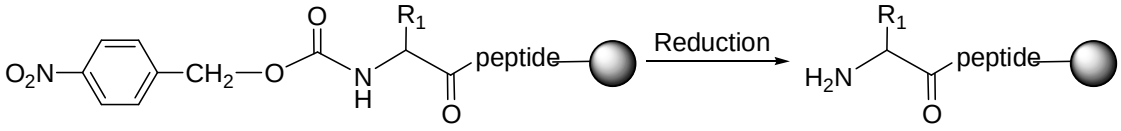
TOC



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[15] Although the existence of this side reaction was not mentioned, Fmoc-N_3 was already proposed as an alternative to the chloroformate in the seminal papers of Carpino and Han (Carpino, L. A.; Han, G. Y. *J. Am. Chem. Soc.* 1970, 92, 5748 and Carpino, L. A.; Han, G. Y. *J. Org. Chem.* 1972, 37, 3404.

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[22] **FALTA REFERENCIAS, DEBEMOS BUSCAR REFERENCIAS DE REDUCCION EN COMP. ORGANICOS.**

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