p-Nitrobenzyloxycarbonyl (pNZ) as a Temporary N^{α}-Protecting Group in Orthogonal Solid-Phase Peptide Synthesis – Avoiding Diketopiperazine and Aspartimide Formation

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Dedicated to Professor Josep Castells on the occasion of his 80th birthday

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p-Nitrobenzyloxycarbonyl (pNZ) was used as a temporary protecting group for α-amino functionalities in solid-phase peptide synthesis. The corresponding derivatives are readily synthesized solids that perform well on solid phase. The pNZ moiety is orthogonal with the most common protecting groups used in peptide chemistry, and is removed under neutral conditions in the presence of catalytic amounts of acid. The use of pNZ derivatives in conjunction with Fmoc chemistry circumvents typical side reactions associated with the use of piperidine, such as DKP and aspartimide formation. The flexibility of pNZ can be exploited for the preparation of libraries of small organic molecules.

Introduction

The solid-phase methodology for the preparation of peptides developed by Bruce R. Merrifield[1] in the late 1950s and early 1960s was crucial for elucidating the utility of peptides as important biochemical tools and, more importantly, their roles in several therapeutic areas.[2] Currently, more than 40 peptide-based drugs are on the market, with four more in registration, 200 in clinical trials, and more than 400 in advanced preclinical development.[3] Solid-phase peptide synthesis (SPPS) strategies are characterized by the lability of both *temporary* (for the N^{α}-amine) and *permanent* (for side chains and for anchoring the *C*-terminal to the solid support through the handle) protecting groups used.[4] The seminal SPPS strategy proposed by Merrifield and fine-tuned over time is based on the graduated acid lability of *tert*-butyloxycarbonyl (Boc), as a *temporary* group, and benzyl-type *permanent* protecting groups. The former is ultimately removed by trifluoroacetic acid (TFA), usually 25–50% in CH_2Cl_2, whereas the latter are removed by strong acids, such as anhydrous HF or trifluoromethanesulfonic acid.[4] Although the Boc-based method has proven successful for the preparation of large numbers of peptides, exposure of the peptide chain to TFA during removal of the Boc group can also cause premature removal of the benzyl protecting group. Furthermore, certain peptides containing fragile residues do not survive the relatively harsh acidic conditions. Finally, HF can be considered a dangerous gas as it must be stored and used in a special Teflon reactor. The aforementioned factors have fueled the development of milder deprotection strategies. Thus, the majority of peptides now produced on solid phase are prepared using the fluorenylmethoxycarbonyl (Fmoc)–*tert*-butyl (*t*Bu) orthogonal protection strategy.[4–6] In this scheme the Fmoc group is normally removed with 20% piperidine in DMF, while the *permanent* protecting groups are removed by TFA in the presence of scavengers. Thus, selective deprotection is governed by alternative cleavage mechanisms rather than by reaction rates. Although this has become the strategy of choice for the preparation of simple peptides, the synthesis of more complex molecules such as cyclic or branched systems may require the use of other protecting groups.[7] Furthermore, the conditions used to remove the Fmoc group are too harsh for certain cases and may be incompatible with several sequences. The main drawbacks associated with the use of Fmoc are that both piperidine, which is a

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base and a rather good nucleophile, and the resulting free amine can provoke side reactions. Thus, an optimal alternative to Fmoc for the protection of N^{α}-amines would be removed under neutral conditions and would leave the resulting amine masked to prevent side reactions. In addition, the protecting group should be orthogonal with *tert*-butyl- and allyl-based protecting groups as well as Fmoc, among others, if it is to be used in the synthesis of complex peptides or other organic molecules.

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

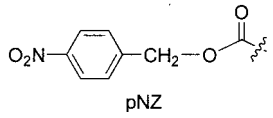

Figure 1. Structure of pNZ protecting group.

The pNZ group, which is a carbamate-type protecting group, was first described by Carpenter and Gish as an alternative to benzyloxycarbonyl (Z).[8] Furthermore, it has been used for the protection of the ε-amino group of Lys.[9] The related *p*-nitrobenzyl (pNB) group has been used to protect the *C*-terminal carboxylic group in side-chain anchoring strategies for the SPS of head-to-tail cyclic peptides,[10] as well as for various functional groups in traditional organic synthesis.[11]

Results and Discussion

Preparation of pNZ-Amino Acids

The classical method for the preparation of N^{α}-carbamate-protected amino acids is via the corresponding chloroformates using Schotten–Baumann conditions, which can generate protected dipeptides as side products.[12] Substantial quantities of protected dipeptide can lead to the insertion of an extra amino acid in the final peptide, which is unacceptable for compounds with therapeutic applications. Several approaches based on the use of activated formates have been proposed to minimize this problematic side reaction.[13] An efficient, competitive and inexpensive procedure is the azide method.[12,14] For the case at hand, an optimized version of a recently described *one-pot* protocol based on the azide was used.[13] Thus, pNZ–Cl was allowed to react with NaN_3, and the resulting pNZ–N_3 was directly treated with free amino acids to afford pNZ–amino acids. These derivatives, which were obtained in relatively high yields (71–94%) and purity, were characterized by HPLC, IR, $^1H/^{13}C$ NMR, and HRMS.

Solubility of pNZ- and Fmoc-Amino Acids

Protected amino acids should be highly soluble, especially for running reactions at high concentration and if automated equipment is to be used to dispense the reagents, a technique often employed for SPPS. In this regard, pNZ-amino acids demonstrate superior solubility in DMF to their corresponding Fmoc derivatives (Table 1).

Table 1. Solubility comparison of pNZ-aa-OH with 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 group can be removed by catalytic hydrogenation as well as other nitro-reducing methods. For the case at hand, the first step was reduction of the nitro group to give the *p*-aminobenzyloxycarbonyl derivative, which suffers spontaneous collapse by 1,6-electron pair shift to afford the quinonimine methide and the carbamic acid. Finally, the carbamic acid decomposes to the corresponding free amine (Figure 2).[15] If this process is carried out in the presence of catalytic amounts of acid, the final product is obtained as an ammonium salt.

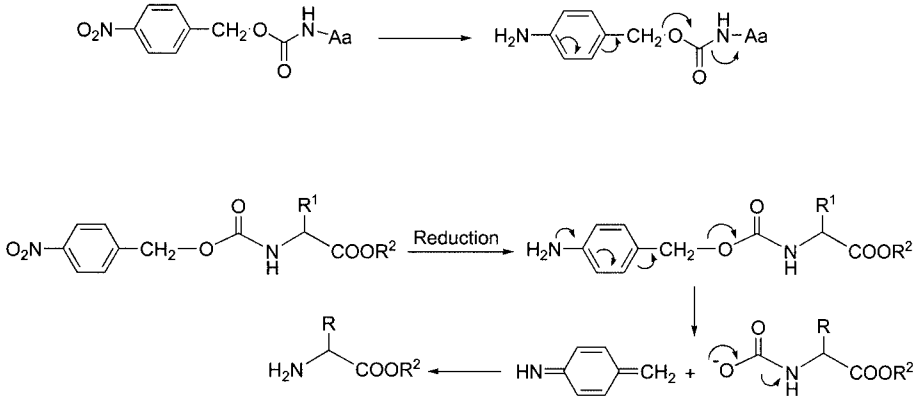

Figure 2. Mechanism of pNZ group removal.

As shown in Figure 2, the key step in the pNZ removal process is the reduction of the nitro group to the corresponding amine. Although the most common method for this transformation is catalytic hydrogention,[16] it is not useful for SPPS. Other common methods are the use of metals such as Zn,[11b] Fe,[17] or Sn[17] in acidic solutions. However, the difficulty of working with metals on solid phase, as well as the highly acidic conditions required for this chemistry, make it problematic for the case at hand. Thus, two reducing reagents envisaged to be compatible with SPS, $Na_2S_2O_4$,[18] and $SnCl_2$,[8b,19,20] were explored.

$Na_2S_2O_4$

Although the effective nitro-reducing agent $Na_2S_2O_4$ is typically used under basic conditions,[21] there are several reports of its application in neutral or nearly neutral media.[18]

The main drawback associated with the use of $Na_2S_2O_4$ on solid phase is its solubility. It is highly insoluble in DMF and other resin-swelling solvents. Furthermore, the reduction of nitro groups by $Na_2S_2O_4$ requires H_2O, which is not a good solvent for most solid supports. In an effort to overcome the water use, 15-crown-5 was used to solubilize $Na_2S_2O_4$. Several attempts at removing the pNZ group of pNZ-Orn(Boc)-OH in solution using DMF as a solvent were first carried out using varying amounts of water and 15-crown-5. It was found by TLC (1% HOAc in EtOAc) that the rate of cleavage increases with the concentration of H_2O and 15-crown-5.

$SnCl_2$

$SnCl_2$ is a good nitro-reducing agent in the presence of catalytic amounts of acid. It was initially probed with HOAc, but non-carboxylic acids were used in latter probes to prevent acetylation on solid phase.

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

The data outlined in Table 2 illustrate that $SnCl_2$ is a superior reducing agent than $Na_2S_2O_4$ for the model case. A 6 M $SnCl_2$ solution is more convenient to use than an 8 M solution, as the latter is supersaturated and some solid $SnCl_2$ may precipitate. HCl (Entry 13) was slightly superior to Tos-OH (Entry 12) and HOAc (Entry 6), while the performance of the alcohols with a relatively acidic hydrogen [HFIP (Entry 10) and TFE (Entry 8)] was inferior.

HCl was thus determined to be the most effective acid. Increasing the concentration of HCl from 1.6 mM (Entry 14) to 64 mM (Entries 15, 16, and 17) did not improve the rate of deprotection. The presence of phenol does not increase the rate of deprotection or the purity of the final product. As might be expected, deprotection occurs faster at high temperature (50 °C) than at room temp. (Entries 18 and 19).

Orthogonality of pNZ-Amino Acids with Fmoc, Boc, and Alloc Groups

The preparation of complex targets such as cyclic or branched peptides, as well as those containing chemicaly fragile moieties, often requires the use of orthogonal protecting groups.[6,7] To demonstrate the orthogonality of pNZ-amino acids with common protecting groups, samples of pNZ-Phe-OH were dissolved in piperidine/DMF (1:4) or TFA (9:1). TLC (1% AcOH in EtOAc) indicated that the pNZ-amino acids are totally stable to both deprotection reagents after 24 h. Likewise, pNZ-Phe-OH was also stable to $Pd(PPh_3)_4$ and phenylsilane in DCM. Furthermore, Boc-

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

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

[a] Experiments were carried out with 10 mg of resin and 0.5 mL of solvent. [b] Yield was calculated by comparing 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 that of the protected peptide, the actual yields are higher than those reported.

Phe-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Orn(Alloc) were stable to a mixture of 6 M $SnCl_2$ and 1.6 mM HCl/dioxane in DMF for 2 h. Minimal degradation was only observed for Boc-Phe-OH after 24 h under these conditions. The aforementioned data hence confirm that the pNZ group is orthogonal with Boc, Fmoc, and Alloc protecting groups.

Solid-Phase Peptide Synthesis Using a pNZ/*tert*-Butyl Strategy

Using pNZ-amino acids with *tert*-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 accomplished with 6 M $SnCl_2$, 1.6 mM HCl/dioxane in DMF (2 × 30 min) at room temperature and at 50 °C, followed by extensive washings with DMF (3 × 30 s), DMF/H_2O (3 × 30 s), THF/H_2O (3 × 30 s), DMF (3 × 30 s), and DCM (3 × 30 s) to remove excess $SnCl_2$ as well as any side products from the protecting group. Before coupling the subsequent pNZ-amino acid, the resin was neutralized with diisopropylethylamine (DIEA)/CH_2Cl_2 (1:9).[22] Couplings were performed using an *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 were obtained in good purity as characterized by HPLC (Figure 3a) and HPLC-MS.

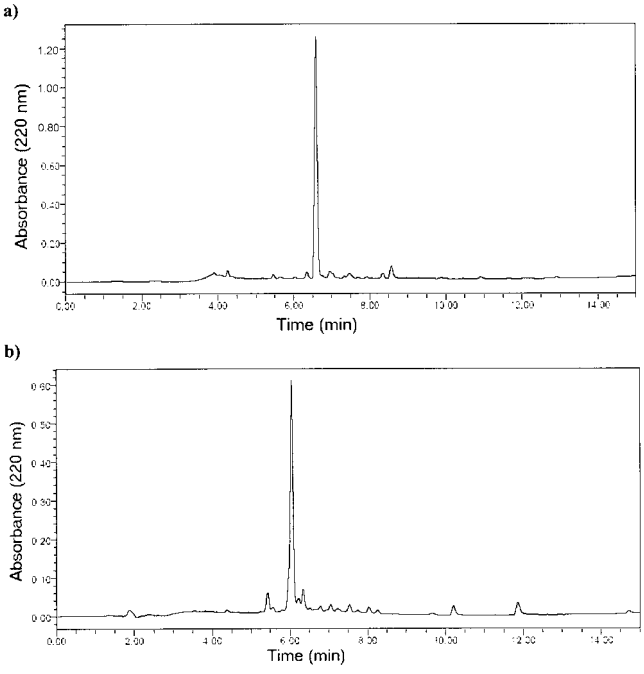

Figure 3. HPLC of (a) Leu-enkephaline and (b) the human phospholipase A2 (18–23) (see Exp. Sect. for chromatographic conditions).

The synthesis of H-Ala-Leu-Ser-Tyr-Gly-Phe-NH_2 [human phospholipase A2 (18–23)] was carried out similarly to the procedure outlined above. The pNZ was removed at room temperature, and the couplings employed an *N*-[(1*H*-3-oxy-4-azabenzotriazol-1-yl)(dimethylamino)methylene]-*N*-dimethyliminium hexafluorophosphate (HATU)/DIEA mediated method. In this case the neutralization step was omitted. After final acidolytic cleavage and workup the crude peptide was obtained in good purity as characterized by HPLC (Figure 3b) and 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 to the use of piperidine, which is an excellent nucleophile and a medium-strength base. Furthermore, after removal of the Fmoc group, the amino functionality remains a free amine. Both, the piperidine and the free amine can cause side reactions such as the formation of diketopiperazines (DKP) and, in the case of Asp-containing peptides, aspartimides.

DKP Formation

The free amino group of a resin-bound dipeptide can attack the peptide-resin anchorage intramolecularly to form cyclic dipeptides or DKPs.[4a,23,24] Thus, the solid-phase synthesis of *C*-terminal peptide acids sometimes requires special protocols for the incorporation of the second and third amino acids (Figure 4).

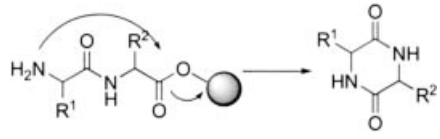

Figure 4. Mechanism of DKP formation.

Although DKP formation is governed by various factors, the side reaction is more severe in Fmoc-based syntheses than in Boc-based syntheses.[25] First of all, piperidine catalyzes the side reaction,[26] secondly, removal of the Boc group is carried out in acidic medium, hence the amine remains protonated and incorporation of the third Boc-amino acid can be done with in situ neutralization, which minimizes DKP formation.[27] DKP formation in Fmoc chemistry can be minimized by various approaches, including the use of sterically hindered resins such as those based on trityl (Trt) or *tert*-butyl (*t*Bu) groups.[28] Likewise, incorporation of the second residue with Trt protection, followed by Trt removal in acidic medium and subsequent incorporation of the third residue with in situ neutralization, can also be employed.[29] Under 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 is carried out with

the corresponding fluoride derivative and is favored over DKP formation.[30]

Owing to its facile removal, it was thought that the pNZ group would be a good candidate for the protection of the second amino acid of a growing peptide on solid phase. To demonstrate this methodology, the tripeptide H-Phe-D-Val-Pro-OH was chosen as a model.[31] 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 {AB linker = 3-[4-(hydroxymethyl)phenoxy]propionic acid}. 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 × 30 min) at room temperature and after extensive washings, the Fmoc-Phe-OH (5 equiv.) was incorporated in the presence of (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) (5 equiv.) and DIEA (10 equiv.) using in situ neutralization.[32]

Aspartimide Formation

The cyclization of Asp residues to form aspartimides is a common side reaction in peptide synthesis.[4a,33] Although this side reaction is favored under various conditions (strong acids, excess coupling reagents or basic medium) and is sequence-dependent, it is most relevant to Fmoc-based syntheses due to the repetitive use of piperidine (Figure 5). Hence, Fmoc strategies not only yield the aspartimide-containing peptide but also generate the corresponding α- and β-peptides (by hydrolysis of α-imides) and α- and β-piperidides (via attack of the aspartimide by piperidine).

The side reaction is more severe when the β-carboxyl group of the Asp is protected with the allyl group, since it forms a better leaving group than *tert*-butyl.[34] Although the side reaction can be minimized to some extent through the use of hindered protecting groups[35] for the Asp and by protecting the preceeding amide,[36] a more convenient and general method is needed.

A possible solution is a hybrid strategy in which Fmoc is used to protect all of the residues preceding an Asp, at which point pNZ is used to protect the Asp and remaining residues. The use of pNZ here would be advantageous as its removal does not require the repetitive use of a base, and the final cleavage and deprotection of the peptide would be carried out with TFA, which is less prone to generate aspartimide than the HF normally used in a Boc/Bzl chemistry strategy.[4a]

The hybrid strategy was studied using the peptide H-Ala-Orn-Asp-Gly-Tyr-Ile-NH_2 model since it contains the sequence Asp-Gly-Tyr-Ile, which is considered to generate a large amount of aspartimides.[33b] Two parallel syntheses were carried out on a Rink-resin. In the first synthesis, Fmoc protection was used for Ile, Tyr, and Gly, and pNZ for Asp(O*t*Bu), Orn(Boc), and Ala, whereas the second synthesis used exclusively Fmoc-amino acids. Furthermore, the peptide H-Ala-Orn-Asp(Gly-Tyr-Ile-NH_2)-OH[37] was synthesized (using Fmoc chemistry) because it is a possible side product of the aspartimide corresponding to the model peptide. Fmoc and pNZ groups were removed with 4 h treatments of 20% piperidine/DMF or 6 M $SnCl_2$/DMF, respectively. While both piperidides were detected in the synthesis using exclusively Fmoc-protection, neither aspartimide nor β-peptide were observed in the synthesis using the Fmoc-pNZ hybrid strategy.

Conclusions

This work has demonstrated the benefits of using pNZ as a temporary protecting group for the α-amines in SPPS. The corresponding derivatives are readily synthesized and, in contrast to other α-amino protecting groups such as Trt and Alloc, they are solids. pNZ is removed under simple, neutral conditions in the presence of catalytic amounts of acid. pNZ is orthogonal to the most common SPPS protecting groups such as *t*Bu/Boc, Fmoc, and Alloc. In addition to its utility for the total elongation of a peptide chain, the pNZ group can be used in conjuction with Fmoc chemistry to overcome side reactions associated with the use of piperidine, such as DKP and aspartimide formation. It is also predicted that the use of pNZ for the preparation of *C*-terminal Cys peptides would circumvent the formation

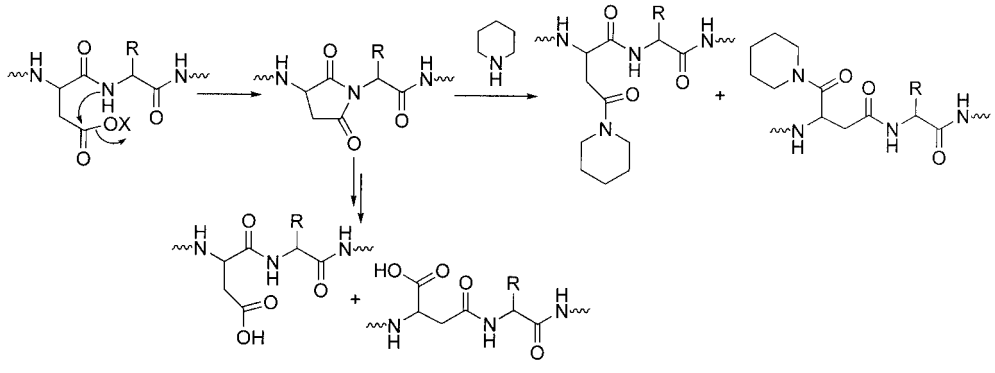

Figure 5. Mechanism of aspartimide formation with typical side products shown.

of *N*-piperidylalanine, a frequent side reaction when Fmoc protection is used.[38] The flexibility of pNZ can be exploited 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 (Läufelfingen, 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 with 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 a flow rate of 1.0 mL/min. MS-HPLC was carried out with 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). NMR spectra were acquired with a Mercury-400 (400 MHz) spectrometer (High Field NMR Unit, Barcelona Science Park), data are given on the δ scale referenced to TMS (see Supporting Information for ^1H and ^{13}C NMR spectra). 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) at 160 °C for 1 h, 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 chloroformate (1.73 g, 8 mmol) was dissolved in 1,4-dioxane (3.5 mL) and a solution of sodium azide (0.624 g, 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 was monitored by TLC (CH_2Cl_2). Gly (0.600 g, 8 mmol), dissolved in 1,4-dioxane/2% aqueous Na_2CO_3 (1:1) (10 mL), was then added dropwise, and the resulting white suspension was stirred for 24 h, keeping the pH between 9 and 10 by adding 10% aqueous Na_2CO_3. At this point, TLC (CH_2Cl_2) showed that there was no remaining azide, H_2O (75 mL) was added and the suspension was washed with methyl *tert*-butyl ether (MTBE) (3×40 mL). The aqueous portion was acidified to pH = 2–3 with 12 N HCl/H_2O (3:1) and a white precipitate appeared, which was filtered off and dried to yield 1.44 g (71%) of the title compound as a white solid.

Method 2: The synthesis was performed as in Method 1 but the acidic suspension was extracted with EtOAc. The organic layers were dried with $MgSO_4$, filtered, and the solvent was removed under reduced pressure to yield an oil, which solidified as white solid by washing with diethyl ether or suspended in acetonitrile (AcCN)/H_2O and lyophilized (H-D-Val-OH and H-L-Ala-OH). The pNZ-amino acids were recrystalized in H_2O or in diethyl ether/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 contained 0.045% of TFA and AcCN contained 0.036% of TFA], HPLC-MS, ^1H and ^{13}C NMR, HR-MS, and IR; for used method and yields see Table 3.

pNZ-Ser(*t*Bu)-OH: M.p. 92.8–93.8 °C. HPLC: R_t = 11.28 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3364, 3170, 2980, 1741, 1697, 1525, 1347 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.21 (d, J = 8.6 Hz, 2 H), 7.61 (d, J = 8.6 Hz, 2 H), 7.45 (d, J = 8.3 Hz, NH), 5.18 (s, 2 H), 4.10 (m, 1 H), 3,55 (m, 2 H), 1.09 (s, 9 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 172.44 (C), 156.42 (C), 147.60 (C), 145.71 (C), 128.78 (CH), 124.15 (CH), 73.50 (C), 64.93 (CH_2), 62.03 (CH_2), 55.61 (CH), 27.84 (CH_3) ppm. HRMS (CI): *m*/*z* calcd. for $C_{15}H_{21}N_2O_7$ [M + H^+] 341.1350, found 341.1359.

pNZ-D-Val-OH: M.p. 125.5–127.5 °C.[8a] HPLC: R_t = 10.5 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3322, 2970, 1693, 1540, 1351 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.22 (d, J = 8.6 Hz, 2 H), 7.63 (d, J = unresolved, NH), 7.61 (d, J = 8.6 Hz, 2 H), 5.17 (s, 2 H), 3.86 (dd, J = 8.5, 5.9 Hz, 1 H), 2.48 (m, 1 H), 0.89 (d, J unresolved, 3 H), 0,87 (d, J unresolved, 3 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 173.82 (C), 156.81 (C), 147.61 (C), 145,75 (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) ppm. HRMS (MALDI-TOF): *m*/*z* calcd. for $C_{13}H_{16}N_2O_6Na$ [M + Na^+] 319.0906, found 319.0889.

pNZ-Gly-OH: M.p. 121.5–122.5 °C (ref.[8a] 122.5–124 °C). HPLC: R_t = 4.50 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3383, 3117, 2941, 1751, 1705, 1522, 1350 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.21 (d, J = 8.7 Hz, 2 H), 7.70 (t, J = 6.1 Hz, NH), 7.59 (d, J = 8.7 Hz, 2 H), 5.18 (s, 2 H), 3.67 (d, J = 6.1 Hz, 2 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 172.12 (C), 156.9 (C), 147.62 (C), 145.7 (C), 128.77 (CH), 124.20 (CH), 64.95 (CH_2), 42.84 (CH_2) ppm. HRMS (CI): *m*/*z* calcd. for $C_{10}H_{11}N_2O_6$ [M + H^+] 255.0618, found 255.0611.

pNZ-Leu-OH: M.p. 81.0–85.5 °C (ref.[8a] 60–61 °C). HPLC: R_t = 7.90 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3421.3, 2961, 1693, 1524, 1345 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.22 (d, J = 8.8 Hz, 2 H), 7.70 (d, J = 8.2 Hz, NH), 7.59 (d, J = 8.8 Hz, 2 H), 5.16 (d, J = 1.96 Hz, 2 H), 3.95 (m, 1 H), 1.63 (m, 1 H), 1.50 (m, 2 H), 0.87 (d, J = 6.6 Hz, 3 H), 0.83 (d, J = 6.5 Hz, 3 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 174.93 (C), 156.55 (C), 147.61 (C), 145.73 (C), 128.75 (CH), 124.19(CH), 64.87 (CH_2), 52.94 (CH), 40.27 (CH_2), 25.00 (CH), 23.54 (CH_3), 21.80 (CH_3) ppm. HRMS (CI): *m*/*z* calcd. for $C_{14}H_{19}N_2O_6$ [M + H^+] 311.1244, found 311.1252.

pNZ-Ile-OH: M.p. 79–83 °C (ref.[8a] 77.5–80 °C). HPLC: R_t = 11.21 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3321, 2967, 1727, 1659, 1538, 1349 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.22 (d, J = 8.7 Hz, 2 H), 7.64 (d, J = 8.5 Hz, NH), 7.61 (d, J = 8.7 Hz, 2 H), 5.17 (s, 2 H), 3.90 (dd, J = 8.5, 6.1 Hz, 1 H), 2.48 (m, 1 H), 1.77 (m, 1 H), 1.38 (m, 1 H), 1.20 (m, 1 H), 0.86 (d, J = 6.8 Hz, 3 H), 0.82 (t, J = 7.4 Hz, 3 H) ppm. ^{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) ppm. HRMS (MALDI-TOF): *m*/*z* calcd. for $C_{14}H_{18}N_2O_6Na$ [M + Na^+] 333.1063 found 333.1063.

pNZ-Tyr(*t*Bu)-OH: M.p. 130–136.5 °C. HPLC: R_t = 9.61 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3351, 2980, 1703, 1522, 1352 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.18 (d, J = 8.7 Hz, 2 H), 7.69 (d, J = 8.6 Hz, NH), 7.45 (d, J = 8.7 Hz, 2 H), 7.13 (d, J = 8.4 Hz, 2 H), 6.84 (J = 8.4 Hz, 2 H), 5.09 (dd, J = 24,1 Hz, 14,2 Hz, 2 H), 4.15 (m, 1 H), 3.04 (dd, J_{gem} = 13.8, J = 4.2 Hz, 1 H), 2.77 (dd, J_{gem} = 13.8, J = 10.7 Hz, 1 H), 1.24 (s, 9 H) ppm. ^{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), 130.35 (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) ppm. HRMS (CI): *m*/*z* calcd. for $C_{21}H_{25}N_2O_7$ [M + H^+] 417.1663, found 417.1665.

Table 3. Method used and yield for the preparation of pNZ-amino acids.

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-Phe-OH: M.p. 129.0–135.1 °C (ref.[8b] 134.5–136.5 °C). HPLC: R_t = 7.90 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3329, 1702, 1516, 1346 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.17 (d, J = 8.7 Hz, 2 H), 7.76 (d, J = 8.5 Hz, NH), 7.48 (d, J = 8.7 Hz, 2 H), 7.25 (m, 5 H), 5.10 (s, 2 H), 4.16 (m, 1 H), 3.08 (dd, J_{gem} = 13.8, J = 4.3 Hz, 1 H), 2.82 (dd, J_{gem} = 13.8, J = 10.6 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 173.88 (C), 156.34 (C), 147.53 (C), 145.76 (C), 138.61 (C), 129.80 (CH), 128.83 (CH), 128.57 (CH), 127.01 (CH), 124.12 (CH), 64.74 (CH_2), 56.33 (CH), 37.20 (CH_2) ppm. HRMS (CI): *m*/*z* calcd. for $C_{17}H_{17}N_2O_6$ [M + H^+] 345.1087, found 345.1079.

pNZ-Ala-OH: Amorphous solid (ref.[8b] 132.5–134 °C). HPLC: R_t = 9.28 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3348, 1697, 1530, 1351 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.21 (d, J = 8.7 Hz, 2 H), 7.74 (d, J = 7.6 Hz, NH), 7.59 (d, J = 8.7 Hz, 2 H), 5.16 (s, 2 H), 3.99 (m, 1 H), 1.26 (d, J = 7.4 Hz, 3 H) ppm. ^{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), 17.70 (CH_3) ppm. HRMS (MALDI-TOF): *m*/*z* calcd. for $C_{11}H_{12}N_2O_6Na$ [M + Na^+] 291.0593, found 291.0599.

pNZ-Orn(Boc)-OH: Amorphous solid. HPLC: R_t = 7.51 min (Gradient B). IR (KBr): $\tilde{\nu}$ = 3405, 2978, 1717, 1523, 1348 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.22 (d, J = 8.6 Hz, 2 H), 7.68 (d, J = 8.0 Hz, NH), 7.60 (d, J = 8.6 Hz, 2 H), 6.77 (t, J = 4.8 Hz, NH), 5.17 (s, 2 H), 3.90 (m, 1 H), 2.91 (m, 2 H), 1.58 (m, 1 H), 1.53 (m, 1 H), 1.42 (m, 2 H), 1.35 (s, 9 H) ppm. ^{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_2) ppm. HRMS (CI): *m*/*z* calcd. for $C_{18}H_{26}N_3O_8$ [M + H^+] 412.1721, found 412.1719.

pNZ-Asp(O*t*Bu)-OH: M.p. 79.5–82.6 °C. HPLC: R_t = 11.37 min (Gradient A). IR (KBr): $\tilde{\nu}$ = 3342, 3157, 1723, 1699, 1521, 1349 cm^{-1}. ^1H NMR (400 MHz, DMSO): δ = 8.21 (d, J = 8.7 Hz, 2 H), 7.76 (d, J = 8.6 Hz, NH), 7.59 (d, J = 8.7 Hz, 2 H), 5.17 (s, 2 H), 4.33 (m, 1 H), 2.68 (dd, J_{gem} = 16.0, J = 5.7 Hz, 1 H), 2.52 (dd, J_{gem} = 16.0, J = 8.4 Hz, 1 H), 1.35 (s, 9 H) ppm. ^{13}C NMR (100 MHz, DMSO): δ = 173.03 (C), 169.87 (C), 156.21 (C), 147.62 (C), 145.62 (C), 128.82 (CH), 124.16 (CH), 80.98 (C), 64.97 (CH_2), 51.32 (CH), 37.88 (CH_2), 28.31 (CH_3) ppm. HRMS (CI): *m*/*z* calcd. for $C_{16}H_{21}N_2O_8$ [M + H^+] 369.1299, found 369.1309.

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

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

pNZ: Unless otherwise indicated, deprotection was accomplished with 6 M $SnCl_2$ and 1.6 mM HCl/dioxane, in DMF (2 × 30 min). The resin was then washed with DMF (3 × 30 s), DMF/H_2O (3 × 30 s), THF/H_2O (3 × 30 s), DMF (3 × 30 s), and DCM (3 × 30 s).

Coupling of Fmoc-Amino Acids and Handles: This was performed by adding the carboxylic acid reagent (4 equiv.) and the coupling reagent [DIPCDI (4 equiv.) and HOBt (4 equiv.), or HATU (3.8 equiv.) and DIEA (12 equiv.)] in DMF (0.5 mL) to the resin and stirring the mixture for 60–90 min. For all cases, reaction progress was monitored by the ninhydrin test[39] and couplings were repeated if a positive test result was obtained. Peptide synthesis transformations and washes were performed at 25 °C unless otherwise indicated.

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 up in HOAc/H_2O (7:3), 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). Couplings DIPCDI/HOBt method. Then, the pNZ was removed and the resin was neutralized with DIEA/CH_2Cl_2 (1:9), washed with DCM (5 × 1 min), and DMF (5 × 1 min) before performing the next coupling. After final acydolitic cleavage and workup, the crude product was analyzed by HPLC [R_t = 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 °C.

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 equiv.), HATU (3.8 equiv.) and DIEA (12 equiv.) in DMF (0.5 mL) were added to the resin and the reaction mixture was stirred for 1 h. Then, the pNZ group was removed and the resin was washed before performing the next coupling without previous neutralization. After final acydolitic cleavage and workup, the crude product was analyzed by HPLC [R_t = 6.05 min; gradient: from H_2O/AcCN (95:5) to H_2O/AcCN (5:95) where H_2O contained 0.045% of TFA and AcCN contained 0.036% of TFA] and HPLC-MS.

Tests of DKP Formation: Aminomethyl resin (100 mg, f = 1.2 mmol/g) was used as the base resin. Fmoc-L-Pro-OH (4 equiv.), DMAP (0.4 equiv.) and DIPCDI (4 equiv.) 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 stirred for 1 h. After washing, the TosCl-PNBP test[41] confirmed the correct coupling of the amino acid. Then the Fmoc group was removed, the resin was divided into 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 hydrolysis of a peptide resin aliquot indicated that the extension of side reactions 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 equiv.), PyAOP (4 equiv.),[42] and DIEA (8 equiv.) 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. The resin was then washed and the amino acid hydrolysis of a peptide resin aliquot indicated that side reactions did not occur.

Tests of Aspartimide Formation: Rink amide resin (200 mg, 0.66 mmol/g) was used and the synthesis was carried out as outlined above using DIPCDI/HOBt-mediated couplings. After removal of the Fmoc group of the Gly, the resin was divided into two parts for the Fmoc and the pNZ synthesis. After acidolytic cleavage and workup, the crude products were analyzed by HPLC [R_t = 9.04 min; gradient: from H_2O to H_2O/AcCN (7:3), where H_2O contained 0.045% of TFA and AcCN contained 0.036% of TFA] and HPLC-MS.

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