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Use of *p*-nitrobenzyloxycarbonyl (*p*NZ) as a permanent protecting group in the synthesis of Kahalalide F analogs

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Abstract—*p*-Nitrobenzyloxycarbonyl (*p*NZ) is used for the permanent protection of ornithine in the synthesis of derivatives of the anti-tumor cyclodepsipeptide Kahalalide F that contain acid labile residues.

1. Introduction

Solid-phase fluorenylmethoxycarbonyl (Fmoc)-*tert*-butyl (^{t}Bu) chemistry is the strategy of choice for the preparation of peptides in basic research and commercial applications.[1,2] In this strategy, the Fmoc group is normally removed with piperidine in DMF, while *permanent* protecting groups are removed by trifluoroacetic acid (TFA) in the presence of scavengers during the cleavage of the peptide from the resin.[1,2] However, certain peptides can be degraded by the treatment with high concentrations of TFA. For these cases, ^{t}Bu-type protecting groups must be substituted with protecting groups that can be removed in neutral or nearly neutral conditions.

Herein, the synthesis of analogs of the anti-tumor cyclodepsipeptide Kahalalide F that contain acid labile substituents (oleanolic and maslinic acids) using *p*-nitrobenzyloxycarbonyl (*p*NZ)[3] as a permanent protecting group for the side chain of Orn is reported (Fig. 1).

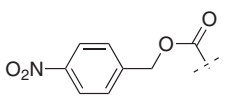

Figure 1. Structure of the *p*NZ protecting group.

2. Results and discussion

Kahalalide F (**1a**), the only member of the kahalalide peptide family to be isolated from the sacoglossan mollusc *Elysya rufescens* and the green alga *Bryopsis* sp.,[4] is in clinical trials for several types of cancers.[4,5] Structurally, Kahalalide F is a head to side-chain cyclic depsipeptide that terminates in an aliphatic acid. The establishment of an efficient solid-phase synthesis of Kahalalide F[6] by our group spawned an in-house program dedicated to creating analogs for SAR.[7]

The original solid-phase synthesis,[6] similar to that outlined in Figure 2, is based on an orthogonal protecting group scheme using a chlorotrityl chloride (ClTrt-Cl, Barlos) resin together with Fmoc and allyloxycarbonyl (Alloc) as temporary protecting groups, and ^{t}Bu and Boc for the side-chain protection of Thr and Orn, respectively.

We envisioned Kahalalide F analogs in which the N-terminal aliphatic acid of the parent compound is substituted with oleanolic acid (3β-hydroxy-12-oleanen-28-oic) or maslinic acid (2α,3β-dihydroxy-12-oleanen-28-oic), pentacyclic triterpenoids widely found in nature.[8]

Keywords: Combinatorial chemistry; Cyclic pepides; Orthogonal protecting group.
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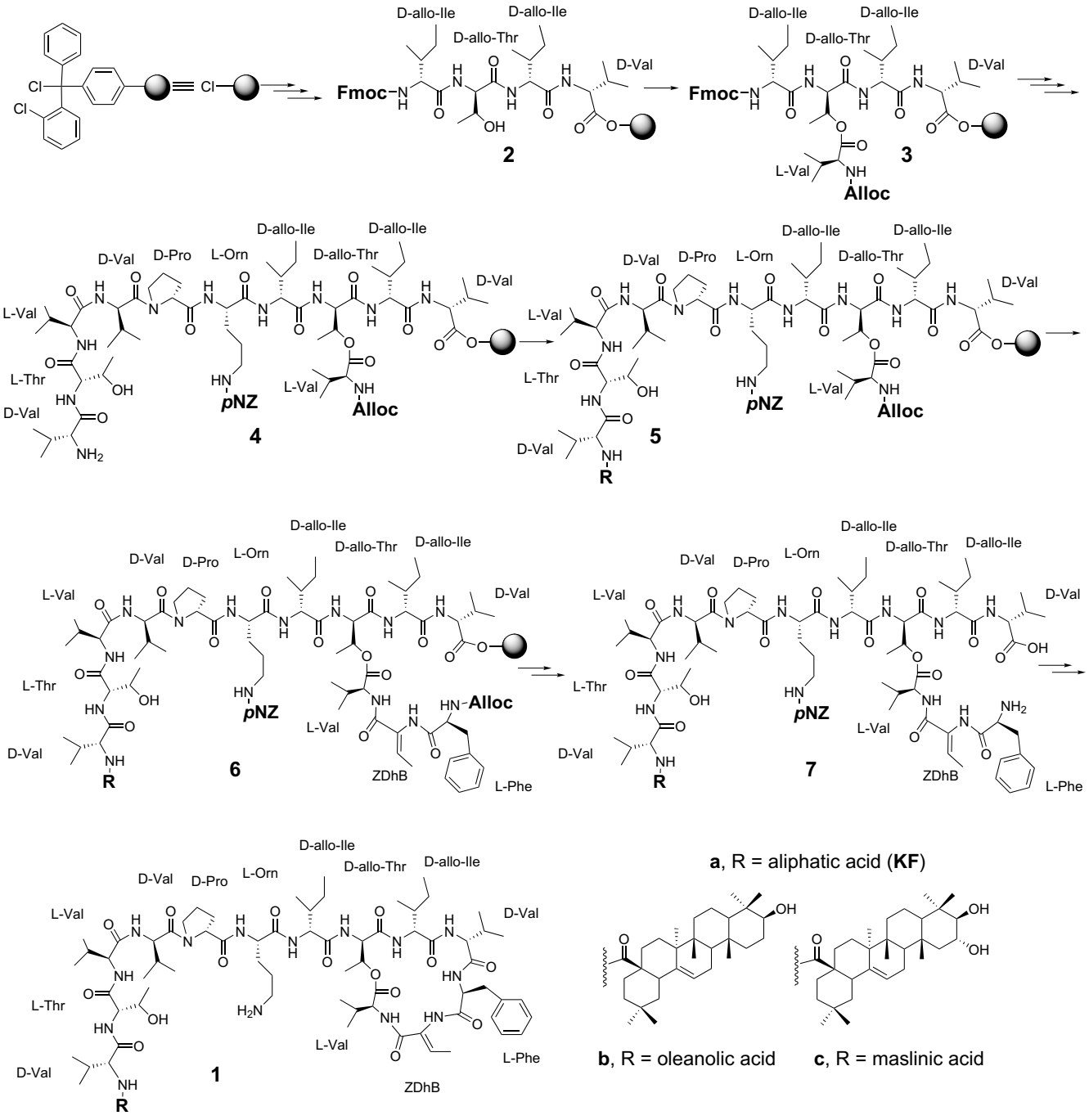

Figure 2. Synthetic strategy developed for the synthesis of KF analogs.

These compounds are isolated in high yields from olive-pressing residues following an extraction process.[9] Both acids, as well as structural analogues, have interesting pharmacological profiles,[10] including in vitro anti-HIV activity[11] and antioxidant properties.[12]

The first attempt at synthesizing the aforementioned analogs progressed smoothly until the last step. Final treatment of the peptidyl-resin with TFA, which should have removed the ^{t}Bu and Boc groups, led to a complex mixture in which the target compound could not be identified by HPLC-MS. It was later established that oleanolic and maslinic acids are not stable to high concentrations of TFA.

We thus sought a new protecting group for the δ-amino function of Orn that would be orthogonal to the Fmoc, Alloc, and Cl-Trt protecting groups.[13] The Thr near the N-terminal can be incorporated with its secondary alcohol function unprotected due to its low nucleophilicity and the few coupling cycles implied in the synthesis.

Specifically, the group had to be stable to piperidine (used for Fmoc removal), Pd(0) in the presence of scav-

engers (used for Alloc removal), and 1% TFA–CH_2Cl_2 (used to cleave the protected peptide from the Cl-Trt resin before cyclization). The *p*NZ group, recently described for the temporary protection of α-functionalities in solid-phase peptide synthesis meets the aforementioned conditions.[3f] The *p*NZ group, removed with $SnCl_2$ in the presence of catalytic amounts of acid, also does not induce side reactions that commonly result from other temporary protecting groups.[3f]

A new synthesis was thus initiated with limited incorporation of Fmoc-D-Val-OH to ClTrt-resin,[14] followed by the stepwise coupling of the next three N^{α}-protected amino acids, with the side chain of D-allo-Thr unprotected. Subsequent incorporation of Alloc-Val-OH using DIPCDI/DMAP allowed formation of the ester bond.[15] At this point, the peptide chain was completed by first introducing Fmoc-Orn(*p*NZ)-OH, followed by the rest of protected amino acids and terminated by either oleanolic or maslinic acid. The incorporation of these two acids required strong coupling conditions, such as benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluoro-phosphate (PyBOP)/1-hydroxy-7-azabenzotriazole (HOAt)/*N*,*N*-diisopropylethylamine (DIEA). The dipeptide Alloc-L-Phe-*Z*Dhb-OH was introduced and the completed linear peptide was cleaved from the resin with TFA–CH_2Cl_2 (1:99), cyclized with *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/hydroxybenzotriazole (HOBt), and deprotected using the *p*NZ removal conditions described above. The target KF analog was obtained with excellent purity and was characterized by MS.[16]

3. Conclusions

This work demonstrates the utility of the *p*NZ group as a permanent protecting group in solid-phase peptide synthesis. *p*NZ can be used with Cl-Trt-resin to prepare peptides having acid sensitive residues, for which ^{t}Bu-type protection is not possible. The *p*NZ group is removed in solution or on-resin with $SnCl_2$ and a catalytic amount of acid.

4. Experimental section

4.1. Fmoc-L-Orn-OH

Fmoc-L-Orn(Boc)-OH (2 g, 4.4 mmol) was dissolved in TFA–DCM (3:7) and stirred for 30 min. After evaporating off the solvent, diethyl ether was added to, and evaporated from, the crude residue five times in order to ensure that all of the TFA had been removed. The final product (1.31 g, 84% yield) was characterized by analytical HPLC (<98% purity) and ES-MS (calcd for $C_{20}H_{22}N_2O_4$: 354.15, found *m*/*z* 355.1 $(M+1)^+$).

4.2. Fmoc-L-Orn(pNZ)-OH

Protection of the side chain of Orn was carried out using the azide method.[17] *p*-Nitrobenzyl chloroformate (0.797 g, 3.7 mmol) was dissolved in dioxane (1.7 mL) and a solution of sodium azide (0.289 g, 4.44 mmol) in H_2O (1.1 mL) was added. The resulting emulsion was stirred for 2 h and the formation of the azide was followed by TLC (CH_2Cl_2). This solution was added dropwise to a suspension of Fmoc-L-Orn-OH (1.31 g, 3.7 mmol) in 6 mL of dioxane–2% aqueous Na_2CO_3 (1:1) and the resulting white suspension was stirred for 24 h with the pH kept between 9 and 10 by adding 10% aqueous Na_2CO_3. At this point, TLC (CH_2Cl_2) results indicated that there was no azide left, H_2O (50 mL) was then added and the suspension was washed with *tert*-butyl methyl ether (MTBE) (3 × 40 mL). The aqueous portion was acidified to pH 1 with 9 N HCl, affording a white precipitate that was filtered off and dried to yield 1.67 g (85% of yield) of the title compound. The white solid was characterized by HPLC (>98% purity), ESMS (calcd for $C_{28}H_{27}N_3O_8$: 533.2, found *m*/*z* 534.4 $(M+1)^+$), ^{1}H and ^{13}C NMR.[18]

4.3. KF analogs

To chlorotrityl resin (500 mg, $f = 1.27$ mmol/g) placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk, was added Fmoc-D-Val-OH (0.7 equiv), DIEA (7 equiv) and dichloromethane, and the resulting slurry was stirred for 1 h. The reaction was then terminated by adding piperidine–DMF (1:4) and the loading was calculated by UV ($f = 0.56$ mmol/g). The chain was elongated by sequentially coupling Fmoc-D-allo-Ile-OH, Fmoc-D-allo-Thr-OH and Fmoc-D-allo-Ile-OH (4 equiv), using DIPCDI (4 equiv) and HOBt (4 equiv) in DMF for 90 min. The completion of all couplings was confirmed with a negative ninhydrin test result. All Fmoc groups were removed as indicated above except the last group, which was not removed. Alloc-Val-OH (5 equiv) was then coupled to the peptide with DIPCDI (5 equiv) and DIEA (1 equiv) in the presence of DMAP (0.5 equiv). This coupling was repeated twice using the same conditions. An aliquot of the resin was then treated with TFA–H_2O (1:99) for 1 min and, after evaporation, the crude product corresponding to the peptide resin **3** was characterized by HPLC (>95% purity) and ESMS (calcd for $C_{30}H_{53}N_5O_9$, 627.4 Found: *m*/*z* 628.2 $[M+H]^+$). Then, the Fmoc group of Fmoc-D-allo-Ile-OH was removed and Fmoc-Orn(*p*NZ)-OH (4 eq), Fmoc-D-Pro-OH (5 equiv), Fmoc-D-Val-OH (4 equiv, a recoupling was needed), Fmoc-L-Val-OH (4 equiv), Fmoc-Thr-OH (5 equiv) and Fmoc-D-Val-OH (4 equiv) were sequentially added to the peptide resin and stirred for 90 min in the presence of equimolar amounts of DIPCDI and HOBt in DMF.

After removing the last Fmoc group, an aliquot of the peptide resin was cleaved as indicated above and the crude product obtained (corresponding to peptide resin **4**) characterized by HPLC (>98% of purity) and MALDI-TOF (calcd for $C_{67}H_{109}N_{13}O_{20}$: 1416.7, found: *m*/*z* 1417.1 $[M+H]^+$, 1439.1 $[M+Na]^+$, 1455.0 $[M+K]^+$). The remaining resin was suspended in DMF and divided into two portions. Oleanolic acid (3 equiv) was coupled to one of the portions, and maslinic acid (3 equiv) was

coupled to the other using PyBOP (3 equiv), HOAt (3 equiv) and DIEA (9 equiv) in DMF. An aliquot of each peptide resins was cleaved and characterized by HPLC (**5b**: >78% of purity, **5c**: >80% of purity) and ESMS (**5b**: *m/z* calcd for $C_{97}H_{155}N_{13}O_{22}$: 1854.1, found *m/z* 1855.6 $[M+H]^+$; **5c**: *m/z* calcd for $C_{97}H_{155}N_{13}O_{23}$: 1870.1, found *m/z* 1871.6 $[M+H]^+$).

The Alloc group of both resins was removed using $Pd(PPh_3)_4$ (0.1 equiv) and $PhSiH_3$ (10 equiv) in DMF (three treatments of 15 min). The resin was then washed with DCM and DMF, followed by 0.02 M sodium diethyldithiocarbamate in DMF (3×15 min). Alloc-Phe-*Z*Dhb-OH (2.5 equiv), HOAt (2.5 equiv) and DIPCDI (2.5 equiv) in DMF were added to each resin, and the mixture was stirred overnight to yield the peptidyl resins **6b** and **6c**. The removal of Alloc group and subsequent washings were carried out as described above.

Both protected peptides were cleaved from the resin with TFA–DCM (1:99) (5×30 s) and successive washings with DCM. Filtrates were collected in H_2O and the solvent was partially removed in vacuo. Acetonitrile (MeCN) was added to dissolve the solid that appeared during H_2O removal, the solutions were lyophilized and the products characterized by HPLC (**7b**: >91% of purity, **7c**: >73% of purity) and ESMS (**7b**: calcd for $C_{106}H_{165}N_{15}O_{22}$, 2001.5, found *m/z* 2002.0 $[M+H]^+$; **7c**: calcd for $C_{106}H_{165}N_{15}O_{23}$, 2017.5, found *m/z* 2018.5 $[M+H]^+$).

The resulting crudes were dissolved in DCM and cyclized in the presence of DIPCDI (3 equiv), HOBt (4 equiv), and DIEA (3 equiv). After 1 h of stirring, no starting materials remained as indicated by HPLC, so the solvent was removed.

Removal of the *p*NZ groups was carried out by stirring the peptides with 1 M $SnCl_2$ and 1.6 mM HCl–dioxane in DMF for 1 h. The solvent was removed in vacuo, and the crudes were purified by HPLC (Kromasil C_8 5 μm, 205×50 mm) then characterized by HPLC (**1b**: 32 mg, >98% of purity, **1c**: 19 mg, >97% of purity) and MALDI-TOF-MS (1b: calcd for $C_{98}H_{157}N_{14}O_{17}$, 1803.8. Found: *m/z* 1804.8 $[M+H]^+$, 1825.8 $[M+Na]^+$, 1841.8 $[M+K]^+$; **1c**: calcd for $C_{98}H_{158}N_{14}O_{18}$, 1819.2. Found: *m/z* 1820.0 $[M+H]^+$, 1842.9 $[M+Na]^+$, 1857.9 $[M+K]^+$).

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18. ^{1}H NMR (400 MHz, DMSO-d_6): 8.2 (2H, d, $J = 8.4$ Hz, H3′ and H5′); 7.9 (2H, d, $J = 7.2$ Hz, H1 and H8); 7.7 (2H, d, $J = 7.2$ Hz, H4 and H5); 7.6 (2H, d, $J = 8.8$ Hz, H2′ and H6′); 7.4 (2H, dd, $J_1 = J_2 = 7.2$ Hz, H3 and H6); 7.3 (2H, dd, $J_1 = J_2 = 7.2$ Hz, H2 and H7); 5.1 (2H, s, –O–CH_2–4-nitrophenyl); 4.2 (3H, m, H9 and fluorene–CH_2–O–); 3.8 (1H, m, α̃CH Orn); 3.0 (2H, m, α̃CH_2 Orn); 1.7 (1H, m, α̃CH_2 Orn); and 1.6 (1H, m, α̃CH_2 Orn); 1.4 (2H, m, α̃CH_2 Orn). ^{13}C NMR (100 MHz, DMSO-d_6): 174.5 (–*C*O–OH); 156.6 and 156.5 (–N–*C*O–O–) 147.6 (C4′); 146.1 (C1′); 144.6 and 144.5 (C12 and C13); 141.4 (C10 and C11); 128.7 (C2′ and C6′); 128.3 (C2 and C7); 127.8 (C3 and C6); 126.0 (C4 and C5); 124.2 (C3′ and C5′); 120.8 (C1 and C8); 66.2 (fluorene–*C*H_2O–); 64.6 (–O–*C*H_2–4-nitrophenyl); 54.8 (α̃CH Orn); 47.36 (C9); 40.8 (α̃CH_2 Orn); 29.4 (α̃CH_2 Orn); 26.7 (α̃CH_2 Orn).