Pergamon

Tetrahedron Letters 43 (2002) 7813–7815

TETRAHEDRON LETTERS

Undesired removal of the Fmoc group by the free ε-amino function of a lysine residue

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Received 15 July 2002; revised 26 July 2002; accepted 2 August 2002

Abstract—In solid-phase peptide synthesis, a side-reaction consisting of the premature and undesired removal of the Fmoc group has been detected. This can be caused by a primary amine of sufficient basicity, such as the ε-amino of the Lys, present in the peptide resin. This side-reaction, which is not promoted by either the β-amino side-chain of the Dapa residue or the α-amino group, can be prevented by a coupling/neutralization protocol in the case of Mtt protection or by a tandem deprotection–coupling reaction in the case of Alloc protection. The same kind of side-reaction has been detected when amino side-chain functions of Orn or Daba are free in the peptide resin.

Lysine is a key amino acid for the synthesis of complex peptides, such as cyclic,[1] branched,[2] and modified systems with labels or probe molecules.[3] Furthermore, it is a useful scaffold for the preparation of small molecules in drug discovery programs.[4] In all these cases, the two amino functions of Lys can be used for anchoring different building blocks. For this type of process it is necessary to have both amino functions protected with orthogonal groups.[5,6] In an Fmoc-based strategy, the most common orthogonal protecting groups are 4-methyltrityl (Mtt)[7] and allyloxycarbonyl (Alloc).[8] While Mtt is removed by low concentrations of TFA in the presence of a scavenger, Alloc is removed by an allyl transfer reaction to an allyl scavenger, usually $PhSiH_3$[9] or $H_3N{\cdot}BH_3/Me_2NH{\cdot}BH_3$,[10] in the presence of Pd(0).

In some of our programs, we have observed a double incorporation of an acyl compound in an N^{α}-Fmoc, N^{ε}-protected Lys-containing structure anchored to a solid support after removal of the ε-protecting group of the lysine and its subsequent reaction with an acylating reagent. This fact could indicate that the Fmoc group had also been removed.[11] In the case of the ε-Alloc compound, the premature Fmoc removal could be interpreted in terms of the abstraction of the H of the Fmoc group by the hydride of the scavenger. However, this explanation does not apply in the case of Mtt. Thus, we decided to undertake a systematic study to investigate the scope of this side-reaction as well as methods to overcome it.

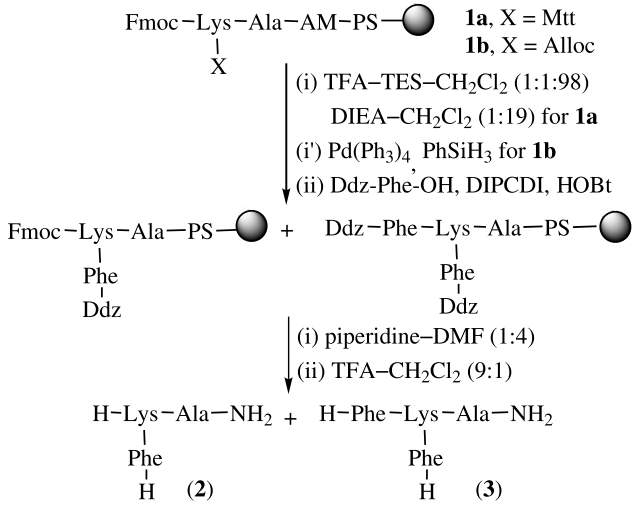

Figure 1. Strategy used for the determination of the extent of side-chain reaction for Lys-containing peptides.

Abbreviations: Ac, acetyl; AM, *p*-[*R*,*S*]-α-[1-(9-fluorenyl)methoxyformamido]-2,4-dimethoxybenzyl]phenoxyacetic acid; DIEA, *N*,*N*-diisopropylethylamine; Ddz, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *N*-{(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene}-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, hydroxybenzotriazole; PS, polystyrene; PyAOP, 7-azabenzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; amino acid symbols denote the L-configuration.

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0040-4039/02/$ - see front matter
PII: S0040-4039(02)01605-2

Firstly, Fmoc–Lys(X)–Ala–AM–PS–resin (X = Mtt, Alloc) was chosen as a model system and the protocol shown in Fig. 1 was followed. Treatment of the model peptide resin with the corresponding deprotection reagent, incorporation of Ddz–Phe–OH,[12] removal of the Fmoc group from the α-amino function, and cleavage with TFA led to two possible peptides: H–Lys(Phe-H)–Ala–NH_2 (**2**) (no premature removal of the Fmoc group) and H–Phe–Lys(Phe-H)–Ala–NH_2 (**3**) (premature removal of the Fmoc group).[13] The ratio between these two peptides was calculated by amino acid analysis (AAA) and HPLC. The presence of **3** was corroborated by MALDI-TOF-MS.

In the case of **1a**, 80% of **2** and 20% of **3** were obtained but for **1b**, the results showed 65% of **2** and 35% of **3**.

A similar experiment was carried out with Fmoc–Ala–Lys(Alloc)–Ala–AM–PS–resin (**4**) to assess whether the side-reaction is dependent on the position of the Fmoc group with respect to the ε-amino function. In this case, 14% premature removal of the Fmoc group [H–Phe–Ala–Lys(Phe-H)–Ala–NH_2] was detected by HPLC and AAA, demonstrating that the side-reaction was not particularly dependent on the relative positions of the two groups.

A similar experiment was then carried out with Fmoc–Dapa(Alloc)–Ala–AM–PS–resin (**5**) and Alloc–Dapa-(Fmoc)–Ala–AM–PS–resin (**6**). Compound **5** was similar to **1b**, where the Lys (4 methylene groups in the side-chain) was substituted by N^{α}-Fmoc-N^{β}-Alloc-L-diaminopropionic acid (1 methylene group in the side-chain), and **6** was similar to **5**, where the two protecting groups of the Dapa residue had been switched. In neither case was premature elimination of the Fmoc group detected.

At that moment it was already clear that the premature removal of the Fmoc group was not related to either the protecting group or the method used to remove it, but was influenced by the basicity of the amino function liberated. It is well known that an ε-amino group, when adjacent to a carbon containing both a carboxamido and a carbamate function (as in **1**), is more basic than both a β-amino group, in the same environment as above (as in **5**), and an α-amino group (as in **6**).

In order to assess the scope of the side-reaction, the test experiment was carried out with Fmoc–Daba(Alloc)–Ala–AM–PS–resin (**7**) (Daba, α,β-L-diaminobutyric acid; 2 methylene groups in the side-chain) and Fmoc–Orn(Alloc)–Ala–AM–PS–resin (**8**) (3 methylene groups in the side-chain). In the case of **7**, 19% of the peptide containing two residues of Phe was obtained, whereas for **8** 21% of the side-product was formed.

A further experiment was carried out to show that the side-reaction was caused by the presence of the free amino function. Thus, resin **1b** was treated with Pd(0) in the presence of the scavenger and then the resin containing the free amine function was shaken in DMF for 16 h. After this time, the incorporation of Ddz–Phe–OH resulted in 95% of double incorporation of Phe.

These results are consistent with the pK_a values of these amino functions in the model compounds shown in Table 1. Thus, while the pK_a values of the side-amino functions of Lys, Orn, and Daba are very close, the pK_a of Dapa is lower by one unit, making this amino function less basic than the another derivatives. The same explanation applies for the α-amino function.

Finally, alternative strategies were tested to circumvent this side-reaction. In order to achieve this goal, it is necessary to avoid the presence of the free ε-amino group in a similar way to methods used to avoid other side-reactions, such as the formation of diketopipezarines (DKP).[9,14,15]

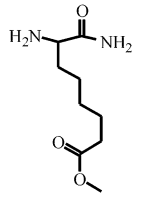
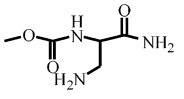
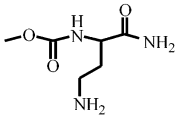
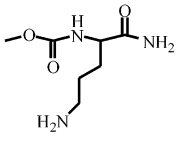
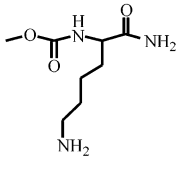
For the Mtt-containing peptides, the protecting group is removed with TFA/TES/CH_2Cl_2 (1:1:99) and then the incorporation of the Ddz–Phe–OH is carried out, without a prior neutralization step, using PyAOP as the coupling reagent in a similar way to the method used to avoid the formation of DKP's.[15–17]

For the Alloc-containing peptides, a tandem deprotection–coupling reaction, in which the removal of the Alloc group with $Pd(PPh_3)_4/PhSiH_3$ is carried out in the presence of Ddz–Phe–F, avoids this side-reaction.[9]

In both cases, double incorporation was not detected neither by HPLC and MALDI-TOF-MS.

In conclusion, in the solid phase the N^{α}-Fmoc protecting group can be prematurely removed by a primary amine of sufficient basicity, such as the ε-amino group of Lys, present in the peptide resin. This side-reaction,

Table 1. pK_a of amino function according to the *p*Kalc module (PALLAS version 2.0, CompuDrug)

				
*p*Ka: 8.04	*p*Ka: 8.49	*p*Ka: 9.45	*p*Ka: 10.00	*p*Ka: 10.09

which is not promoted by either the β-amino side-chain of the Dapa residue or the α-amino group, can be prevented by a coupling/neutralization protocol in the case of Mtt protection or by a tandem deprotection–coupling reaction in the case of Alloc protection.

Experimental protocols

Standard SPPS protocols

The Fmoc group was removed by treatment with piperidine/DMF (1:5) (2×15 min). Incorporation of protected amino acids (5 equiv.) was performed with DIPCDI (5 equiv.) and HOBt (5 equiv.) in DMF for 2 h.

Removal of the Mtt group was carried out with TFA/TES/CH_2Cl_2 (1:1:98) (2×10 min).

Removal of the Alloc group was achieved with $Pd(PPh_3)_4$ (0.1 equiv.) in the presence of $PhSiH_3$ (20 equiv.) in CH_2Cl_2 under Ar (2×20 min, 25°C).

Cleavage of peptides from the resin was carried out with TFA/CH_2Cl_2 (9:1) for 2 h at 25°C.

Circumventing premature removal of Fmoc

The Mtt-containing peptide resin was treated with TFA/CH_2Cl_2 (1:99) (3×1 min), washed with CH_2Cl_2 (5×30 s), and the protected amino acid (10 equiv.), PyAOP (10 equiv.), and DIEA (20 equiv.) were sequentially added and the mixture left with occasional stirring for 2 h at 25°C.

The Alloc-containing peptide resin was washed with CH_2Cl_2 (5×30 s) under Ar, and the active species of the protected amino acid (10 equiv.), $Pd(PPh_3)_4$ (0.10 equiv.), and $PhSiH_3$ (10 equiv.) in CH_2Cl_2 were added and the mixture left with occasional stirring for 2 h at 25°C.

Acknowledgements

We thank Dr. Victor Segarra from Almirall-Prodesfarma for the pK_a calculations. M.R. is a 'Ramon y Cajal' fellow (MCyT, Spain). This work was partially supported by CICYT (BQU2000-0235), Generalitat de Catalunya (Grup Consolidat, and Centre de Referència en Biotecnologia).

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