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Fmoc-2-Mercaptobenzothiazole (MBT), for the Introduction of the Fmoc Moiety Free of Side-Reactions

Abstract*: A double side-reaction, consisting in the formation of Fmoc-β-Ala-OH and Fmoc- β-Ala-AA-OH, during the preparation of Fmoc protected amino acids (Fmoc-AA-OH) with Fmoc-OSu is discussed. Furthermore, the new Fmoc-2-MBT reagent is proposed for avoiding these side-reactions as well as the formation of the Fmoc-dipeptides (Fmoc-AA-AA-OH) and even tripeptides, which is another important side-reaction when chloroformates such as Fmoc-Cl is used for the protection of the α-amino function of the amino acids.*

Keywords: ***Alloc, Fmoc-dipeptides, Fmoc-OSu, p-NZ, protecting group, side-reaction,***

INTRODUCTION

Carbamates such as Boc,[1,2] Fmoc,[3] Z,[4] and in minor extension Alloc,[5,6] pNZ,[7] and Troc[8] are the most efficient way to mask the nucleophilicity of the amino function during peptide synthesis.[9,10] The introduction of the alkoxycarbonyl moiety was

first carried out under weakly basic conditions via the Schotten-Baumann reaction using the corresponding chloroformates (also known as chloride).[3] However, our group[11] and others[12,13,14,15] described in the early eighties that during the preparation of Fmoc-amino acids from Fmoc-Cl, the most part of the amino acids maybe contaminated with significant levels (1-20%) of their corresponding Fmoc-dipeptide and even tripeptide. As an example, even when the relatively hindered Alloc-Val-OH was prepared in a laboratory scale, 14% of the corresponding dipeptide was obtained.[9] 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 the preparation of an API. This side-reaction can be avoided by carrying out an *in situ* bis-trimethylsilylation protection followed by reaction with the chloride.[14,16] The fact that the temporal protection of the carboxylic function avoids the side-reaction reinforces that the mechanism goes through a asymmetrical anhydride (Figure 1).

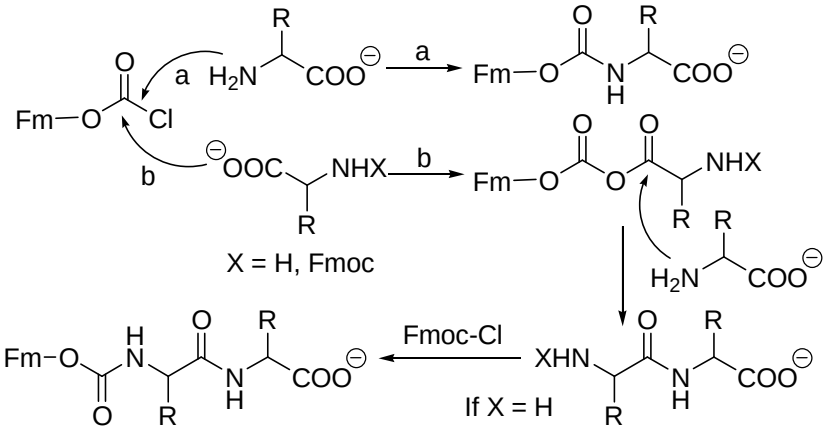

Figure 1. Mechanism for the formation of protected dipeptides during the protection of amino acids

As alternative to the chloride and as this side-reaction is associated to the quality of the leaving, we proposed the use of the less reactive azide derivative, which can be readily prepared and isolated from the chloroformate[3,11] or prepared *in situ*

before reacting with the amino acid.[17] Several approaches based in the use of others less reactive species such as the 1,2,2,2-tetrachloroethyl,[18,19] the 5-norbornene-2,3-dicarboximido,[20] the pentafluorophenyl,[21] and the symmetrical pyrocarbonates[22] have been proposed, but the hydroxysuccinimido (Su)[12,13,15,23,24] ester has been considered the reagent of choice for the introduction of the protecting moiety.

However, lastly the use of Fmoc-OSu has been questioned, because it has been showed that commercial Fmoc-amino acids prepared from Fmoc-OSu contains Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH as contaminants (0.1-0.4%).[25] Although, these are tiny amounts, they are unacceptable in the manufacture of a drug substance.

Herein, a discussion regarding the formation of this double side-reaction is carried out as well as an alternative for the preparation of protected amino acids free of side reactions.

EXPERIMENTAL SECTION

General

2-MBT was obtained from ARIEL (City, Country). HPLC was performed on a reversed-phase C_{18} (4.6 × 150 mm, 5 μm) with a linear gradient of 0.045% TFA and 0.036% in CH_3CN (from 0 % to 100%) at a flow rate of 1.0 mL/min and detection of 220 nm.

Fmoc-2-MBT

MBT ·DCHA salt:

To a solution of 2-MBT (5g, 29.9 mmol) in 250 mL of EtOAc, DCHA was added and the resulting suspension was stirred overnight. The precipitate obtained was isolated by filtration washed with ethyl acetate and dried in vacuo yielding a white solid (9.44 g, 91% of yield).

Fmoc-2-MBT:

To a solution of Fmoc-Cl (7.01g, 27.1 mmol) in 70 mL of $CHCl_3$, MBT·DCHA salt (9.44 g, 27.1 mmol) was slowly added and stirred overnight. The suspension was filtered and the solid washed with 2 x 10 mL of $CHCl_3$. The filtrate was washed with 10% of aq. citric acid (2 x 30 mL), 10 % of aq. $NaHCO_3$ (2 x 30 mL), H_2O (2 x 30 mL), dried over $MgSO_4$ and evaporated to dryness to yield the title compound as a white solid (8.53 g, 81.9 % of yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ= 8.01 (d, 1H, *J*= 8.2 Hz), 7.85 (d, 1H, *J*= 8.0 Hz), 7.72 (d, 2H Fmoc, *J*= 7.5 Hz), 7.53 (d, 2H Fmoc, *J*= 7.5 Hz), 7.47 (dd, 1H, *J*= 8.0 Hz, *J'*= 7.4 Hz), 7.39 (m, 2H Fmoc and 1H MBT), 7.28 (dd, 2H Fmoc, *J*= 7.5 Hz, *J'*= 7.5 Hz), 4.60 (d, 2H, *J*= 7.3 Hz), 4.27 (t, 1H, *J*= 7.3 Hz).

^{13}C NMR (100 MHz, $CDCl_3$): δ= 166.47, 157.49, 152.40, 143.00, 141.53, 136.99, 128.33, 127.53, 126.72, 125.99, 125.28, 123.47, 121.45, 120.40, 70.96, 46.78.

Fmoc-amino acids using Fmoc-2-MBT

Fmoc-glycine

H-Gly-OH (318 mg, 4.24 mmol) was suspended in 24 mL of a mixture of 1% aquous Na_2CO_3-dioxane (1:1, v/v) and cooled with and ice bath. A mixture of Fmoc-MBT (1.5 g, 3.85 mmol) and 4 mL of dioxane was slowly added keeping the pH at 9.5 with 10% aq. Na_2CO_3. The ice bath was removed and the suspension stirred at room temperature keeping the pH at 9.5. The course of the reaction was followed by TLC. After 24 h of stirring 50 mL of H_2O were added to the reaction mixture, the pH was adjusted to 8 with 1N HCl and washings with MTBE (3 x 30mL) were carried out. The aq. phase was acidified to pH 1 with HCl-H_2O (1:2) and extracted with EtOAc (3 x 40 mL). The organic fractions were dried with $MgSO_4$, evaported to dryness and the resulting yellow solid was washed with DCM to give Fmoc-Gly (627 mg, 55% of yield, yield not optimized) as a white solid. The product was characterized by HPLC and ESMS.

RESULTS AND DISCUSSION

A possible explanation for the presence of this double side-reaction (formation Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH) are depicted in Figure 2. One of the cornerstones of this process involves the presence of a nucleophile and herein two nucleophiles are present: ⁻OSu and ⁻OH.

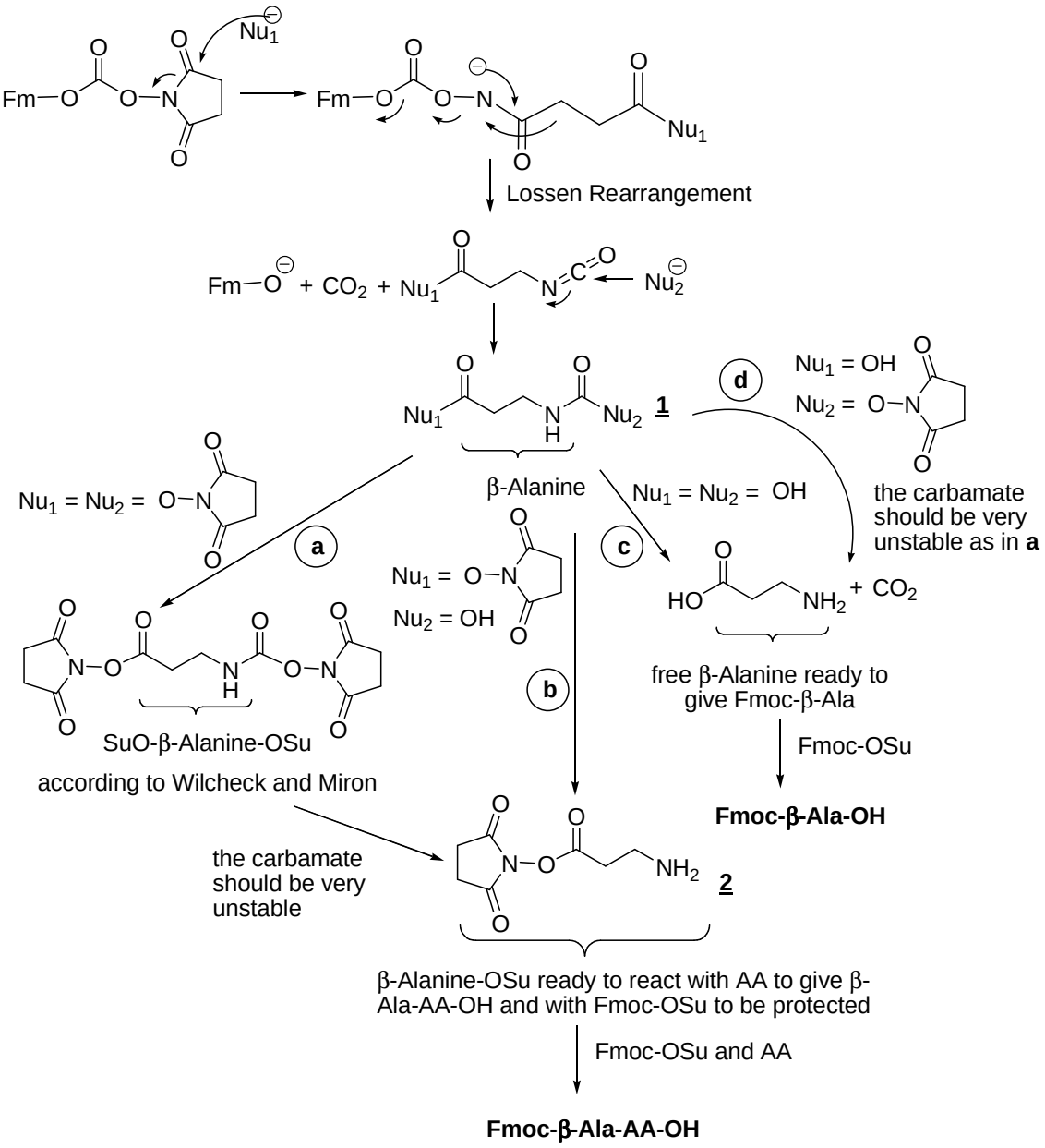

Figure 2. Mechanism for the formation of Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH during the protection of amino acids

The β-Ala structure (**1**) was formed through a Lossen rearrangement after the attack of a nucleophile, probably $^{-}$OSu, to one of the carbonyls of the HOSu moiety presented in the Fmoc-OSu. A similar pathway for this first part of the mechanism was first reported by during the reaction of HOSu with DCC,[26] and then by Wilcheck and Miron.[27] Furthermore, similar β-Ala impurities have been found by Zalipsky during the use PEG-OSu as pegylation reagent.[28]

The formation of the free amine of the β-alanine is coming from the carbamic acid (Nu_2=OH, pathways b and c) or from the rather unstable *O*-*N*-succinimide carbamate (Nu_2=Su, pathways a and d). Furthermore, the formation of the Su ester of β-Ala (**2**), which is susceptible of reacting with the free amino acid to give the dipeptide, is formed when Nu_1=Su (pathways a and b). This mechanism also interprets the major content found by Hlebowicz et al.[25] (Table 9, Arg) of β-Ala by amino acid analysis than those found by HPLC (Fmoc-β-Ala-Arg-OH + Fmoc-β-Ala-OH). This higher amount can be interpreted from **2**, which can give polymerization and therefore the corresponding peaks can be more difficult to be identified. Furthermore, this scheme agrees with the no formation of Fmoc-AA-β-Ala-OH as pointed out by Hlebowicz et al.[25]

Formation of H-β-Ala-OH or H-β-Ala-OSu can take place either during the preparation of Fmoc-OSu or during the preparation of the protected amino acids. However, the HPLC analysis of commercial Fmoc-OSu has revealed the absence of Fmoc-β-Ala-OH and therefore indicated that the side-reaction is taking place during the protection of the amino acids.

Thus, if a substitute to Fmoc-OSu wants to be found, this should be of a reactivity similar to OSu esters and therefore lower that other active species such as OBt, which

leads to the formation of protected peptides or tripeptides.[12] In this regard, the 2-MBT recently proposed by Evans as additive to the carbodiimides based coupling in substitution of the HOBt called our attention.[29] Thus, pKa calculations carried out with ChemAxon software showed that the pKa of both tautomers of 2-MBT (*p*Ka's: NH tautomer, 10.90; SH tautomer, 7.49) was higher than the one of HOSu (*p*Ka HOSu, 7.19) making it 2-MBT derivatives potentially less reactive than OSu ones.

One additional advantage of 2-MBT is the price as it is broadly used as ARIEL SOME INFORMATION?.

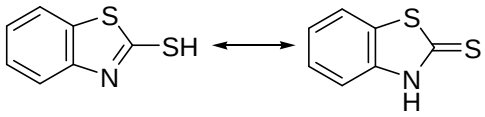

Figure 3. Structure of 2-MBT

Preparation of Fmoc-2-MBT was readily achieved by reaction of 2-MBT with Fmoc-Cl following a well reported method described for another reagents.[23] Although, in the literature is described that 2-MBT active species are presented in a mixed of *S*- and *N*-regioisomers, the structure obtained by X-ray diffraction of the Fmoc-2-MBT showed to be just the *S*-regioisomer (Figure 4).

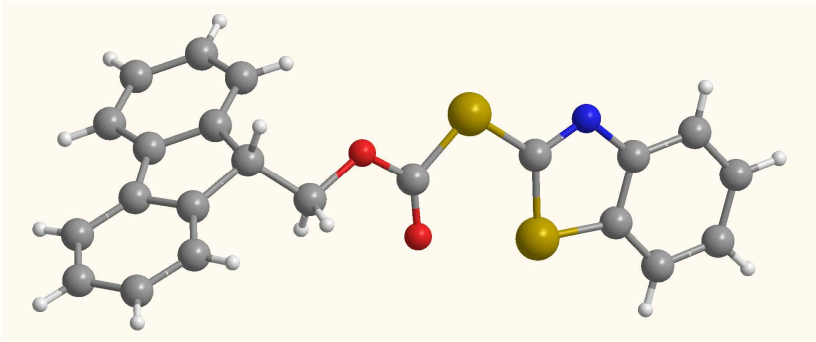

Figure 4. Structure of the Fmoc-2-MBT obtained by X-Ray Difraction.

Fmoc-2-MBT reacts smoothly with amino acids to render the corresponding Fmoc-AA-OH. It is worthy to indicate that the acylation reaction is slower when it is carried

out with Fmoc-2-MBt than when Fmoc-OSu is used (24 h *vs* 0.5 h to accomplish a total conversion). This rather slow conversion indicates than 2-MBT active species are less reactive than OSu ones and therefore that the acylation presumably is taking place without the formation of Fmoc-dipeptides, because as it was discussed earlier, its formation is associated to the effectivity of the active specie. The 2-MBT liberated during the reaction can be removed by washing the final product with DCM. HPLC analysis of the crude Fmoc-amino acids obtained following this method showed that no formation of dipeptide took place and therefore that they are free of side-products (Figure 5), confirming the first hypothesis about the poor reactivity of the 2-MBT derivatives.

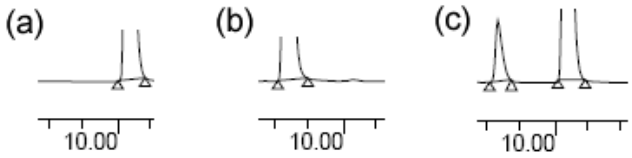

Figure 5. HPLC analysis: (a) Fmoc-Gly-OH obtained, (b) Fmoc-Gly-Gly-OH, (c) coinjection of Fmoc-Gly-OH and Fmoc-Gly-Gly-OH.

CONCLUSIONS

The use of the new Fmoc-2-MBT reagent allows the preparation of Fmoc-amino acids free of the three side-products: (a) Fmoc-dipeptides associated to the quality of the leaving group and that takes place when Fmoc-Cl is used; (b) Fmoc-β-Ala-OH; and (c) Fmoc-β-Ala-AA-OH. These two side-products is produced when Fmoc-OSu is used as acylating reagent. A mechanism for the presence of the side-products has been also proposed.

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ABBREVIATIONS

Abbreviations used for amino acids and the designations of peptides follow the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem*. **1982**, *247*, 977−983. Amino acid symbols denote L-configuration.
API, active pharmaceutical ingredient; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DCM, dichloromethane; EtOAc, ethyl acetate, ESMS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, hydroxybenzotriazole; HPLC, high performance liquid chromatography; 2-MBT, 2-mercaptobenzothiazole; MTBE, *tert*-butylmehtyl ether; OSu, *O*-succinimidyl; PEG, polyethylene glycol; polystyrene solid support; *p*NZ, *p*-nitrobenzyloxycarbonyl; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; Troc, 2,2,2-Trichloroethyloxycarbonyl.

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