A comparison between the yields, purity and reaction time for the preparation of aspartame (H-Asp-Phe-OMe) by the ball-milling process under conditions, using different activating esters of N^{α}, $COOH^{\beta}$-diprotected aspartic acid

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A comparison between the yields, purity and reaction time for the preparation of Aspartame (H-Asp-Phe-OMe) by the ball-milling process under conditions, using different activating esters of N^{α}, $COOH^{\beta}$-diprotected aspartic acid: *The paper describes the comparison between the yields, purity and reaction time for the preparation of the artificial sweetener aspartame by the ball-milling process, using a variety of activating esters of protected aspartic acid. These results, juxtaposed between the various activating esters prove that their negative induction effect correlated well with the yield and reaction time of the prepared product.*

Key words: *Ball-milling reaction, Aspartame, Peptide synthesis, Activating esters, Green chemistry.*

INTRODUCTION

In the previous article [1] was described the convenient synthesis of the artificial sweetener aspartame by the ball-milling process, using a variety of activating esters of N^{α}, $COOH^{\beta}$-diprotected aspartic acid. The synthesis was carried out in the absence of solvents, leading to the production of the final compound in good yields and purity in general. A new synthetic approach for synthesis was developed, supporting the green synthesis approach, without using any solvents.

Herein we describe a comparison between the yields, purity and reaction time for the preparation of Aspartame (H-Asp-Phe-OMe) by the ball-milling process under conditions, using different activating esters of N^{α}, $COOH^{\beta}$-diprotected aspartic acid. The N-protective groups don't have any influence on the reaction time, yields and purity. This was the reason to avoid this factor from the others, relying to its low importance.

LAYOUT

In general, ball-milling process has a widespread influence for synthesis of nanoparticles, used in nanotechnology. Ding Chen, Yingzhe Zhang and Chuanjun Tu demonstrated the preparation of high saturation magnetic $MgFe_2O_4$ nanoparticles by microwave-assisted ball milling process [2]. Zhejuan Zhang, Z. Sun, Yiwei Chen reported the process, assisting carbon nanotubes treated by ball-milling reaction [3]. S.L.A. Hennart, M.C. Domingues, W.J. Wildeboer, P. van Hee, G.M.H. Meesters described the study of the process of stirred ball milling of poorly water soluble organic products using factorial design [4]. Also J.L. Li, L.J. Wang, G.Z. Bai and W. Jiang produced carbon tubes during high-energy ball milling process [5]. X.L. Shi et al. succeeded to synthesize $(Mg_{0.476}Mn_{0.448}Zn_{0.007})(Fe_{1.997}Ti_{0.002})O_4$ powder and sintered ferrites by high energy ball-milling process [6].

I their article Ohara S. et. demonstrated novel mechanochemical synthesis of fine $FeTiO_3$ nanoparticles by a high-speed ball-milling process [7]. In the article of Xueqing Yue, the effect of expansion temperature of expandable graphite on microstructure evolution of expanded graphite during high-energy ball-milling is described [8]. Jaewoo Kim et al. described in their article the synthesis and growth of boron nitride nanotubes by a ball milling–annealing process [9] and Youngseok Oh et al. revealed the effects of ball milling process on the diameter dependent fracture of single walled carbon nanotubes [10].

In the previous article [1], we mentioned that the product (aspartame) was prepared in different yields, depending on the sort of the activating ester. When N-hydroxybenzotriazole active ester was used, the yield was the highest (98%, for 6 hours), after that the N-hydroxysuccinimide activated ester (95% yield, 8 hours), the p-nitrophenyl activating ester (93% yield, 9 hours), and the N-hydroxymaleimide activated ester (91%

yield, 10 hours) are the following compounds, prepared in high yields (Table 1). Other esters had lower yields, due to the lower reactivity of the activating esters. 2,4,5-trinitrophenyl and 2,4,5-trichlorophenyl esters exhibited this low reactivity, probably because of the sterically hindering of these groups and inaccessibility of the attacking nucleophile (the alpha-amino group of H-Phe-OMe) to the reactive centre of the carboxyl group (the carbon atom) (Table 1).

Nevertheless, these two activating esters (2,4,5-trinitrophenyl and 2,4,5-trichlorophenyl esters) describe good yields of the reaction products, prepared by their participation in the condensation reaction, despite the prolonged reaction time: 73% and 71% for 18 hours accordingly (Table 1). As it can be seen from the table, the N-hydroxymaleimide ester has a very good reactivity and the prepared aspartame by this activated ester has high yields (yield 91% for 10 hours). This fact probably is attributed to the structural similarity of this reagent to the N-hydroxysuccinimide activated ester. Another compounds, that described a high reactivity as activating esters were 2-hydroxy 1,2-propyl ester and 2-hydroxy ethyl ester (ethylene glycol esther and 1,2-propylene glycol ester): 86% for 20 hours and 84% for 15 hours, which gives the considerable possibility for using these esters in the large scale preparation of peptide products. This high reactivity is due to the fact, that the *cis*-vicinal 2-OH group plays the role as a catalyst, allowing to the acceleration of the electrophilicity in the carbonyl carbon atom from the ester group and its higher susceptibility to the nucleophilic attack from the external nucleophile. This is the main reason the 2-methoxy ethyl ester (in which the vicinal hydroxyl group is methylated) to exhibit the lower reactivity and lower yield in the resulting product by the reaction with this activated ester: 79% for 16 hours. By the same reason the 2,2,2-trichloroethyl activating ester describes a lower reactivity, despite the strong inductive effect, which trichloroethyl group shows, due to three chlorine atoms. The same fact explains the low reactivity of cyanomethyl ester (yield 65% for 24 hours), vinyl ester (yield 64% for 20 hours) and acetylenyl ester (acetylene ester, ethine ester) (yield 59% for 24 hours).

The above noted compounds (as activated esters) were employed for the synthesis of aspartame for a different period, described in the Table 1. The reaction time was varying from 6 hours to 24 hours due to the fact, that the longer reaction time leads to the side products formation and deterioration of the product purity, monitored by HPLC. From another hand, the reaction period of 6 hours was sufficient for the product preparation, applying the N-hydroxybenzotriazole ester, providing by this way aspartame in high yield and purity. At the same time for other activating esters, the reaction had to be monitored to choice the optimal time as for the high yields achievement, as well as for the realization of high purity, by the prevention of a possibility from unwanted side reactions.

All of the above mentioned activating esters, used in our originally developed procedure for ball-milling green peptide synthesis, can also be applied in the synthesis of other peptides with important biological properties and application in the pharmaceutical industry, by the same methodology. Another peptides were synthesized by the authors, using the same procedure, and the results will be published elsewhere [11]. Moreover, the kinetics of the reaction for a preparation of the concrete product will be studied, employing the different activating esters of N^{α}, $COOH^{\beta}$-diprotected aspartic acid with a goal – to reveal the reaction mechanism as well as – for control of the reaction.

And finally, this methodology will be developed for a large scale preparation of peptides, by the selection of activating esters of the carboxyl component, that describe the highest yields and purity during the carrying out of the reaction in laboratory conditions. The authors will aim to reduce the reaction time – to achieve the lowest reaction durability and energy consumption that have an important influence in the large tonnage manufacturing, necessary for the chemical and pharmaceutical industry. Hopefully, the idea and methodology for peptide synthesis by ball-milling, which avoids the using of solvents, will meet the support of many professionals, who will start to apply this procedure for the preparation of many products (not only peptides), and will use this methodology for

realizing of many reactions with a general importance in the chemical and pharmaceutical industry. In general, the reaction time and the yield of the prepared aspartame, using a variety of activating esters of di-protected aspartic acid, correlated well with the negative induction effect (electron withdrawing effect) of the ester groups, determining their function and properties (role) as good leaving groups (nucleophuges).

Moreover, for deprotection of these functionalities, together with the conventional procedures, a ball-milling procedure was used, in particular - for deprotection of Fmoc- (OFm) and Boc- (OBu^t) protecting groups. The yields of the prepared aspartame were within 70-85% depending on the protecting groups used.

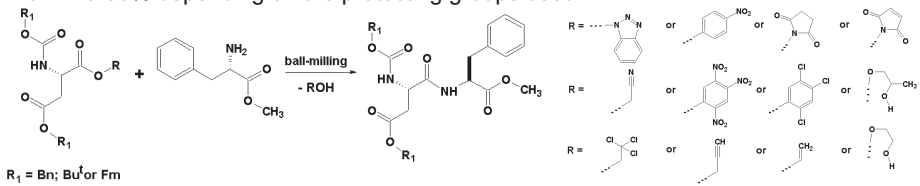


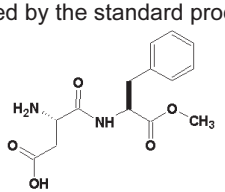
Fig.1. Scheme of the ball-milling peptide synthesis, demonstrating by aspartame preparation, using a variety of activating esters of di-protected aspartic acid and H-Phe-OMe

EXPERIMENTAL PART

A General procedure for ball-milling deprotection of aspartame functionalities:

The alpha-amino group and beta-carboxyl group of di-protected aspartic acid from aspartame were deprotected as follows:

The protected aspartame was mixed with $NaHCO_3/Na_2CO_3$ (in the case of Fmoc-, OFm-protecting groups) or $NaHSO_4$ (when Boc- and Bu^t-protections were used) and allowed to react in the ball-milling laboratory machine. Depending on the type of the protective group, the reaction time varied from 1 to 24h (the reaction was controlled by HPLC monitoring and TLC analyses of aliquots from the reaction mixture). At the end of the reaction time the deprotected aspartame was isolated by the standard procedure and crystallized**.**



Aspartame (H-Asp-Phe-OMe): ^{1}H NMR (600 MHz, DMSO-d_6, 25°C): δ = 2.571-2.753(dd, 2H, β-CH_2, Asp), 2.776-3.012(dd, 2H, β-CH_2, Phe), 3.591 (s, 3H, OCH_3), 4.783(t, 1H, α-CH, Phe), 4.867(q, 1H, α-CH, Asp), 7.321(m, 5H, CH, Ar, Phe), 7.913(s, broad, 3H, CONH, α-NH of Phe, α-NH_2 of Asp and β-COOH, Asp). ^{13}C NMR (150 MHz, DMSO-d_6, 25^{0}C): δ = 36.37(β-CH_2, Asp), 38.23(β-CH_2, Phe), 49.76(α-CH, Asp), 52.21(α-CH, Phe), 52.74(OCH_3, Phe), 126.65(4-CH, Ar, Phe), 128.47(3-CH and 5-CH, Ar, Phe), 129.53(2-CH and 6-CH, Ar, Phe), 137.26(1-C, Ar, Phe), 169.53(α-CONH, Asp), 172.15($COOCH_3$, Phe), 173.08(β-COOH, Asp).

Elemental analysis: Anal. Calculated for $C_{14}H_{18}N_2O_5$: (M_w = 294.3 g/mol); C-57.135%, H-6.165%, N-9.519%; found: C-57.128%, H-6.156%, N-9.557%.

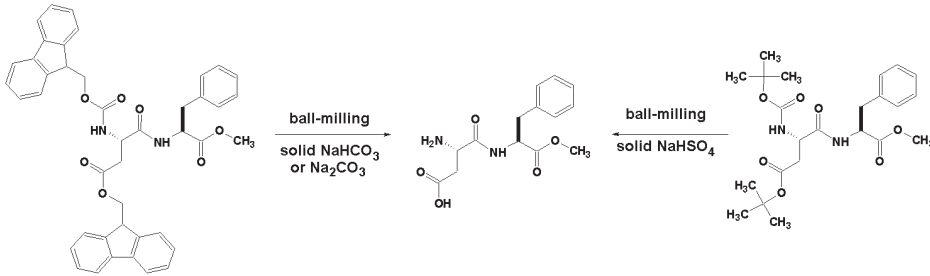
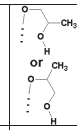


Fig.2. Scheme of the ball-milling deprotection of aspartame

Table 1. Yields, purity and reaction time for the preparation of Aspartame (H-Asp-Phe-OMe) by the ball-milling process, under conditions, using different activated esters of N^{α}, $COOH^{\beta}$-diprotected aspartic acid

Activating esters							
Yield (%)	**98**	**93**	**95**	**91**	**65**	**73**	**71**
Purity, HPLC (%)	**98**	**96,5**	**97**	**95,7**	**93**	**98,6**	**97,3**
Reaction Time (h)	**6**	**9**	**8**	**10**	**24**	**18**	**18**

Table 1. Yields, purity and reaction time for the preparation of Aspartame (H-Asp-Phe-OMe) by the ball-milling process, under conditions, using different activated esters of N^{α}, $COOH^{\beta}$-diprotected aspartic acid

Activating esters						
Yield (%)	**86**	**78**	**59**	**64**	**84**	**79**
Purity, HPLC (%)	**96,9**	**96**	**91**	**97**	**98,6**	**98,9**
Reaction Time (h)	**20**	**24**	**24**	**20**	**15**	**16**

CONCLUSIONS AND FUTURE WORK

The convenient ball-milling solvent-free synthesis of aspartame was achieved, describing the previously reported protocol. The comparison between the yields, purity and reaction time suggests, that the most successful synthesis was achieved using N-oxybenzotriazole, N-oxysuccinimide and p-nitrophenyl activating esters of the diprotected aspartic acid as an acyl component.

This methodology allows for the spreading of the protocol to large-scale synthesis of a variety of small peptides without solvent, and for industrial purposes.

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The paper is reviewed.