Think Green: Ball-Milling Peptide Synthesis

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Think Green: Ball-Milling Peptide Synthesis: *A novel technology for peptide (dipeptide) synthesis by the ball-milling process was realized and developed, which was demonstrated by the preparation of the commercial artificial dipeptide sweetener Aspartame (H-Asp-Phe-OMe). The reaction was carried out using a variety of activating esters of N^{α}, $COOH^{\beta}$-diprotected aspartic acid, that have influenced on the product yield and purity, and on the reaction time. This reaction strategy probably would find a wide application in the laboratory and industrial peptide synthesis, because it avoids the using of solvents and strives to keep and maintain our environment clean.*

Key words: *Ball mill, peptide synthesis, Aspartame, activating (activated) esters, Aspartic acid (Asp), Phenylalanine (Phe), methyl ester.*

INTRODUCTION

A ball milling process is a type of grinder widely used to grind materials into extremely fine powder for use in mineral dressing processes, paints, pyrotechnics, and ceramics. Devices for shaking materials along with hard balls might be old, but it was not until the industrial revolution and the invention of steam power that a machine could be built. It is reported to have been used for grinding flint for pottery in 1870 [1]. Aside from common ball mills there is a second type of ball mill called Planetary Ball Mill. Planetary ball mills are smaller than common ball mills and mainly used in laboratories for grinding sample material down to very small sizes. A planetary ball mill consists of at least one grinding jar which is arranged eccentrically on a so-called sun wheel. The direction of movement of the sun wheel is opposite to that of the grinding jars (ratio: 1:-2 or 1:-1 or else). The grinding balls in the grinding jars are subjected to superimposed rotational movements, the so-called Coriolis forces. The difference in speeds between the balls and grinding jars produces an interaction between frictional and impact forces, which releases high dynamic energies. The interplay between these forces produces the high and very effective degree of size reduction of the planetary ball mill. This process was used recently in the organic synthesis (e.g. for synthesis of catenanes and rothaxanes) [2], but it has insignificant application in the peptide synthesis. Whereas the microwave peptide synthesis has a general and wide application in the peptide synthesis, the ball-milling process is not so a broad utility in the organic synthesis.

Here we describe a pioneer method for ball-milling peptide synthesis, which can be applied to the large-scale production of short peptides (dipeptides, tripeptides and tetrapeptides).

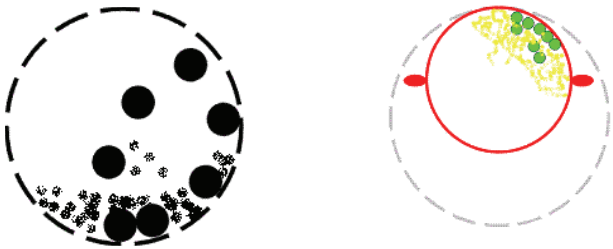
EXPOSITION

A ball mill, a type of grinder, is a cylindrical device used in grinding (or mixing) materials like ores, chemicals, ceramic raw materials and paints. Ball mills rotate around a horizontal axis, partially filled with the material to be ground plus the grinding medium. Different materials are used as media, including ceramic balls, flint pebbles and stainless steel balls. An internal cascading effect reduces the material to a fine powder. Industrial ball mills can operate continuously, fed at one end and discharged at the other end. Large to medium-sized ball mills are mechanically rotated on their axis, but small ones normally consist of a cylindrical capped container that sits on two drive shafts (pulleys and belts are used to transmit rotary motion). A rock tumbler functions on the same principle. Ball mills are also used in pyrotechnics and the manufacture of black powder, but cannot be used in the preparation of some pyrotechnic mixtures such as flash powder because of their sensitivity to impact. High-quality ball mills are potentially expensive and can grind mixture particles to as small as 5 nm, enormously increasing surface area and reaction rates. The grinding works on the principle of critical speed. The critical speed can be understood as

that speed after which the steel balls (which are responsible for the grinding of particles) start rotating along the direction of the cylindrical device; thus causing no further grinding.

Some principles of ball-milling process are important, and some features of the grinding process have to be taken under consideration:

- Where the color of the finished product is important, the color and material of the grinding media must be considered.
- Where low contamination is important, the grinding media may be selected for ease of separation from the finished product (i.e.: steel dust produced from stainless steel media can be magnetically separated from non-ferrous products). An alternative to separation is to use media of the same material as the product being ground.
- Flammable products have a tendency to become explosive in powder form. Steel media may spark, becoming an ignition source for these products. Either wet-grinding, or non-sparking media such as ceramic or lead must be selected.
- Some media, such as iron, may react with corrosive materials. For this reason, stainless steel, ceramic, and flint grinding media may each be used when corrosive substances are present during grinding.



Ball mill High-energy ball-milling

Fig.1 Scheme, which demonstrates the basic principle of the ball-milling process, and the principle of the high-energy ball-milling.

Fig.2 Laboratory scale ball milling equipment

Ball mills are used extensively in the Mechanical alloying process [3] in which they are not only used for grinding but for cold welding as well, with the purpose of producing alloys from powders [4]. The ball mill is a key piece of equipment for grinding crushed materials, and it is widely used in production lines for powders such as including cement, silicates, refractory material, fertilizer, glass ceramics, etc. as well as for ore dressing of both ferrous non-ferrous metals. The ball mill can grind various ores and other materials either wet or dry. There are two kinds of ball mill, grate type and overfall type due to different ways of discharging material. There are many types of grinding media suitable for use in a ball mill, each material having its own specific properties and advantages. Key properties of grinding media are size, density, hardness, and composition.

- Size: The smaller the media particles, the smaller the particle size of the final product. At the same time, the grinding media particles should be substantially larger than the largest pieces of material to be ground.

- Density: The media should be denser than the material being ground. It becomes a problem if the grinding media floats on top of the material to be ground.
- Hardness: The grinding media needs to be durable enough to grind the material, but where possible should not be so tough that it also wears down the tumbler at a fast pace.
- Composition: Various grinding applications have special requirements. Some of these requirements are based on the fact that some of the grinding media will be in the finished product. Others are based in how the media will react with the material being ground.

EXPERIMENTAL

Material and Methods

All of the reagents for the activation of the alpha-carboxyl group in Asp, as well as for the protection of the alpha-amino group and beta-carboxyl group in Asp (Boc, Bu^t, Fmoc, Fm, Z, Bn), thionyl chloride and sulfuric acid, methanol were purchased from Merck. All reagents and solvents were purchased and used without further purification. TLC analyses were performed on silica plates UV_{260}, purchased from Merck, where for the spots labeling and virtual detection on TLC plates, a 5% solution of H_2SO_4 in methanol or ethanol was employed, and also – an alcohol solution of ninhydrin was used, as well as a solution of phosphorus-molybdenum acid. For TLC analyses - CH_2Cl_2 : MeOH (9.5:0.5) was employed as a solvent system. The reverse phase HPLC analyses were performed on a Waters Liquid Chromatograph equipped with an absorbance detector model 441 set at 280 nm and a column Nucleosil 100-5C_{18} (12.5 cm x 4.6 mm) for analytical runs. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II+ 600MHz spectrometer in $CDCl_3$, using BBO or TBI probeheads. Chemical shifts are expressed in ppm and coupling constants in Hz. The precise assignments of the ^1H and ^{13}C NMR spectra were accomplished by measurement of 2D homonuclear correlation (COSY), DEPT-135 and 2D inverse detected heteronuclear (C–H) correlations (HSQC and HMBC). Chemical shifts are reported in δ (ppm). The analysis of first order multiplets in ^1H NMR spectra was speed up by the use of FAFOMA program [5]. For NMR data, Bruker Avance II+ NMR spectrometer operating at 600 MHz for ^1H and at 150 MHz for ^{13}C NMR was used. The elemental analysis was carried out and organic compounds were determined using the automatic analyzers: Carlo Erba Elemental Analyzer Model 1106 with automatic sampler for 53 samples (Carlo Erba, Milan, Italy) and Perkin-Elmer Elemental Analyzer Model 240 (Perkin-Elmer Corp., Norwalk, Connecticut).

Experimental part

A General procedure for Ball-milling peptide synthesis, presented by the Aspartame synthesis, using activated esters of the protected aspartic acid (Asp):

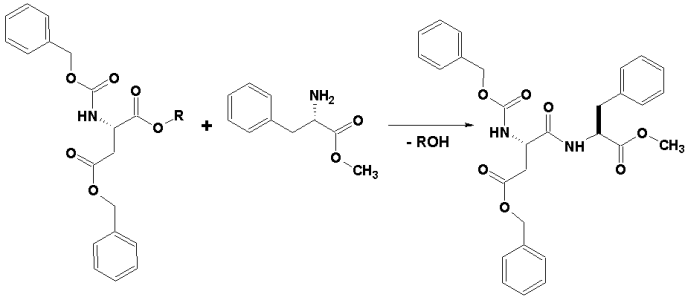
The alpha-amino group and beta-carboxyl group di-protected aspartic acid activated esters (1equiv.) were mixed with the phenylalanine methyl ester (H-Phe-OMe) (1,05 equiv.) and allowed to react in the ball-milling laboratory machine. Depending on the type of the activated aspartic acid ester, the reaction 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 the protected Aspartame was deprotected, using the standard protocols for deprotection of the conventional protecting group**°.**

° *Also for deprotection of these functionalities was used a ball-milling procedure, but the results will be published elsewhere.*

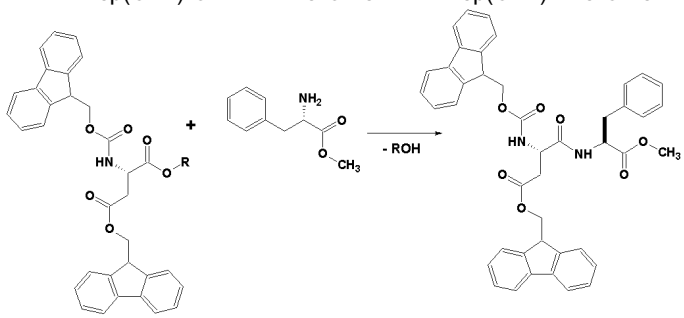
RESULTS AND DISCUSSION

As was mentioned above, the yields of the product were different, depending on the type of the activating ester. In the case of using of N-hydroxybenzotriazole activated ester, the yield was the highest (98%, for 6 hours), after that the N-hydroxysuccinimide activated ester (95% yield, 8 hours), the p-nitrophenyl activated ester (93% yield, 9 hours), and the N-hydroxymaleimide activated ester (91% yield, 10 hours) are the following compounds, prepared with high yields. Other esters had lower yields, due to the lower reactivity of the

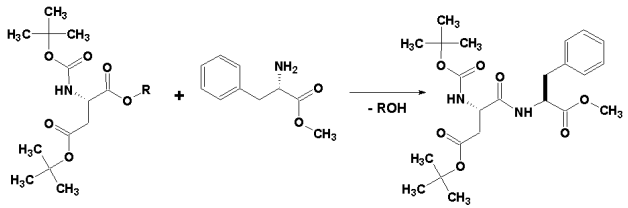
activating esters. 2,4,5-trinitrophenyl and 2,4,5-trichlorophenyl esters exhibited this low reactivity, probably because of the sterically hindering of these groups and inaccessibility of the attacking nucleophile (the alpha-amino group of H-Phe-OMe) to the reactive centre of the carboxyl group (the carbon atom).



Z-Asp(OBn)-OR + H-Phe-OMe → Z-Asp(OBn)-Phe-OMe



Fmoc-Asp(OFm)-OR + H-Phe-OMe → Fmoc-Asp(OFm)-Phe-OMe



Boc-Asp(OBu^t)-OR + H-Phe-OMe → Boc-Asp(OBu^t)-Phe-OMe

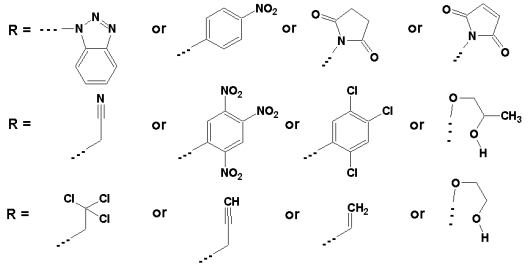
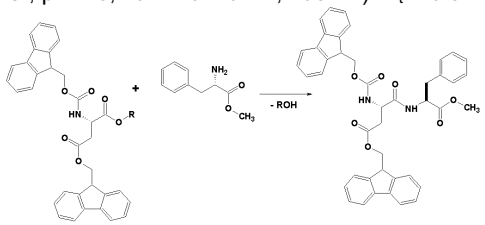


Fig.3. Scheme of the ball-milling Aspartame (peptide) synthesis, using a variety of activating esters of protected aspartic acid and H-Phe-OMe.

Z-Asp(OBn)-Phe-OMe: ^{1}H NMR (600 MHz, $CDCl_3$, 25^{o}C): δ = 2.771-2.832(dd, 2H, β-CH_2, Asp), 3.013-3.132(dd, 2H, β-CH_2, Phe), 3.629 (s, 3H, OCH_3), 4.371(q, 1H, α-CH, Asp), 4.873(t, 1H, α-CH, Phe), 5.091(s, 2H, $PhCH_2$, Z, Asp), 5.121(s, 2H, $PhCH_2$, OBn, Asp), 7.312(m, 5H, CH, Ar, Phe), 7.321-7.409(m, 10H, Ar: Z and Bn), 7.413(s, broad, 1H, BnOCONH, α-NH of Asp), 7.503(s, broad, 1H, CONH, α-NH of Phe). ^{13}C NMR (150 MHz, $CDCl_3$, 25^{0}C): δ = 35.67(β-CH_2, Asp), 38.02(β-CH_2, Phe), 49.53(α-CH, Phe), 51.69(α-CH, Asp), 52.95(OCH_3, Phe), 66.84($PhCH_2$, Z, Asp), 67.03($PhCH_2$, OBn, Asp), 126.03(CH-Ar, Z: 2-CH, 3-CH, 4-CH, 5-CH, 6-CH; Asp and 4-CH, Ar, Phe), 128.23(3-CH and 5-CH, Ar, Phe), 128.56(2-CH and 6-CH, Ar, OBn, Asp), 128.58(4-CH, Ar, OBn, Asp), 128.74(3-CH and 5-CH, Ar, OBn, Asp), 129.75(2-CH and 6-CH, Ar, Phe), 136.57(1-C, Ar, Z, Asp), 137.29(1-C, Ar, Phe), 137.75(1-C, Ar, OBn, Asp), 157.07(OCONH, Z, Asp), 168.32(α-CONH, Asp), 171.08(β-COO, Asp), 171.24($COOCH_3$, Phe).

Elemental analysis: Anal. Calculated for $C_{29}H_{30}N_2O_7$: (M_w = 518.561 g/mol); C-67.169%, H-5.831%, N-5.402%; found: C-67.071%, H-5.956%, N-5.621%.

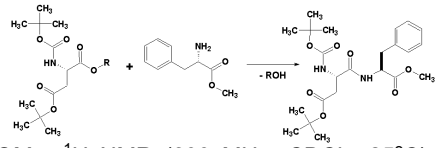
Rf-0.786 (CH_2Cl_2 : MeOH (9.5:0.5)). Analytical RP-HPLC (70% CH_3CN in 0.02M KH_2PO_4/K_2HPO_4 buffer, pH 7.0; flow 1.0 ml/min, 298.2K): R_t = 6.3 min.



Fmoc-Asp(OFm)-Phe-OMe: ^{1}H NMR (600 MHz, $CDCl_3$, 25^{o}C): δ=2.781-2.921(dd, 2H, β-CH_2, Asp), 3.001-3.131(dd, 2H, β-CH_2, Phe), 3.605 (s, 3H, OCH_3), 4.214(d, 2H, OCH_2, Fmoc, Asp), 4.263(t, 1H, 9-CH, OFm, Asp), 4.303(t, 1H, 9-CH, Fmoc, Asp), 4.353(t, 1H, α-CH, Asp), 4.412(d, 2H, OCH_2, OFm, Asp), 4.864(t, 1H, α-CH, Phe), 7.073(t, 2H, 2-CH and 7-CH, Fmoc, Asp), 7.193(t, 2H, 3-CH and 6-CH, OFm, Asp), 7.232(t, 2H, 3-CH and 6-CH, Fmoc, Asp), 7.297-7.326(m, 5H, CH, Ar, Phe), 7.392(d, 2H, 1-CH and 8-CH, OFm, Asp), 7.491(s, broad, 1H, FmOCONH, α-NH of Asp), 7.542(s, broad, 1H, CONH, α-NH of Phe), 7.573(d, 2H, 1-CH and 8-CH, Fmoc, Asp), 7.744(d, 2H, 4-CH and 5-CH, Fmoc, Asp), 7.877(d, 2H, 4-CH and 5-CH, OFm, Asp), . ^{13}C NMR (150 MHz, $CDCl_3$, 25^{0}C): δ = 35.42(β-CH_2, Asp), 37.97(β-CH_2, Phe), 47.07(9-CH, Fmoc, Asp), 47.58(9-CH, OFm, Asp), 49.56(α-CH, Phe), 51.67(α-CH, Asp), 52.92(OCH_3, Phe), 66.24(OCH_2, OFm, Asp), 67.01(OCH_2, Fmoc, Asp), 119.95(4-CH and 5-CH, Fmoc, Asp), 120.21(4-CH and 5-CH, OFm, Asp), 124.93(1-CH and 8-CH, OFm, Asp), 125.24(1-CH and 8-CH, Fmoc, Asp), 126.03(4-CH, Ar, Phe), 127.23(2-CH and 7-CH, Fmoc, Asp), 127.57(2-CH and 7-CH, OFm, Asp), 127.74(3-CH and 6-CH, Fmoc, and 3-CH and 6-CH, OFm, Asp), 128.19(3-CH and 5-CH, Ar, Phe), 129.71(2-CH and 6-CH, Ar, Phe), 137.36(1-C, Ar, Phe), 141.35(4a-C and 4b-C, Fmoc, Asp), 141.72(4a-C and 4b-C, OFm, Asp), 143.32(8a-C and 9a-C, OFm, Asp), 143.85(8a-C and 9a-C, Fmoc, Asp), 156.37(OCONH, Fmoc, Asp), 168.23(α-CONH, Asp), 170.83(β-COO, Asp), 171.21($COOCH_3$, Phe).

Elemental analysis: Anal. Calculated for $C_{43}H_{38}N_2O_7$: (M_w = 694.775 g/mol); C-74.335%, H-5.513%, N-4.032%; found: C-74.283%, H-5.576%, N-4.061%.

Rf-0.813 (CH_2Cl_2 : MeOH (9.5:0.5)). Analytical RP-HPLC (70% CH_3CN in 0.02M KH_2PO_4/K_2HPO_4 buffer, pH 7.0; flow 1.0 ml/min, 298.2K): R_t = 7.7 min.



Boc-Asp(OBu^t)-Phe-OMe: ^{1}H NMR (600 MHz, $CDCl_3$, 25^{o}C): δ=1.398(s, 9H, CH_3, Boc, Asp), 1.463 (s, 9H, CH_3, OBu^t, Asp), 2.781-2.921(dd, 2H, β-CH_2, Asp), 3.021-3.145(dd, 2H, β-CH_2, Phe), 3.635 (s, 3H, OCH_3), 4.357(t, 1H, α-CH, Asp), 4.837(t, 1H, α-CH, Phe), 7.271-7.323(m, 5H, CH, Ar, Phe), 7.363(s, broad, 1H, Bu^{t}OCONH, α-NH of Asp), 7.385 (s, broad, 1H, CONH, α-NH of Phe). ^{13}C NMR (150 MHz, $CDCl_3$, 25^{0}C**):** δ = 27.78(CH_3, Boc, Asp), 28.04(CH_3, OBu^t, Asp), 36.29 (β-CH_2, Asp), 37.72(β-CH_2, Phe), 50.37(α-CH, Phe), 51.64(α-CH, Asp), 52.93(OCH_3, Phe), 79.93(tert-C, Boc, Asp), 81.07(tert-C, OBu^t, Asp), 126.82(4-CH, Ar, Phe), 128.54(3-CH and 5-CH, Ar, Phe), 130.06(2-CH and 6-CH, Ar, Phe), 136.59(1-C, Ar, Phe), 155.93(OCONH, Boc, Asp), 168.27(α-CONH, Asp), 170.72(β-COO, Asp), 172.29($COOCH_3$, Phe).

Elemental analysis: Anal. Calculated for $C_{23}H_{34}N_2O_7$: (M_w = 450.528 g/mol); C-61.317%, H-7.607%, N-6.218%; found: C-61.197%, H-7.576%, N-6.261%.

Rf-0.867 (CH_2Cl_2 : MeOH (9.5:0.5)). Analytical RP-HPLC (70% CH_3CN in 0.02M KH_2PO_4/K_2HPO_4 buffer, pH 7.0; flow 1.0 ml/min, 298.2K): R_t = 6.9 min.

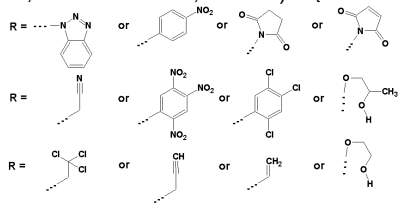


Fig.3. Scheme of the ball-milling Aspartame (peptide) synthesis, using a variety of activating esters of protected aspartic acid and H-Phe-OMe.

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 with 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 [6]. 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.

CONCLUSION

The novel methodology for green peptide synthesis by ball-milling, without solvents utilization was applied and developed. The applicability of this procedure was demonstrated by the successful synthesis of the widespread artificial sweetener Aspartame (H-Phe-Asp-OMe), using the conventional functional group protection in the amino acids, and applying the method of the activated esters. For example, depending on the type of activating ester, the prepared product had a different purity and yield, and the reaction time, which was necessary, varied in some extent. Nevertheless, this procedure allows for the development of the methodology for the large-scale peptide preparation, necessary for the medical industry in the future.

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