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New synthetic strategy for *o*-NBS protected amino acids and their use in synthesis of mono-benzylated peptides

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Abstract—A synthetic strategy to prepare *o*-NBS protected Fmoc-amino acids under mild conditions, in a rapid and efficient way, characterised by high yields and excellent purity of the final products has been developed. The *o*-NBS protected Fmoc-amino acids are employed in solid phase peptide synthesis to prepare peptidomimetics carrying mono-benzylated moieties on peptide side chains.

The choice of amine protecting groups is critical for the chemical synthesis of amino acid derivatives and of bioorganic compounds in general. Among the different possibilities,[1] the 2-nitrobenzenesulfonamide (*o*-NBS) group as received increasing attention.[2–6] As a protecting group for primary and secondary amines, it is stable under both acidic and basic conditions. Moreover, its attachment to primary amines greatly increases the acidity of the amine hydrogen enough to allow selective amine mono-alkylations via Mitsunobu conditions in addition to more traditional approaches (K_2CO_3, halide).[2]

As the 2-nitrobenzenesulfonamide group is stable under standard amino acid/peptide chemistry protocols, both in solution and on solid phase,[7] it can also be a suitable protecting group for amino functions of amino acid derivatives used in peptide or peptidomimetic synthesis. When bound to the side-chain amino group, 2-nitrobenzenesulfonamide group remains stable during peptide assembly and can be removed at the end of the peptide synthesis. Moreover, it allows the mono-alkylation during the peptide chain elongation in solid phase. For example, by using this strategy, it can be possible to obtain peptidomimetics, which contain amino benzyl derivatives of several amino acid residues, such as lysine, ornithine (Orn), di-aminobutyric acid (Dab) or di-aminopropionic acid (Dap), in which the benzyl moiety is at different distances from the peptide backbone. In fact, rational-designed peptidomimetics often contain no natural amino acids able to fit the hydrophobic binding pocket of macromolecular receptors. By changing the length of amino acidic side chains, it is possible to accommodate the ligand in the binding site of the receptor and meet the topochemical array of the pharmacophore model.

The aim of this work has been to develop a synthetic strategy to synthesise Fmoc-protected amino acids (Fmoc = 9-fluorenylmethoxycarbonyl) suitable to prepare mono-benzylated peptides. Fmoc-amino acid derivatives containing 2-nitrobenzenesulfonamide on the side-chain amino function result in very attractive building blocks to use in solid phase peptide and peptidomimetic synthesis.

Although synthetic methods for amine protection by using 2-nitrobenzensulfonyl chloride are well established,[5] the introduction of *o*-NBS onto amino acid amine function in aqueous solution of NaOH 1 N[8,9] is incompatible with the presence of a basic labile protecting group such as Fmoc.

Herein, we describe a novel and efficient procedure to obtain Fmoc-amino acid derivatives carrying *o*-NBS group on the side-chain amino group. Moreover, the use of these residues in solid phase peptide synthesis to prepare amino mono-benzylated peptides is also reported.

Keywords: *o*-NBS protected amino acids; Mono-benzylated peptides; Building blocks for peptide synthesis.
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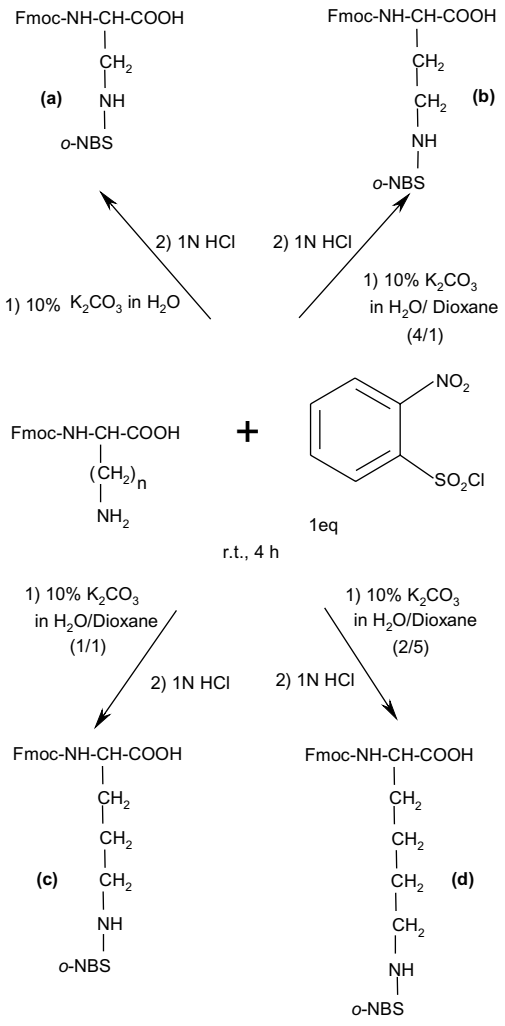
Standard procedures for the introduction of *o*-NBS group onto primary or secondary amines in good yield consist of the reaction of *o*-NBS chloride in organic solvent, such as dichloromethane and dimethylformamide, in the presence of an organic base (triethylamine, *di*-isopropylethylamine or pyridine).[5] These methods are hardly suitable in the case of Fmoc-amino acid derivatives containing an amine function on the side chain and a free carboxylic function such as Fmoc-Lys-OH, Fmoc-Orn-OH, Fmoc-Dab-OH and Fmoc-Dap-OH. It was previously reported that the introduction of *o*-NBS as an amino protecting group onto amino acids in organic solvent requires the amino acids to be used as methyl ester derivatives.[7] In fact, the amphipatic property of the *o*-NBS protected derivatives, due to the presence of aromatic groups (Fmoc and *o*-NBS) and of free carboxylic function, makes the separation of the desired product from the organic ammonium salt and the final chromatographic purification difficult, thus giving a very low yield of the pure product.

We propose a synthetic strategy to prepare Fmoc-Lys(*o*-NBS)-OH, Fmoc-Orn(*o*-NBS)-OH, Fmoc-Dab(*o*-NBS)-OH and Fmoc-Dap(*o*-NBS)-OH, which avoids the synthetic problems described above in the standard procedures.

Our method of synthesis uses potassium carbonate aqueous solution or a mixture of this solution and 1,4-dioxane as a system solvent to protect the side-chain amine with *o*-NBS group. Mild conditions and inexpensive reagents are used to obtain the desired compounds in excellent yield and purity.

In Scheme 1, the synthetic routes employed to obtain each *o*-NBS protected Fmoc-amino acid derivatives are summarised in detail. After dissolving the Fmoc-amino acid derivative in 10% aqueous solution of K_2CO_3 and, for some of them, a small amount of 1,4-dioxane, the mixture is stirred in an ice-water bath and 1 equiv of *o*-NBS chloride is added in small portion. The resulting solution is stirred at room temperature for about 4 h. Any unreacted *o*-NBS chloride is easily removed by extraction with diethyl ether and the final product is recovered as a pale-yellow precipitate by acidifying the aqueous layer with 1 N HCl. Any unreacted Fmoc-amino acid remains soluble in the acidic aqueous solution, while the carbonate is completely neutralised. By using this strategy, the *o*-NBS protected Fmoc-amino acid derivatives are collected in high yield and in excellent purity,[10] as assessed by analytical RP-HPLC, LC-ES-MS and ^{1}H NMR spectroscopy, and any chromatographic purification is not needed.[11] In Scheme 1, yield and purity data for the four amino acid derivatives prepared are reported, while their full characterisation is summarised in the references and notes.[12]

The proposed synthetic pathway presents additional advantages when the amino acid derivatives obtained are intended to be used as building blocks for Fmoc solid phase peptide synthesis. The introduction of *o*-NBS onto amine groups during the solid phase peptide synthesis is a well-established reaction.[7] Nevertheless, the procedure to introduce *o*-NBS onto side-chain amine groups of a peptide chain bound to a solid support is supposed to employ expensive Fmoc-amino acid derivatives. In fact, such derivatives contain an orthogonal amine protecting group, which is selectively removed during the peptide assembly, in order to allow the introduction of *o*-NBS group.

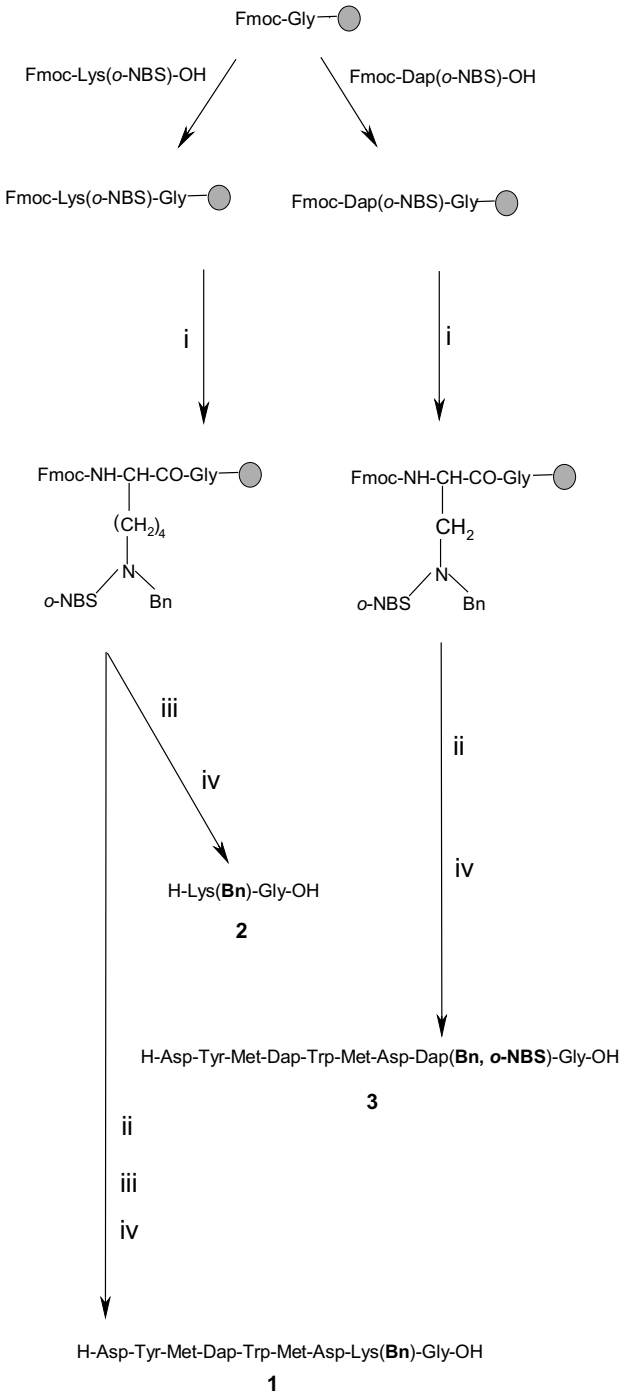


(**a**) Fmoc-Dap(*o*-NBS)-OH, yield 84 %, purity 98 %;
(**b**) Fmoc-Dab(*o*-NBS)-OH, yield 65 %, purity 98 %;
(**c**) Fmoc-Orn(*o*-NBS)-OH, yield 64 %, purity 98 %;
(**d**) Fmoc-Lys(*o*-NBS)-OH, yield 80 %, purity 98 %;

Scheme 1. Synthetic routes to obtain *o*-NBS protected Fmoc-amino acid derivatives.

It is clear that the use of *o*-NBS protected Fmoc-amino acid derivatives as building blocks in peptide synthesis simplifies the procedure to synthesise mono-alkylated peptide derivatives under conventional conditions (K_2CO_3, halide). Such a modification of peptides is particularly interesting, since it could allow the introduction of conformational constraints desired in rational design approach. On this regard, solid phase preparation of mono-benzylated peptides results in a valuable applicability of the N-alkylation proposed procedure.

We synthesised two peptides in which the newly developed *o*-NBS protected Fmoc-amino acids were incorporated into the peptide chain by using Fmoc solid phase standard methods, as shown in Scheme 2.[13] The first example is given by the synthesis of the nonapeptide (**1**) containing in position 8 a lysine residue functionalised on the ε-NH_2 with the benzyl group. Having the Fmoc-Lys(*o*-NBS)-OH bound to the growing peptide chain, the benzylation reaction was performed under conventional conditions as the next step. After the complete assembly of the peptide onto the solid support the *o*-NBS protecting group was removed by using 2-mercapto-ethanol in combination with DBU. In order to verify the absence of any side reactions on the *o*-NBS protected amines during peptide elongation and the efficiency of the benzylation reaction performed at the end of the peptide synthesis, we repeated the synthesis of (1) by inverting the synthetic steps i and ii. The final compound was isolated and analysed by RP-HPLC and LC-ES-MS.[14] Both strategies provided peptide (1) in similar yields. The dipeptide H-Lys(Bn)-Gly-OH (**2**) was isolated during the solid phase peptide synthesis and completely characterised by RP-HPLC, LC-ES-MS and ^{1}H NMR.[15] The mono-benzylated dipeptide was obtained in almost quantitative yield, which proved the high efficiency of the synthetic procedure employed.



Scheme 2. Peptide synthesis according to the Fmoc solid phase protocol. Reagents and conditions: Wang resin, loading of 0.99 mmol g^{-1}; (i) K_2CO_3 (2 equiv), benzyl bromide (2 equiv), in DMF, 1 h; (ii) protected amino acids, PyBop/HOBt/DIPEA, in DMF, 1 h; (iii) DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) (10 equiv), 2-mercapto-ethanol (5 equiv), in DMF, 30 min; (iv) TFA/H_2O/TIS (TIS = tris-*iso*-propylsilane) (97:2:1), 1.5 h. Synthesis of (1) can also be obtained by inverting steps i and ii.

The second example is another nonapeptide (**3**) containing a benzyl group on the β-NH_2 of di-aminopropionic acid introduced in position 8 of the sequence. In this case, we did not remove the *o*-NBS group onto the peptide, in order to check its stability under mildly basic coupling conditions (0.12 M *di*-isopropylethylamine), Fmoc removal conditions (20% piperidine), and also the acidic treatment necessary for cleavage and deprotection of the peptide (95% TFA). By ESI mass analysis, it was assessed that the final peptide was obtained in high yield and retained the complete amount of the *o*-NBS moiety onto the β-NH_2 of Dap residue.[16]

In conclusion, we describe a synthetic strategy to prepare *o*-NBS protected Fmoc-amino acids under mild conditions, in a rapid and efficient way, characterised by high yields and excellent purity of the final products. The *o*-NBS protected Fmoc-amino acids are very useful building blocks to be employed in solid phase peptide synthesis, since the *o*-NBS group is compatible with many protecting groups widely used in peptide/organic synthesis. Their use allows the synthesis of peptidomimetics modified with mono-benzylated functions on peptide side chains, without any modification of the synthesis protocols.

Acknowledgements

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10. General procedure for the synthesis of (**a**), (**b**), (**c**) and (**d**). Approximately 1 mmol of each Fmoc-amino acid was suspended in a mixture of 10% aqueous solution of K_2CO_3 and 1,4-dioxane. To dissolve Fmoc-Dab-OH, Fmoc-Orn-OH and Fmoc-Lys-OH the following volume (mL) of 10% bicarbonate in water/1,4-dioxane were used, respectively: 4:1, 4:4 and 2:5; 4.5 mL of 10% bicarbonate in water was used only to dissolve Fmoc-Dap-OH. The solution was stirred and cooled in an ice-water bath while 0.9 equiv (~200 mg) of *o*-nitrobenzensulfonyl chloride, dissolved in a small portion of dioxane (150 μL), was added over a period of 5 min. The stirring was continued at room temperature for about 4 h. The resulting reaction mixture was than poured into water (80 mL) and extracted with diethyl ether (40 mL in two portions). The organic layer was washed twice with basic water to recover some of the final product, which had passed into the ether. TLC analysis (eluent: dichloromethane/methanol, 9:1) of the aqueous layer showed a quite clean formation of the final protected product (d) ($R_f = 0.2$), with only a small spot of the Fmoc-amino acid on the TLC start line. The aqueous solution is cooled in an ice-water bath and acidified under vigorous stirring with 1 N HCl in water. This allows to precipitate the desired compound (d) as a pale-yellow solid. After 1 h, the mixture is filtered and the dry product was isolated with high purity, as assessed by HPLC and ^{1}H NMR analysis.
11. Analytical RP-HPLC runs were carried out on a HP Agilent Series 1100 apparatus using a Phenomenex (Torrance, CA) C18 column, 4.6 × 250 mm with a flow rate of 1.0 mL min^{-1}. Preparative RP-HPLC was carried out on a Shimadzu 8A apparatus equipped with an UV Shimadzu detector using a Phenomenex (Torrance, CA) C18 column, 22 × 250 mm with a flow rate of 20 mL min^{-1}. The system solvent used was H_2O 0.1% TFA (A) and CH_3CN 0.1% TFA (B), with a linear gradient from 5% to 70% B in 30 min (gradient 1) or from 30% to 95% B in 20 min (gradient 2). LC-ES-MS data were obtained using a Finnigan Surveyor MSQ single quadrupole electrospray ionisation mass spectrometer coupled with a Finnigan Surveyor HPLC (Finnigan/Thermo Electron Corporation San Jose, CA, USA). HR-ES-MS were run on a Micromass QTOF mass spectrometer. ^{1}H NMR spectra were recorded on a Varian INOVA-Unity 500 MHz spectrometer. Specific rotation (in deg mL/g dm) is measured by using a Perkin–Elmer model 141 polarimeter.
12. N_α-(9-Fluorenylmethoxycarbonyl)-N_β-2-nitrobenzen-sulfonyl-L-2,3-di-aminopropionic acid (**a**). HPLC: t_R = 15.49 min (gradient 2); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.20 (br, 1H, *o*-NBS-N*H*), 7.98 and 7.85 (2m, 4H, *o*-NBS-$H_{3,4,5,6}$), 7.89 and 7.71 (2d, J = 7.3 Hz, 4H, Fmoc-$H_{1,4,5,8}$), 7.53 (d, J = 7.3 Hz, 1H, N*H*), 7.42 and 7.33 (2t, J = 7.3 Hz, 4H, Fmoc-$H_{2,3,6,7}$), 4.26 (d, J = 6.5 Hz, 2H, Fmoc-CH_2), 4.22 (t, J = 6.7 Hz, 1H, Fmoc-C*H*), 3.98 (m, 1H, Dap-CH^α), 3.3 (overlaps HDO signal, 2H, Dap-CH_2^β); ES-MS: calcd (M+H$^+$), 512.1; found, *m/z* 512.3; HS-MS: calcd (M+H$^+$ = $C_{24}H_{22}O_8N_3S^+$), 512.1128; found, *m/z* 512.1135; $[\alpha]_D^{20}$ −15.5 (*c* 1.05, CH_3OH).
N_α-(9-Fluorenylmethoxycarbonyl)-N_γ-2-nitrobenzen-sulfonyl-L-2,4-di-aminobutyric acid (**b**). HPLC: t_R = 15.57 min (gradient 2); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.23 (br, 1H, *o*-NBS-N*H*), 7.96 and 7.83 (2m, 4H, *o*-NBS-$H_{3,4,5,6}$), 7.88 and 7.69 (2d, J = 7.3 Hz, 4H, Fmoc-$H_{1,4,5,8}$), 7.60 (d, J = 7.2 Hz, 1H, N*H*), 7.40 and 7.30 (2t, J = 7.3 Hz, 4H, Fmoc-$H_{2,3,6,7}$), 4.25 (d, J = 6.5 Hz, 2H, Fmoc-CH_2), 4.21 (t, J = 6.7 Hz, 1H, Fmoc-C*H*), 3.98 (br, 1H, Dab-CH^α), 2.97 (m, 2H, Dab-CH_2^γ), 1.91 and 1.77 (2m, 2H, Dab-CH_2^β); ES-MS: calcd (M+H$^+$), 526.1; found, *m/z* 526.3; HS-MS: calcd (M+H$^+$ = $C_{25}H_{24}O_8N_3S^+$), 526.1284; found, *m/z* 526.1291; $[\alpha]_D^{20}$ −7.2 (*c* 1.04, CH_3OH).
N_α-(9-Fluorenylmethoxycarbonyl)-N_δ-2-nitrobenzen-sulfonyl-L-ornithine (**c**). HPLC: t_R = 15.83 min (gradient 2); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.12 (br, 1H, *o*-NBS-N*H*), 7.96 and 7.83 (2m, 4H, *o*-NBS-$H_{3,4,5,6}$), 7.89 and 7.70 (2d, J = 7.3 Hz, 4H, Fmoc-$H_{1,4,5,8}$), 7.51 (d, J = 7.5 Hz, 1H, N*H*), 7.40 and 7.31 (2t, J = 7.3 Hz, 4H, Fmoc-$H_{2,3,6,7}$), 4.26 (d, J = 6.8 Hz, 2H, Fmoc-CH_2), 4.20 (t, J = 6.8 Hz, 1H, Fmoc-C*H*), 3.84 (m, 1H, Orn-CH^α), 2.88 (t, 2H, Orn-CH_2^γ), 1.71 and 1.56 (2m, 2H, Orn-CH_2^β),1.48 (m, 2H, Orn-CH_2^δ); ES-MS: calcd (M+H$^+$), 540.1; found, *m/z* 540.2; HS-MS: calcd (M+H$^+$ = $C_{26}H_{26}O_8N_3S^+$), 540.1441; found, *m/z* 540.1449; $[\alpha]_D^{20}$ −3.4 (*c* 1.03, CH_3OH).
N_α-(9-Fluorenylmethoxycarbonyl)-N_ε-2-nitrobenzen-sulfonyl-L-lysine (**d**). HPLC: t_R = 16.26 min (gradient 2); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.12 (br, 1H, *o*-NBS-N*H*), 7.97 and 7.86 (2m, 4H, *o*-NBS-$H_{3,4,5,6}$), 7.89 and 7.72 (2d, J = 7.3 Hz, 4H, Fmoc-$H_{1,4,5,8}$), 7.60 (d, J = 7.8 Hz, 1H, N*H*), 7.41 and 7.32 (2t, J = 7.3 Hz, 4H, Fmoc-$H_{2,3,6,7}$), 4.28 (d, J = 6.8 Hz, 2H, Fmoc-CH_2), 4.22 (t, J = 6.8 Hz, 1H, Fmoc-C*H*), 3.87 (m, 1H, Lys-CH^α), 2.88 (m, 2H, Lys-CH_2^ε), 1.64 and 1.56 (2m, 2H, Lys-CH_2^β), 1.42 (m, 2H, Lys-CH_2^δ), 1.31 (m, 2H, Lys-CH_2^γ); ES-MS: calcd (M+H$^+$), 554.1; found, *m/z* 554.0; HS-MS: calcd (M+H$^+$ = $C_{27}H_{28}O_8N_3S^+$), 554.1597; found, *m/z* 554.1589; $[\alpha]_D^{20}$ −2.7 (*c* 1.02, CH_3OH).
13. The peptides synthesis was carried out by solid phase method using the standard Fmoc procedure. The first amino acid was loaded on Wang resin according to literature protocol (Sheppard, R. C.; Williams, B. J. *Int. J. Peptide Protein Res.* **1982**, *20*, 451–454) and the loading was evaluated by Fmoc test. Amino acid couplings were monitored by Kaiser test (Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598).
14. H-Asp-Tyr-Met-Dap-Trp-Met-Asp-Lys(Bn)-Gly-OH (**1**). Yield: 32–36%; HPLC: t_R = 27.35 min (gradient 1); ES-MS: calcd (M+H$^+$=$C_{56}H_{76}O_{15}N_{12}S_2^+$), 1220.5; found, *m/z* 1220.5.
15. H-Lys(Bn)-Gly-OH (**2**): Yield: 78%; HPLC: t_R = 13.52 min (gradient 1); ^{1}H NMR (500 MHz, D_2O): δ 7.50–7.41 (m, 5H, Lys-Ar*H*), 4.22 (m, 2H, Gly-CH_2^α), 3.95–3.88 (2m, 3H, Lys-CH_2-Ar and Lys-CH^α), 2.95 (t, J = 7.6 Hz, 2H, Lys-CH_2^ε), 1.91 (m, 2H, Lys-CH_2^β), 1.66 (m, 2H, Lys-CH_2^δ), 1.44 (m, 2H, Lys-CH_2^γ); ES-MS: calcd (M+H$^+$=$C_{15}H_{23}O_3N_3^+$), 294.2; found, *m/z* 294.6.
16. H-Asp-Tyr-Met-Dap-Trp-Met-Asp-Dap(Bn, *o*-NBS)-Gly-OH (**3**). Yield: 35%; HPLC: t_R = 31.31 min (gradient 1); ES-MS: calcd (M+H$^+$=$C_{59}H_{74}O_{19}N_{13}S_3^+$), 1364.4; found, *m/z* 1364.6.