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ORIGINAL ARTICLE

Rapid acidolysis of benzyl group as a suitable approach for syntheses of peptides naturally produced by oxidative stress and containing 3-nitrotyrosine

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Abstract 3-Nitrotyrosine (Nit) belongs to products of oxidative stress and could probably influence conformation of neurodegenerative proteins. Syntheses of peptides require availability of suitable synthon for introduction of Nit residue. Common phenolic protection groups are more acid labile, when they are attached to Nit residue. We have found that Fmoc–Nit(Bn)–OH is a good building block for syntheses of Nit containing peptides by Fmoc/tBu strategy. Interestingly, the peptides containing multiple Nit residues can be available solely by use of Fmoc–Nit(Bn)–OH synthon. Bn is removed rapidly with ca 80 % trifluoroacetic acid in dark. The cleavage of Bn from Fmoc–Nit(Bn)–OH proceeds via pseudo-first order mechanism with activation barrier 32 kcal mol^{-1} and rate $k = 15.3\ s^{-1}$ at 20 °C. This rate is more than 2,000,000 times faster than that for cleavage of benzyl from Tyr(Bn).

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Standard abbreviations have been followed throughout this paper (J Peptide Sci 12:1–12, 2006). Unless stated otherwise, amino acids are of L-configuration.

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Introduction

The aging population is progressively affected by neurodegenerative diseases such as Alzheimer's disease (Butterfield et al. 2011), Parkinson's disease (Gurry et al. 2013) and amyotrophic lateral sclerosis (Abe et al. 1995; Beal et al. 1997; Strong et al. 1998). Moreover, transmissible neurodegenerative diseases (Prusiner 1998) emerged as a potential threat of entire society independently on the age. In many of the above mentioned diseases, conformational changes of proteins are considered to play the detrimental role (Gurry et al. 2013; Prusiner 1998). Another detrimental factor for neurodegenerative diseases is an oxidative stress (Butterfield et al. 2011; Radi 2013), which can affect the system on molecular level. The attacked resources are not only membrane lipids and proteins (Radi 2013; Bartesaghi et al. 2010) but also intracellular machinery responsible for metabolic transformations. The oxidized proteins can both gain or lose their functions and thus negatively influence fragile equilibrium in life (Butterfield et al. 2011; Radi 2013).

There are many free radical reactions responsible for protein modifications (Radi 2013; Robinson and Evans 2012). Among them, the nitration of tyrosine (H–Tyr–OH) residue is quite abundant (Radi 2004, 2013; Bartesaghi et al. 2010; Robinson and Evans 2012). However, only several structures containing 3-nitrotyrosine (H–Nit–OH) were deposited in Protein Data Bank (Sept/1 2015). The described structures mostly belong to family of oxidoreductases, i.e. proteins directly involved in redox reactions and oxidative stress. Unfortunately, none of the deposited

structures belongs to the neurodegenerative proteins despite many of them contain tyrosine residues (Fig. 1). In past, the excessive nitration of proteins was observed during transmissible spongiform encephalopathies (Guentchev et al. 2000; Dear et al. 2007; Fernández et al. 2007), Alzheimer's disease (Butterfield et al. 2011; Lüth et al. 2002; Sacksteder et al. 2006), Parkinson's disease (Sacksteder et al. 2006; Pennathur et al. 1999; Giasson et al. 2000), and amyotrophic lateral sclerosis (Abe et al. 1995; Beal et al. 1997; Strong et al. 1998).

Nitration of proteins significantly alters their properties such as hydrophobicity and acidity (Radi 2013, 2004). Moreover, it significantly changes the structure of proteins (Butterfield et al. 2011). Thus, recognition of proteins by cellular receptor can be affected. Prion proteins can play an important role in transmembrane transport and signaling (Laurén et al. 2009). It is hypothesized that neurotrophic functions depend on protein–protein interactions, where the prion protein is one of the interacting members (Martins et al. 2010). Hence, the nitration of tyrosine in prion protein can change its role as the receptor.

Several nitro amino acids belong to family of tailor-made amino acids (Sorochinsky et al. 2013a; Aceña et al. 2014), which can influence protein conformation and/or lead to 3D-defined structures of proteins (Soloshonok et al. 1999; Sorochinsky et al. 2013a, b; Aceña et al. 2014).

Based on the previous findings, we can see that alpha-synuclein and prion proteins are suitable targets for protein nitration. Nitration can affect both conformationally ordered and unordered regions. The influence of nitration on conformational changes of these proteins can have practical impact on the understanding of neurodegenerative diseases.

To understand the influence of particular tyrosine nitration on neurodegenerative protein structure, the synthesis of selectively nitrated peptides and proteins should be available. For solid-phase peptide synthesis, a few protections of the nitrotyrosine have been employed so far: Boc–Nit(Bn)–OH, Fmoc–Nit–OH and Fmoc–Nit(Trt)–OH (Hanson and Law 1965; Mittoo et al. 2003; Song et al. 2006). These strategies have some limitations for syntheses of neurodegenerative peptides and another protection of Nit is required.

We have observed facile and fast cleavage of benzyl protection from nitrotyrosine in trifluoroacetic acid (Fig. 2). This cleavage is ca 2,000,000 times faster than that from Tyr(Bn) (Erickson and Merrifield 1973). Thus, Fmoc–Nit(Bn)–OH (**1**) appears to be a good synthon for syntheses of nitrotyrosine containing peptides by Fmoc/tBu strategy. We suggest that the facile cleavage of benzyl from nitrotyrosine containing peptides proceeds by neighboring group assistance.

Experimental part

General methods

TLC was performed on silica gel-coated aluminium plates. The compounds were visualized by exposure to UV light

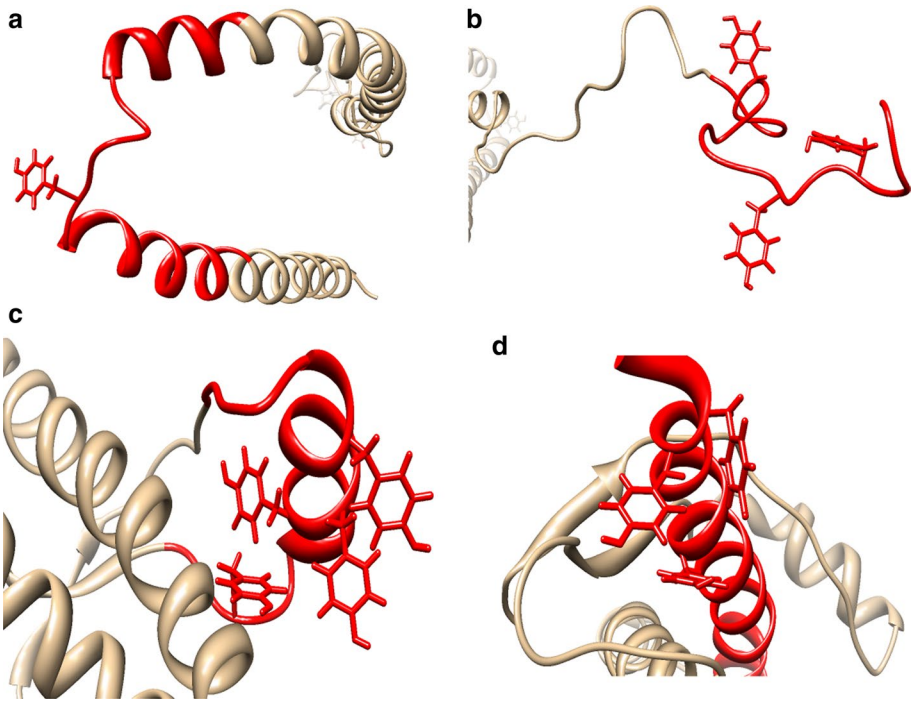

Fig. 1 Tyrosine is quite abundant in certain parts of "neurodegenerative" proteins with exception of model **a**, where only one residue is presented. Selected model peptides (*red*) in the structures of alpha-synuclein (**a**, **b**) and prion protein (**c**, **d**) (protein databank access nos. 1XQ8 and 1QM2, respectively). The tyrosine residues are emphasized by all atom models. **a** alpha-synuclein (25–53), **b** alpha-synuclein (118–140), **c** human prion protein (137–159) and **d** human prion protein (200–228) (color figure online)

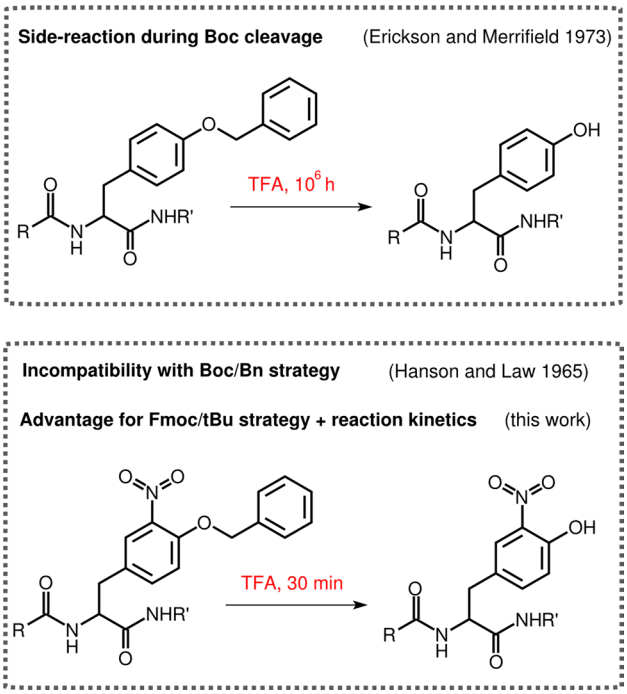

Fig. 2 Cleavage of benzyl group from tyrosine or nitrotyrosine in synthetic peptides

at 254 nm or by ninhydrin spraying. The products were dried in a vacuum drying box at room temperature for 16 h. During the syntheses, the molecular weights of the peptide fragments were determined using a matrix assisted laser desorption ionization and electrospray ionization mass spectroscopies (MALDI–TOF and ESI, respectively). For HPLC, an instrument with a quaternary pump, thermostat, diode array detector and reverse-phase C_{18} columns were used. The peptides were purified by semi-preparative HPLC on the VYDAC 250 × 10 mm, 10 µm Vydac RP-18 column flow rate 3 mL/min using gradient 0–100 % ACN (acetonitrile) in 0.05 % aqueous TFA. The analytical HPLC was carried out on the Poroshell 120 SB-C18 2.7 mm, 3.0 × 50 mm column, flow rate 1 mL/min and diode array detection using method A with gradient 10–10–100 % of ACN in 0.05 % aqueous TFA within 0–1–10 min or method B with gradient 5–5–100 % of ACN in 0.05 % aqueous TFA within 0–1–21 min. Unless specified, the method A was used.

***O*-Benzyl-*N*-(9-fluorenylmethoxycarbonyl)-3-nitro-L-tyrosine 2.25 hydrate—Fmoc–Nit(Bn)–OH *2.25 H_2O (1)** We have combined and modified the procedures for syntheses of H–Tyr(Bn)–OH (Bodanszky and Bodanszky 1994) and H–Tyr[Bn(2,6-Cl_2)]–OH (Erickson and Merrifield 1973; Yamashiro and Li 1973). To commercially available H–Nit–OH (4.0 g, 17.7 mmol) in 2 M NaOH (17.7 mL, 35.4 mmol), a solution of $CuSO_4$ $5H_20$ (2.2 g in 10 mL H_2O, 8.84 mmol) was added. The mixture was shortly heated to 60 °C, then it was cooled to rt, diluted with MeOH (80 mL) and made more alkaline by addition of 2 M NaOH (2.66 mL, 5.32 mmol). Then benzyl bromide (2.1 mL, 17.7 mmol) was added and the mixture was vigorously stirred at rt. After 1.5 h, second portion of benzyl bromide (2.1 mL, 17.7 mmol) was added and the mixture was stirred at rt for 1 h. The blue precipitate was collected and washed with *n*-hexane (3 × 20 mL) and dried 60 min under vacuum. The complex [Cu(H–Nit(Bn)–O)$_2$] (3.5 g, 5.04 mmol) was dissolved in water (15 mL) with Chelaton III (10.6 g, 28.5 mmol) and stirred on ice bath for 10 min. Subsequently, Fmoc–OSu (3.52 g, 10.4 mmol) in dioxane (15 mL) was added. The pH was maintained at 9.2 with 10 % aqueous Na_2CO_3 for 6 h at room temperature. The suspension was washed with ether (2 × 50 mL). The aqueous layer was acidified with aqueous $KHSO_4$ to pH 3. The aqueous solution was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were collected, washed with brine and dried over anhydrous Na_2SO_4. Solvent was removed in vacuo, the product was obtained as a yellow solid (5.2 g, content of **1** according to HPLC 220 nm was 90 %) which was recrystallized from hot EtOAc/*n*-hexane to give **1** as a yellow crystal. **Yield**: 4.0 g, 42 %. **Rf** ($CHCl_3$–CH_3OH 9:1) 0.45; **Rf** (CH_3OH–toluene 3:10) 0.39; **Rf** (EtOAc–CH_3OH 16:1) 0.24. HPLC **Rt** (method B, 220 nm, 95 % purity) 12.7 min; **UV–Vis**: λ_{maxNO2} = 338 nm. **EA** calcd. for $C_{31}H_{26}N_2O_7$ * 2.25 H_2O: C, 64.30; H, 5.31; N, 4.84. Found C, 64.87; H, 4.91; N, 4.76. **ESI HRMS** (*m*/*z*): for $[M + Na]^+$ $C_{31}H_{26}N_2O_7^{23}Na$ calcd. 561.16322; found 561.16325 (0.05201 ppm). **Mp** 103–105 °C. **Specific rotation** $[\alpha]_D^{20}$ 16.9 (c 0.3, acetone). **IR** ($CHCl_3$, sat. sol., cm^{-1}): ν(C=O) 1715 s, $\nu_{as}(NO_2)$ 1533 vs, $\nu_s(NO_2)$ 1352 m. **^{1}H-NMR** (400 MHz, DMSO-*d6*) δ 7.87 (d, J = 7.5 Hz, 2H, $H4_{Fmoc}$), 7.84 (d, J = 2.2 Hz, 1H, $H2_{Nit}$), 7.78 (d, J = 8.6 Hz, 1H, NH), 7.62 (d, J = 7.5 Hz, 2H, $H1_{Fmoc}$), 7.55 (dd, J = 8.7, 2.2 Hz, 1H, $H6_{Nit}$), 7.46–7.24 (m, 10H, $H2_{Fmoc}$ + H2–4_{Bn} + $H3_{Fmoc}$ + $H5_{Nit}$), 5.24 (s, 2H, $CH_{2,Bn}$), 4.18 (m, 4.2 Hz, 4H, $CH\alpha_{Nit}$ + CH_{2Fm} + $H9_{Fmoc}$), 3.12 (dd, J = 13.9, 4.4 Hz, 1H, $CH\beta_{Nit}$), 2.88 (dd, J = 13.9, 10.7 Hz, 1H, $CH\beta_{Nit}$). **^{13}C-NMR** (100 MHz, DMSO-*d6*) δ 173.44 ($COOH_{Nit}$), 156.44 ($NHCOO_{Fmoc}$), 150.12 ($C4_{Nit}$), 144.18 ($C9a_{Fmoc}$), 141.15 ($C4a_{Fmoc}$), 139.61 ($C3_{Nit}$), 136.5 ($C1_{Bn}$), 135.60 ($C6_{Nitt}$), 131.22 ($C1_{Nit}$), 128.97 ($C3_{Bn}$), 128.51 ($C4_{Bn}$), 128.09 ($C2_{Fmoc}$), 127.84 ($C2_{Bn}$), 127.50 ($C3_{Fmoc}$), 126.00 ($C2_{Nit}$), 125.65 ($C1_{Fmoc}$), 120.56 ($C4_{Fmoc}$), 115.78 ($C5_{Nit}$), 70.87 ($CH_{2,Bn}$), 66.14 (CH_{2Fmoc}), 55.59 ($CH\alpha_{Nit}$), 47.01 ($H9_{Fmoc}$), 35.43 ($CH\beta_{Nit}$).

***N*-(9-Fluorenylmethoxycarbonyl)-3-nitro-L-tyrosine–Fmoc–Nit–OH (2)** was prepared according to combination of known procedures (Song et al. 2006; Fields and Noble 1990; Carpino and Han 1972). To a stirred solution of 1.0 g (44.2 mmol) of H–Nit–OH and 15 mL of water was

added Fmoc–OSu (1.64 g, 48.6 mmol) in 15 mL of acetone at 0 °C. The pH was maintained at 9.2 with 10 % aqueous Na_2CO_3 for 6 h at room temperature. The suspension was washed with ether (2 × 50 mL). The aqueous layers were acidified with aqueous $KHSO_4$ to pH 3. The aqueous solution was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were collected and dried over anhydrous Na_2SO_4. Solvent was removed in a vacuum, and the product was obtained as a yellow solid. **Yield**: 1.8 g, 90 %. **Rf** ($CHCl_3$–CH_3OH, 9:1) 0.53; **Rf** (EtOAc–CH_3OH, 16:1) 0.43. HPLC **Rt** (gradient B, 220 nm, 95 %) 12.1 min.; **UV–Vis**: λ_{maxNO2} 358 nm. **EA** calcd. for $C_{24}H_{20}N_2O_7$: C, 64.28; H, 4.50; N, 6.25. Found C, 64.57; H, 4.56; N, 5.86. **ESI HRMS** (*m*/*z*): for $[M + Na]^+$ $C_{24}H_{20}N_2O_7Na$ calcd. 471.11627; found 471.11627 (0.09827 ppm). **Mp** 143–144 °C. **Specific rotation** $[\alpha]_D^{20}$ −3.7 (c 0.3, acetone). **IR** ($CHCl_3$, sat. sol., cm^{-1}): ν(C=O) 1721 vs, $\nu_{as}(NO_2)$ 1541 s, $\nu_s(NO_2)$ 1324 s. **^{1}H-NMR** (400 MHz, DMSO-*d*6) δ 10.81 (s, 1H, OH), 7.87 (d, J = 7.5 Hz, 2H, $H4_{Fmoc}$), 7.84 (d, J = 2.2 Hz, 1H, $H2_{Nit}$), 7.76 (d, J = 8.6 Hz, 1H, NH_{Nit}), 7.61 (dd, J = 7.5, 3.8 Hz, 2H, $H1_{Fmoc}$), 7.46 (dd, J = 8.6, 2.2 Hz, 1H, $H6_{Nit}$), 7.40 (t, J = 7.5 Hz, 2H, $H3_{Fmoc}$), 7.28 (dt, J = 8.7, 7.5, 1.1 Hz, 2H, $H2_{Fmoc}$), 7.04 (d, J = 8.6 Hz, 1H, $H5_{Nit}$), 4.21–4.13 (m, 4H, $CH\alpha_{Nit}$ + $CH_{2,Fmoc}$ + CH_{Fmoc}), 3.07 (dd, J = 13.9, 4.4 Hz, 1H, $CH\beta_{Nit}$), 2.84 (dd, J = 13.9, 10.7 Hz, 1H, $CH\beta_{Nit}$). **^{13}C-NMR** (100 MHz, DMSO) δ 173.02 ($COOH_{Nit}$), 155.96 ($NHCOO_{Fmoc}$), 150.85 ($C4_{Nit}$), 143.67 ($C9a_{Fmoc}$), 140.68 ($C4a_{Fmoc}$), 136.22 ($C6_{Nit}$ + $C3_{Nit}$), 129.17 ($C1_{Nit}$), 127.63 ($C3_{Fmoc}$), 127.03 ($C2_{Fmoc}$), 125.47 ($C2_{Nit}$), 125.21 ($C1_{Fmoc}$), 120.12 ($C4_{Fmoc}$), 118.98 ($C5_{Nit}$), 65.65 ($CH_{2,Fmoc}$), 55.23 ($C\alpha_{Nit}$), 46.55 (CH_{Fmoc}), 35.01 ($C\beta_{Nit}$).

Attempt to prepare *O*-trityl-*N*-(9-fluorenylmethoxycarbonyl)-3-nitro-L-tyrosine—Fmoc–Nit(Trt)–OH (3), Fmoc–Nit–O–Trt (4) and Fmoc–Nit(Trt)–O–Trt (5) Compound Fmoc–Nit–OH (**2**, 1 g, 2.5 mmol) and trityl chloride (700 mg, 2.5 mmol) were suspended in 50 mL of anhydrous acetonitrile, and redistilled triethylamine (0.7 mL, 5 mmol) was added dropwise (Song et al. 2006). The mixture was stirred at room temperature for 5 h, then the solvent was evaporated and the crude product was stirred with 50 mL water and ice. The yellow precipitate was filtered and dried in a vacuum for 3 h (Figure SI 1). In our hands, the attempt to prepare compound **3** was not successful (Song et al. 2006), we obtained mixture of Fmoc–Nit–OH, Fmoc–Nit(Trt)–OH, Fmoc–Nit–OTrt, Fmoc–Nit(Trt)–OTrt, and Trt–OH as confirmed by HPLC (Figure SI 1), UV–Vis and MS. Any attempts to separate this mixture lead to removal of Trt protection from the phenolic group. Even silica pretreated with various bases, e.g. pyridine or triethylamine leads to decomposition of reaction mixture to starting material and Trt–OH. We can trap the Fmoc–Nit(Trt)–OH by HPLC; however, its life time was very short. E.g. 0.05 % TFA caused complete conversion of Nit(Trt) compounds to corresponding Nit within 20 min, thus re-running of purified peak provided mixture of Fmoc–Nit–OH and Trt–OH. To analyze each compound from the reaction mixture, we used HPLC separation with collecting of peaks in 0.01 M NaOH and subsequent MS. However, during prolonged storage, compounds spontaneously decompose to Fmoc–Nit–OH and Trt–OH. The UV–Vis λ_{maxNO2} can serve as a proof of phenolic group protection (see section UV–Vis spectra of Nit derivatives in Supplementary Information). **Fmoc–Nit(Trt)–OH (3): HPLC Rt** (gradient B) 16.9 min; **UV–Vis**: λ_{maxNO2} 328 nm. **ESI HRMS** (*m*/*z*): for $[M + H]^+$ $C_{43}H_{34}N_2O_7Na$ calcd. 713.22582, found 713.22611 (0.40786 ppm). **Fmoc–Nit–O–Trt (4): HPLC Rt** (gradient B) 17.9 min; **UV–Vis**: λ_{maxNO2} 358 nm. **ESI HRMS** (*m*/*z*): for $[M + H]^+$ $C_{43}H_{34}N_2O_7Na$ calcd. 713.22582, found 713.22599 (0.23193 ppm). **Fmoc–Nit(Trt)–O–Trt (5): HPLC Rt** (gradient B) 21.3 min; **UV–Vis**: λ_{maxNO2} 328 nm. **ESI HRMS** (*m*/*z*): for $[M + H]^+$ $C_{62}H_{48}N_2O_7Na$ calcd. 955.33537, found 955.33583 (0.48096 ppm).

Hydrochloride of methyl 3-nitro-L-tyrosinate—HCl.H–Nit–OMe (6) was prepared according to published method (Bodanszky and Bodanszky 1994). To a stirred suspension of H–Nit–OH (1.0 g, 4.42 mmol) and 10 mL of dry methanol was added thionyl chloride (1 mL, 13.3 mmol) dropwise at −10 °C. Subsequently, 20 mL of methanol was added. The mixture was then refluxed 6 h and stirred overnight at room temperature. The solution was concentrated in a vacuum. The product **6** (1.18 g, 4.27 mmol) was obtained as a yellow–brown oil. **Yield**: 1.18 g, 96 %. **Rf** ($CHCl_3$–CH_3OH, 9:1) 0.55. HPLC **Rt** (gradient B, 220 nm, 95 %) 4.24 min. **ESI HRMS** (*m*/*z*): for $[M + H]^+$ $C_{10}H_{12}N_2O_5$ calcd. 241.08190; found 241.08194 (0.17764 ppm). **Mp** 183–184 °C, (195–197 °C)0.[20] **^{1}H-NMR** (400 MHz, DMSO-*d*6) δ 7.58 (d, J = 2.3 Hz, 1H, $H2_{Nit}$), 7.11 (dd, J = 8.6, 2.4 Hz, 1H, $H6_{Nit}$), 6.77 (d, J = 8.6 Hz, 1H, $H5_{Nit}$), 3.58 (s, 3H, OCH_3), 3.49 (m, 1H, $CH\alpha_{Nit}$), 2.73 (dd, J = 13.6, 6.0 Hz, 1H, $CH\beta_{Nit}$), 2.65 (dd, J = 13.5, 7.0 Hz, 1H, $CH\beta_{Nit}$). **^{13}C-NMR** (100 MHz, DMSO) δ 175.39 ($COOH_{Nit}$), 158.64 ($C4_{Nit}$), 135.89 ($C6_{Nit}$ + $C3_{Nit}$), 125.67 ($C2_{Nit}$), 123.37 ($C5_{Nit}$), 122.59 ($C1_{Nit}$), 55.64 ($C\alpha_{Nit}$), 51.39 (OCH_3), ($C\beta_{Nit}$, hidden by solvent, identified by HSQC).

Methyl *N*-(9-fluorenylmethoxycarbonyl)-3-nitro-L-tyrosinate—Fmoc–Nit–OMe (7) synthesis was adapted according to published procedure (Sever and Wilker 2001). Fmoc–OSu in 40 mL of dioxane (9.35 g, 27.7 mmol) was added dropwise to emulsion of H–Nit–OMe (5 g, 20.8 mmol) in a mixture of 10 % Na_2CO_3 (42.5 mL) and dioxan (20 mL) at 0 °C. After 20 h of stirring with gradual warming to rt, the solution was poured into ice/water (500 mL) and extracted with EtOAc (3 × 70 mL). The

extract was dried (Na_2SO_4), and evaporated to an oil, which crystallized from EtOAc/hexane or from methanol as yellow crystals. **Yield**: 6.52 g, 68 %. **Rf** (EtOAc–hexan, 1:3) 0.20; **Rf** (EtOAc–hexan, 1:1) 0.61; **Rf** (CH_3OH–toluene, 2:5) 0.77; **Rf** (CH_3OH–toluene, 2:4) 0.75; **Rf** ($CHCl_3$–CH_3OH, 9:1) 0.92. HPLC **Rt** (gradient B, 220 nm, 95 %) 12.32 min.; **UV–Vis**: λ_{maxNO2} 358 nm. **EA** calcd. for $C_{25}H_{22}N_2O_7$: C, 64.96; H, 4.80; N, 6.06. Found C, 64.95; H, 4.54; N, 5.95. **ESI HRMS** (*m/z*): for $[M + H]^+$ $C_{25}H_{23}N_2O_7$ calcd. 463.14975; found 463.14998 (−0.49160 ppm); for $[M + H]^+$ $C_{25}H_{22}N_2O_7$ ^{23}Na calcd. 485.13180, found 485.13192 (−0.25306 ppm). **Mp** 133–134 °C. **Specific rotation** $[\alpha]_D^{20}$ −14.6 (c 0.3, acetone). **IR** (KBr, cm^{-1}): ν(C=O) 1724 s, 1691 vs, $\nu_{as}(NO_2)$ 1536 vs, $\nu_s(NO_2)$ 1335 vs. **^{1}H-NMR** (400 MHz, DMSO-*d*6) δ 10.83 (s, 1H, OH), 7.90 (d, J = 8.4 Hz, 1H, NH_{Nit}), 7.87 (d, J = 7.6 Hz, 2H, $H4_{Fmoc}$), 7.83 (d, J = 2.2 Hz, 1H, $H2_{Nit}$), 7.61 (dd, J = 7.6, 2.4 Hz, 2H, $H1_{Fmoc}$), 7.47–7.36 (m, 3H, $H6_{Nit}$ + $H3_{Fmoc}$), 7.29 (q, J = 7.9 Hz, 2H, $H2_{Fmoc}$), 7.05 (d, J = 8.5 Hz, 1H, $H5_{Nit}$), 4.30–4.20 (m, 3H, $CH_{2,Fmoc}$ + CH_{Fmoc}), 4.17 (m, 1H, $CH\alpha_{Nit}$), 3.64 (s, 3H, OCH_3), 3.05 (dd, J = 13.9, 4.9 Hz, 1H, $CH\beta_{Nit}$), 2.86 (dd, J = 13.9, 10.4 Hz, 1H, $CH\beta_{Nit}$). **^{13}C-NMR** (100 MHz, DMSO-*d*6) δ 172.31 (COO_{Nit}), 156.18 ($NHCOO_{Fmoc}$), 151.18 ($C4_{Nit}$), 143.89 ($C9a_{Fmoc}$), 140.94 ($C4a_{Fmoc}$), 136.45 ($C3_{Nit}$ + $C6_{Nit}$), 128.94 ($C1_{Nit}$), 127.91 ($C3_{Fmoc}$), 127.28 ($C2_{Fmoc}$), 125.77 ($C2_{Nit}$), 125.38 ($C1_{Fmoc}$), 120.37 ($C4_{Fmoc}$), 119.29 ($C5_{Nit}$), 65.93 ($CH_{2,Fmoc}$), 55.43 ($C\alpha_{Nit}$), 52.31 (OCH_3), 46.82 (CH_{Fmoc}), 35.20 ($C\beta_{Nit}$).

Methyl *N*-(9-ethoxycarbonyl)-3-nitro-L-tyrosinate—Etoc–Nit–OMe (8) synthesis was based on published method (Song et al. 2006). A stirred solution of 0.8 g (2.9 mmol) of H–Nit–OMe.HCl and 10 mL of water was neutralized by NaOH to pH 7. Subsequently, 0.45 g of $NaHCO_3$ (5.35 mmol) was added. The reaction mixture was cooled and stirred in an ice bath and solution of ethyl chloroformate (0.44 mL, 4.62 mmol) in chloroform (10 mL) was slowly added. The mixture was kept at rt for 4 h. The organic layer was washed with Na_2SO_4, and dried over anhydrous Na_2SO_4 and solvent was removed in a vacuum. The product was obtained as a yellow solid. Yield (0.51 g, 56.2 %). **Rf** ($CHCl_3$–CH_3OH, 9:1) 0.82. HPLC **Rt** (gradient B, 220 nm—95 %) 8.3 min; **UV–Vis**: λ_{maxNO2} 358 nm. **EA** calcd. for $C_{13}H_{16}N_2O_7$: C, 50.00; H, 5.16; N, 8.97. Found C, 50.13; H, 5.13; N, 8.77. **ESI HRMS** (*m/z*): for $[M + H]^+$ $C_{13}H_{17}N_2O_7$ calcd. 313.10307, found 313.10303 (0.12106 ppm), $[M + H]^+$ $C_{13}H_{16}N_2O_7Na$ calcd. 335.08507, found 335.08497 (0.28521 ppm). **Mp** 75–77 °C. **Specific rotation** $[\alpha]_D^{20}$ +3.6 (c 0.3, CH_3OH) lit. (Song et al. 2006) $[\alpha]_D^{20}$ +10.1 (c 0.6, $CHCl_3$). **IR** (KBr, cm^{-1}): ν(C=O) 1745 vs (ester), 1721 vs, 1621 vs, $\nu_{as}(NO_2)$ 1539 vs, $\nu_s(NO_2)$ 1333 vs. **^{1}H NMR** (401 MHz, DMSO-*d*6) δ 7.77 (d, J = 2.0 Hz, 1H, NH_{Nit}), 7.63 (d, J = 8.2 Hz, 1H, $H2_{Nit}$), 7.43 (dd, J = 8.5, 2.2 Hz, 1H, $H6_{Nit}$), 7.04 (d, J = 8.5 Hz, 1H, $H5_{Nit}$), 4.27–4.17 (m, 1H, $CH\alpha_{Nit}$), 3.97–3.87 (m, 2H, $CH_{2,Et}$), 3.62 (s, 3H, OCH_3), 3.01 (dd, J = 13.9, 4.8 Hz, 1H, $CH\beta_{Nit}$), 2.81 (dd, J = 13.8, 10.4 Hz, 1H, $CH\beta_{Nit}$), 1.09 (t, J = 7.1 Hz, 3H, $CH_{3,Et}$). **^{13}C-NMR** (101 MHz, DMSO-*d*6) δ 172.43 (COO_{Nit}), 156.38 ($NHCOO_{Etoc}$), 151.15 ($C4_{Nit}$), 136.49 ($C3_{Nit}$ + $C6_{Nit}$), 128.92 ($C1_{Nit}$), 125.71 ($C2_{Nit}$), 119.28 ($C5_{Nit}$), 60.29 ($CH_{2,Et}$), 55.41 ($CH\alpha_{Nit}$), 52.24 ($OCH_{3,Nit}$), 35.29 ($CH\beta_{Nit}$), 14.75 ($CH_{3,Et}$).

Attempts for tert.-butylation of Fmoc–Nit–OMe (7) and Etoc–Nit–OMe (8)

Fmoc–Nit(tBu)–OMe, method A

Isobutene (70 mL, 733.5 mmol) was condensed to the thick-walled, well-stoppered Champagne bottle with mixture of Fmoc–Nit–OMe (**7**, 1.0 g, 2.16 mmol), DCM (30 mL) and H_2SO_4 (60 µL, 96 %, 1.08 mmol). The mixture was shaken for 4 days at rt (Beyerman and Bontekoe 1962). The bottle was carefully and slowly cooled in CO_2 (s)/ethanol bath, the cooled bottle was opened, subsequently $NaHCO_3$ (0.188 g, 2.16 mmol) was added and the reaction mixture was gradually warmed to rt. Following careful degassing, the rest of isobutene gas and DCM was evaporated, and the residue was dissolved in 20 mL MeOH. The precipitate was filtered off and dried in a vacuum for 3 h. The filtrate was concentrated and dried in a vacuum. Only the starting material was recovered as a yellow solid compound (0.8 g) as confirmed by HPLC, TLC and NMR. Yield 0 %.

Fmoc–Nit(tBu)–OMe, method B

Attempt to prepare Fmoc–Nit(tBu)–OMe was inspired by the method described (Pícha et al. 2013; Mathias 1979). Diisopropylcarbodiimide (3.15 mL, 20 mmol) and CuCl (20 mg, 0.21 mmol) were added to tert.-butyl alcohol (1.9 mL, 20 mmol), and the mixture was stirred for 12 h at room temperature to allow the formation of *O*-tert-butyl-*N,N*-diisopropylurea. A solution of Fmoc–Nit–OMe (**7**, 85 mg, 0.18 mmol) in DCM (5 mL) was slowly added to the isourea upon cooling to 0 °C, and the reaction mixture was refluxed overnight. After the reaction mixture cooled, the precipitated urea was filtered off, the filtrate was evaporated in a vacuum, and then the filtrate was redissolved in toluene (20 mL). The second crop of urea was filtered off, and the solvent was evaporated in a vacuum again. The crude material (brown–yellow oil) contained only starting compound as was confirmed by HPLC, TLC and NMR. Yield 0 %.

Etoc–Nit(tBu)–OMe

Isobutene (34 mL, 356.3 mmol) was condensed to the thick-walled, well-stoppered Champagne bottle with mixture of Etoc–Nit–OMe (**8**, 3.4 g, 11 mmol), DCM (26 mL) and H_2SO_4 (0.5 mL, 96 %, 0.09 mmol). The mixture was shaken for 4 days at rt (Beyerman and Bontekoe 1962). The bottle was carefully and slowly cooled in CO_2(s)/ethanol bath, the cooled bottle was opened, and the reaction mixture was gradually warmed to rt. Following careful degassing, the rest of isobutene gas and DCM was evaporated, and the residue was dissolved in 50 mL EtOAc. The solution was washed with 1 M $NaHCO_3$ (3 × 50 mL). The organic layer was washed with sat. Na_2SO_4 (3 × 30 mL), water (3 × 30 mL) and dried over Na_2SO_4. The solvent was removed in a vacuum. To the resultant yellow oil, hexane was added and the precipitate was filtered and dried in a vacuum for 3 h. The starting material was recovered as a yellow solid (2 g) as confirmed by HPLC, TLC and NMR. Yield 0 %.

Reactivity study

The reaction kinetics of benzyl cleavage from Fmoc–Nit(Bn)–OH (**1**) were investigated according to adapted procedure for acridine reactions (Zawada et al. 2011). Briefly, Fmoc–Nit(Bn)–OH (ca. 1 mg) was dissolved in acetonitrile (200 μL). The solution was stirred for 10 min to ensure temperature equilibration. In another working vial, trifluoroacetic acid (180 μL) and anisole (20 μL) were stirred for 10 min to ensure temperature equilibration. After addition of 20 μL of Fmoc–Nit(Bn)–OH (**1**) solution to TFA system, the mixture was vigorously stirred for 20 s and then 20 μL of reaction mixture was injected into the HPLC apparatus in desired time. The reaction mixture was analyzed by HPLC at 301 nm with the following solvent gradient: 20–20–70–100–100 % ACN within 0–1–7–10–12 min. The purity of the Hamilton syringe was crucial; after each injection it was rinsed ten times with ACN (full volume of the syringe), the syringe was dried with dry air for around 20 s, and the piston was wiped dry. The temperature of the syringe and piston was allowed to partially equilibrate with that of the reaction mixture. Before injection, the Hamilton syringe was rinsed three times with the reaction mixture. The time (τ) dependence of the amount of reactant (n) was fitted by a first-order kinetic model, $dn/d\tau = A \times \exp(-k\tau)$. By using the Arrhenius relation [$k = P \times \exp(-E^*/RT)$], the activation energy E^* was calculated from the rate constants (k) obtained at five different temperatures (T) within the interval 273–298 K; R is the universal gas constant and P is a constant independent of temperature.

Calculations

In the calculations of heat of formation of cations (Fig. 4, compounds **9a–c** and their cations **10a–c**), tyrosine was simplified to the p-cresol (**11**) and 3-nitrotyrosine as 2-nitro-p-cresol (**12**). Geometries were optimized and energies were calculated using Gaussian09 program (Frisch et al. 2009). The energies were obtained using B3LYP functional (Becke 1993; Vosko et al. 1980; Lee et al. 1988; Miehlich et al. 1989), 6-31 + G^{**} basis set, and CPCM (Barone and Cossi 1998; Cossi et al. 2003; Tomasi et al. 2005) solvent model (which is the Gaussian implementation of the COSMO model (Klamt and Schürmann 1993)) with parameters of acetic acid as a solvent.

Peptide synthesis

The peptides were synthesized by the Fmoc/tBu method (Fields and Noble 1990) by automatic solid-phase synthesizer ABI 433A (Applied Biosystems) using the FastMoc 0.1 mmol program (SynthAssist™ version 3.1) with a single coupling; 10 eq of an excess of protected amino acids and HBTU coupling reagent and 20 eq of an excess of DIPEA were used. Nitrotyrosine containing peptides were prepared using either Fmoc–Nit–OH or Fmoc–Nit(Bn)–OH for compounds **14a, 16a–22a** and **14b, 16b–22b**, respectively. The peptides were cleaved from the resin by mixture of TFA (4.5 mL), H_2O (150 μL), EDT (150 μL), thioanisole (150 μL) and TIS (50 μL) for 4 h. If benzylated products were detected, the cleavage was repeated with the same mixture for another 4 h (**20b**). All the peptides were prepared in more than 95 % purity. Retention times are summarized in Table 1.

Alpha-synuclein(25–53)—H–Gly–Val–Ala–Glu–Ala–Ala–Gly–Lys–Thr–Lys–Glu–Gly–Val–Leu–Tyr–Val–Gly–Ser–Lys–Thr–Lys–Glu–Gly–Val–Val–His–Gly–Val–Ala–OH (**13**)—For $C_{125}H_{209}N_{35}O_{40}$ (2840.54) found MALDI-MS, m/z: 2841.6 ($[M + H]^+$); 2864.6 ($[M + Na + H]^+$); 2881.6 ($[M + K + H]^+$). Amino acid analysis: Ala 4.11 (4), Glu 3.50 (3), Gly 6.00 (6), Tyr 1.20 (1), Thr 1.88 (2), Ser 0.94 (1), His 1.73 (1), Lys 4.26 (4), Leu 1.12 (1), Val 5.27 (6).

[Nit^{39}]Alpha-synuclein(25–53)—H–Gly–Val–Ala–Glu–Ala–Ala–Gly–Lys–Thr–Lys–Glu–Gly–Val–Leu–Nit–Val–Gly–Ser–Lys–Thr–Lys–Glu–Gly–Val–Val–His–Gly–Val–Ala–OH (**14a**)—For $C_{125}H_{208}N_{36}O_{42}$ (2885.52) found MALDI-MS, m/z: 2887.6 ($[M + 2H]^+$); 2909.6 ($[M + Na + H]^+$); 2925.6 ($[M + K]^+$). Amino acid analysis: Ala 4.02 (4), Glu 3.50 (3), Gly 6.00 (6), Thr 1.94 (2), Ser 0.91 (1), His 1.10 (1), Lys 4.03 (4), Leu 1.12 (1), Val 5.21 (6), Nit 0.84 (1).

[Nit^{39}]Alpha-synuclein(25–53)—H–Gly–Val–Ala–Glu–Ala–Ala–Gly–Lys–Thr–Lys–Glu–Gly–Val–Leu–

Table 1 Yields and retention times of synthesized peptides

Peptide	Yield (%)	HPLC RT (min)
Alpha-synuclein (25–53) (13)	4[a]	3.2
[Nit[39]]Alpha-synuclein (25–53) (**14a**, **14b**)	3[b], 3[c]	4.5
Alpha-synuclein (118–140) (**15**)	2[a]	3.3
[Nit[125]]Alpha-synuclein (118–140) (**16a**, **16b**)	4[b], 2[c]	4.0
[Nit[133]]Alpha-synuclein (118–140) (**17a**, **17b**)	1[b], 3[c]	3.3
[Nit[136]]Alpha-synuclein (118–140) (**18a**, **18b**)	3[b], 6[c]	3.5
[Nit[125], Nit[133]]Alpha-synuclein (118–140) (**19a**, **19b**)	5[b], 4[c]	4.2
[Nit[125], Nit[136]]Alpha-synuclein (118–140) (**20a**, **20b**)	4[b], 5[c,d]	4.2
[Nit[133], Nit[136]]Alpha-synuclein (118–140) (**21a**, **21b**)	4[b], 6[c]	4.2
[Nit[125], Nit[133], Nit[136]]Alpha-synuclein (118–140) (**22a**, **22b**)	0[b,e], 7[c]	4.3

[a] Synthesis with Fmoc–Tyr(tBu)–OH

[b] Synthesis with Fmoc–Nit–OH

[c] Synthesis with Fmoc–Nit(Bn)–OH

[d] After 4 h cleavage with TFA, only mono- and bis-benzylated products were isolated with overall yields 2 and 3 %, respectively. In another batch, the desired peptide was obtained using 2 × 4 h cleavage

[e] The deletion peptide lacking one Nit residue was isolated with overall yield 3 %

Nit–Val–Gly–Ser–Lys–Thr–Lys–Glu–Gly–Val–Val–His–Gly–Val–Ala–OH (**14b**)—For $C_{125}H_{208}N_{36}O_{42}$ (2885.52) found MALDI-MS, *m/z*: 2887.4 ($[M + 2H]^+$); 2909.4 ($[M + Na + H]^+$); 2925.4 ($[M + K]^+$). Amino acid analysis: Ala 4.02 (4), Glu 3.50 (3), Gly 6.00 (6), Thr 1.94 (2), Ser 0.90 (1), His 1.10 (1), Lys 4.03 (4), Leu 1.12 (1), Val 5.21 (6), Nit 0.84 (1).

Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Tyr–Glu–Met–Pro–Ser–Glu–Glu–Gly–Tyr–Gln–Asp–Tyr–Glu–Pro–Glu–Ala–OH (**15**)—For $C_{114}H_{157}N_{25}O_{48}S$ (2676.03) found MALDI-MS, *m/z*: 2699.0 ($[M + Na]^+$); 2716.0 ($[M + K]^+$). Amino acid analysis: Ala 2.00 (2), Asp + Asn 3.62 (4), Glu + Gln 7.34 (7), Gly 0.98 (1), Pro 3.22 (3), Tyr 3.14 (3), Ser 0.78 (1), Met 0.82 (1), Val 0.87 (1).

[Nit[125]]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Nit–Glu–Met–Pro–Ser–Glu–Glu–Gly–Tyr–Gln–Asp–Tyr–Glu–Pro–Glu–Ala–OH (**16a**)—For $C_{114}H_{156}N_{26}O_{50}S$ (2721.02) found MALDI-MS, *m/z*: 2745.1 ($[M + H + Na]^+$), 2761.1 ($[M + K]^+$). Amino acid analysis: Ala 1.94 (2), Asp + Asn 3.89 (4), Glu + Gln 7.44 (7), Gly 0.96 (1), Pro 3.00 (3), Tyr 1.95 (2), Ser 0.82 (1), Met 0.81 (1), Val 1.00 (1), Nit 1.04 (1).

[Nit[125]]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Nit–Glu–Met–Pro–Ser–Glu–Glu–Gly–Tyr–Gln–Asp–Tyr–Glu–Pro–Glu–Ala–OH (**16b**)—For $C_{114}H_{156}N_{26}O_{50}S$ (2721.02) found MALDI-MS, *m/z*: 2744.9 ($[M + H + Na]^+$), 2760.9 ($[M + K]^+$). Amino acid analysis: Ala 1.88 (2), Asp + Asn 3.74 (4), Glu + Gln 7.32 (7), Gly 1.00 (1), Pro 2.67 (3), Tyr 2.04 (2), Ser 0.80 (1), Met 0.76 (1), Val 0.90 (1), Nit 0.91 (1).

[Nit[133]]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Tyr–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Tyr–Glu–Pro–Glu–Ala–OH (**17a**)—For $C_{114}H_{156}N_{26}O_{50}S$ (2721.02) found MALDI-MS, *m/z*: 2744.9 ($[M + H + Na]^+$); 2760.9 ($[M + K]^+$). Amino acid analysis: Ala 1.90 (2), Asp + Asn 4.07 (4), Glu + Gln 7.29 (7), Gly 0.89 (1), Pro 3.07 (3), Tyr 2.00 (2), Ser 0.77 (1), Met 0.69 (1), Val 1.00 (1), Nit 0.80 (1).

[Nit[133]]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Tyr–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Tyr–Glu–Pro–Glu–Ala–OH (**17b**)—For $C_{114}H_{156}N_{26}O_{50}S$ (2721.02) found MALDI-MS, *m/z*: 2745.1 ($[M + H + Na]^+$); 2761.1 ($[M + K]^+$). Amino acid analysis: Ala 1.88 (2), Asp + Asn 3.68 (4), Glu + Gln 7.24 (7), Gly 1.00 (1), Pro 2.85 (3), Tyr 2.01 (2), Ser 0.78 (1), Met 0.78 (1), Val 0.82 (1), Nit 0.94 (1).

[Nit[136]]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Tyr–Glu–Met–Pro–Ser–Glu–Glu–Gly–Tyr–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH (**18a**)—For $C_{114}H_{156}N_{26}O_{50}S$ (2721.02) found MALDI-MS, *m/z*: 2745.0 ($[M + H + Na]^+$); 2761.0 ($[M + K]^+$). Amino acid analysis: Ala 1.85 (2), Asp 4.05 (4), Glu 7.22 (7), Gly 0.87 (1), Pro 3.00 (3), Tyr 2.04 (2), Ser 0.80 (1), Met 0.75 (1), Val 1.06 (1), Nit 0.70 (1).

[Nit[136]]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Tyr–Glu–Met–Pro–Ser–Glu–Glu–Gly–Tyr–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH (**18b**)—For $C_{114}H_{156}N_{26}O_{50}S$ (2721.02) found MALDI-MS, *m/z*: 2745.0 ($[M + H + Na]^+$), 2760.9 ($[M + K]^+$). Amino acid analysis: Ala 2.03 (2), Asp + Asn 3.78 (4), Glu + Gln 7.26 (7), Gly 1.07 (1), Pro 3.11 (3), Tyr 2.18 (2), Ser 0.86 (1), Met 0.85 (1), Val 0.88 (1), Nit 1.00 (1).

[Nit[125], Nit[133]]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Nit–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Tyr–Glu–Pro–Glu–Ala–OH

(**19a**)—For $C_{114}H_{155}N_{27}O_{52}S$ (2766.00) found MALDI-MS, *m/z*: 2790.1 ($[M + H + Na]^+$); 2806.1 ($[M + K]^+$). Amino acid analysis: Ala 2.00 (2), Asp + Asn 3.77 (4), Glu + Gln 7.67 (7), Gly 0.96 (1), Pro 2.87 (3), Tyr 1.02 (1), Ser 0.79 (1), Met 0.61 (1), Val 0.96 (1), Nit 1.61 (2).

[Nit^{125}, Nit^{133}]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Nit–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Tyr–Glu–Pro–Glu–Ala–OH (**19b**)—For $C_{114}H_{155}N_{27}O_{52}S$ (2766.00) found MALDI-MS, *m/z*: 2790.1 ($[M + H + Na]^+$); 2806.1 ($[M + K]^+$). Amino acid analysis: Ala 1.91 (2), Asp 3.79 (4), Glu 7.54 (7), Gly 0.94 (1), Pro 3.01 (3), Tyr 1.00 (1), Ser 0.79 (1), Met 0.84 (1), Val 0.85 (1), Nit 1.62 (2).

[Nit^{125}, Nit^{136}]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Nit–Glu–Met–Pro–Ser–Glu–Glu–Gly–Tyr–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH (**20a**)—For $C_{114}H_{155}N_{27}O_{52}S$ (2766.00) found MALDI-MS, *m/z*: 2790.1 ($[M + H + Na]^+$); 2806.1 ($[M + K]^+$). Amino acid analysis: Ala 1.90 (2), Asp + Asn 3.72 (4), Glu + Gln 7.38 (7), Gly 0.91 (1), Pro 2.86 (3), Tyr 1.00 (1), Ser 0.76 (1), Met 0.76 (1), Val 0.90 (1), Nit 1.54 (2).

[Nit^{125}, Nit^{136}]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Nit–Glu–Met–Pro–Ser–Glu–Glu–Gly–Tyr–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH (**20b**)—For $C_{114}H_{155}N_{27}O_{52}S$ (2766.00) found MALDI-MS, *m/z*: 2790.1 ($[M + H + Na]^+$); 2806.1 ($[M + K]^+$). Amino acid analysis: Ala 2.00 (2), Asp 4.02 (4), Glu 7.86 (7), Gly 1.01 (1), Pro 3.62 (3), Tyr 1.13 (1), Ser 0.89 (1), Met 0.72 (1), Val 0.94 (1), Nit 1.61 (2).

[Nit^{133}, Nit^{136}]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Tyr–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH (**21a**)—For $C_{114}H_{155}N_{27}O_{52}S$ (2766.00) found MALDI-MS, *m/z*: 2790.1 ($[M + H + Na]^+$); 2806.1 ($[M + K]^+$). Amino acid analysis: Ala 2.00 (2), Asp + Asn 3.84 (4), Glu + Gln 7.72 (7), Gly 0.97 (1), Pro 3.06 (3), Tyr 1.00 (1), Ser 0.83 (1), Met 0.82 (1), Val 0.83 (1), Nit 1.72 (2).

[Nit^{133}, Nit^{136}]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Tyr–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH (**21b**)—For $C_{114}H_{155}N_{27}O_{52}S$ (2766.00) found MALDI-MS, *m/z*: 2790.1 ($[M + H + Na]^+$); 2806.0 ($[M + K]^+$). Amino acid analysis: Ala 1.96 (2), Asp + Asn 3.73 (4), Glu + Gln 7.23 (7), Gly 1.01 (1), Pro 3.10 (3), Tyr 1.03 (1), Ser 0.80 (1), Met 0.79 (1), Val 1.00 (1), Nit 1.79 (2).

Trial for synthesis of [Nit^{125}, Nit^{133}, Nit^{136}]alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Nit–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH (**22a**)—The desired peptide was not obtained. Instead, a peptide without one Nit residue was obtained as confirmed by MS and amino acid analysis: H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH For $C_{105}H_{146}N_{26}O_{50}S$ (2602.94) found MALDI-MS, *m/z*: 2627.1 ($[M + H + Na]^+$); 2643.1 ($[M + K]^+$). Amino acid analysis: Ala 1.69 (2), Asp + Asn 3.84 (4), Glu + Gln 7.59

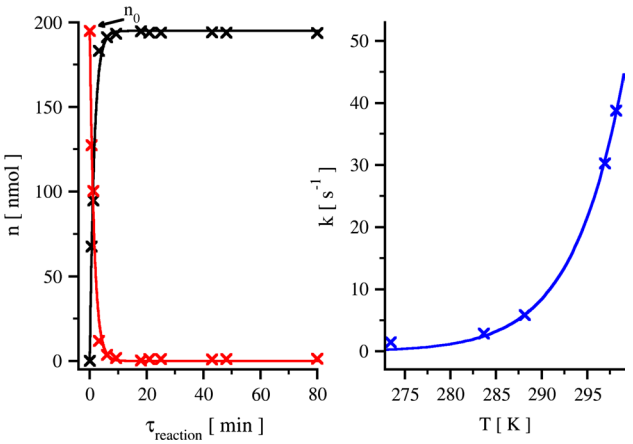

Fig. 3 Kinetic of benzyl removal from Fmoc–Nit(Bn)–OH with ca 80 % TFA at 25 °C (*left*). The amount of Fmoc–Nit(Bn)–OH (**1**) and Fmoc–Nit–OH (**2**) is shown in *red* and *black color*, respectively. The data were fitted with first-order kinetic providing *k* 38.8 s^{-1}. The dependence of rate constant on temperature (*right*) was fitted by Arrhenius equation. The activation energy from the fit is 32 kcal mol^{-1}

Table 2 Reaction rates of protection group cleavage and energies of model cation formation

Reactant	k [s^{-1}][b]	ΔE [kcal mol^{-1}][a]					
		Protonation			Cleavage		
		TFA	HCl	HBr	TFA	HCl	HBr
Tyr(Bn)	6.36×10^{-6} [c]	42	33	30	7	7	7
Nit(Bn)	15.3	33	25	22	8	8	8
Tyr(tBu)	3.12 [d]	36	27	24	8	8	8

[a] Reaction: reactant + acid → protonated cation + anion → product + anion + cleaved cation

[b] In TFA

[c] Erickson and Merrifield (1973) and Tam et al. (1983)

[d] Lundt et al. (1978)

(7), Gly 1.07 (1), Pro 3.00 (3), Ser 0.92 (1), Met 0.91 (1), Val 0.93 (1), Nit 1.82 (2).

[Nit^{125}, Nit^{133}, Nit^{136}]Alpha-synuclein(118–140)—H–Val–Asp–Pro–Asp–Asn–Glu–Ala–Nit–Glu–Met–Pro–Ser–Glu–Glu–Gly–Nit–Gln–Asp–Nit–Glu–Pro–Glu–Ala–OH (**22b**)—For $C_{114}H_{154}N_{28}O_{54}S$ (2810.99) found MALDI-MS, *m/z*: 2835.1 ($[M + H + Na]^+$). Amino acid analysis: Ala 1.91 (2), Asp 3.78 (4), Glu 7.46 (7), Gly 1.00 (1), Pro 3.03 (3), Ser 0.82 (1), Met 0.77 (1), Val 0.82 (1), Nit 2.73 (3).

Results and discussions

For introduction of 3-nitrotyrosine into synthetic peptides, the use of Boc–Nit(Bn)–OH, Fmoc–Nit–OH (**2**) and Fmoc–Nit(Trt)–OH (**3**) was described in literature (Hanson and Law 1965; Mittoo et al. 2003; Song et al. 2006). We have found that the described synthesis (Song et al. 2006) of Fmoc–Nit(Trt)–OH (**3**) does not provide pure Fmoc–Nit(Trt)–OH in our hands but a mixture of Fmoc–Nit–OH (**2**), Fmoc–Nit(Trt)–OH (**3**), Fmoc–Nit–OTrt (**4**), Fmoc–Nit(Trt)–OTrt (**5**) and Trt–OH (see Figure SI 1 in supporting information). The tritylation did not lead to completion; attempts of chromatographic purification using mobile phase with various bases led to Trt cleavage. It appears that Nit(Trt) is even more acid labile than Tyr(Trt) (Barlos et al. 1991). Since Fmoc–Nit–OH can be used for syntheses of peptides on solid phase (Mittoo et al. 2003) and the Fmoc–Nit(Trt)–OH (**3**) is very labile compound, we suppose that the previously prepared peptides (Song et al. 2006) were obtained using mixture of Fmoc–Nit–OH (**2**) and Fmoc–Nit(Trt)–OH (**3**).

We have also tried to protect phenolic group of Fmoc–Nit–OMe (**7**) and Etoc–Nit–OMe (**8**) (Song et al. 2006; Goeshen et al. 2011) using analogous procedure for preparation of Fmoc–Tyr(tBu)–OMe with isobutene (Beyerman and Bontekoe 1962; Adamson et al. 1991); however, we have just recovered the starting materials.

Since the *O*-protected Nit is more vulnerable to acids than analogous *O*-protected Tyr, we have used more acid stable benzyl group commonly cleavable by strong

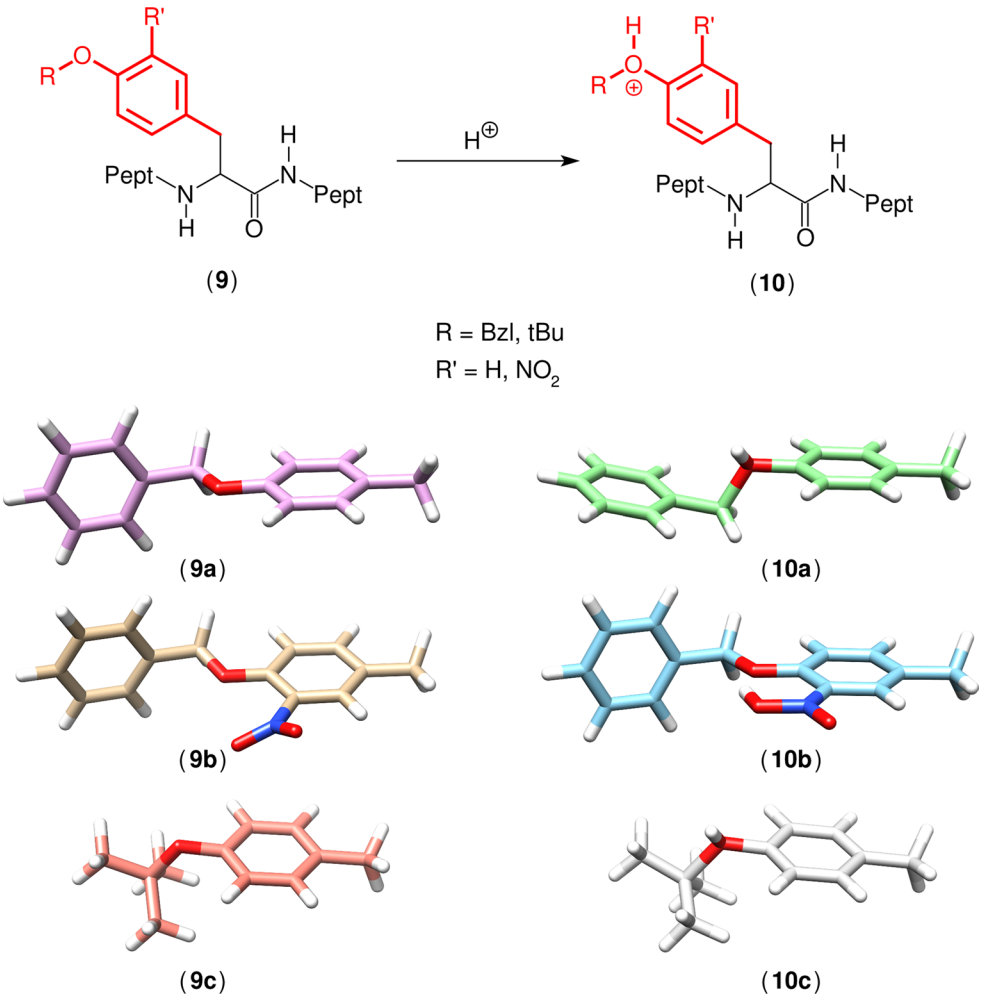

Fig. 4 Formation of cation from protected peptides. Calculated geometries of parent peptide models (considered only *red part*) and their cations. **a** Tyr(Bn), **b** Nit(Bn), and **c** Tyr(tBu) (color figure online)

acids such as liquid HF, TFMSA or HBr/AcOH. We have observed rapid cleavage of benzyl protection with mild trifluoroacetic acid. At 25 °C, 50 % of the benzyl group is removed within 1.1 min with ca 80 % TFA (Fig. 3).

Our observation of rapid cleavage of Nit(Bn) by trifluoroacetic acid confirmed that Nit(Bn) is not compatible with Boc/Bn strategy. This instability of benzyl at Boc–Nit(Bn)–OH during mild acid treatment was already observed (Hanson and Law 1965). Generally, the benzyl can be cleaved with TFA from OBn protected phenols with activating group (Marsh and Goodman 1965; Fletcher and Gunning 2008).

We have determined the activation energy for cleavage of benzyl from Fmoc–Nit(Bn)–OH (**1**) to be 32 kcal mol^{-1}. The reaction fitted to first-order kinetic equation, which corresponds to the same order as for the acidolytic cleavage of benzyl group from Tyr(Bn) (Erickson and Merrifield 1973). For the acidolytic cleavage of Tyr(Bn), the rate constant k was 6.36 × 10^{-6} s^{-1} at 20 °C (Erickson and Merrifield 1973). In the case of Nit(Bn), we have determined k as 15.3 s^{-1} at 20 °C. Thus, the cleavage of benzyl from Nit(Bn) proceeds more than 2,000,000 times faster than that from Tyr(Bn). Although we compare the cleavage in 50 % (Erickson and Merrifield 1973; Tam et al. 1983) and 80 % TFA, the concentration should not influence the rate because the pseudo-first order kinetic is achieved in both cases. Interestingly, since cleavage of tert.-butyl from Z–Tyr(tBu)–OH proceeded with k 3.12 s^{-1} (Lundt et al. 1978), the cleavage of benzyl from Nit(Bn) is ca five times faster. This clearly confirmed that the nitro group made the *O*-benzyl or *O*-tert. butyl protection extremely vulnerable towards acidolysis.

Using simple model systems, where the peptide chain was substituted with hydrogen atoms, energies of cation formation before the benzyl group deprotection (cleavage) were calculated using density functional theory (Table 2). The energy correlates with reaction rate of various tyrosine deprotection reactions (Figure SI 2). Based on the inspection of neutral and protonated states (Fig. 4), the nitro group stabilizes the cation by its protonation (Exner and Böhm 2005) i.e. the nitro group is protonated instead of phenolic oxygen in the cation. However, in transition state, the phenolic oxygen became protonated just before bond cleavage (Fig. 5, SI movies 1 and 2). Transition state is stabilized by neighboring nitro group via hydrogen bonding.

The experimental evidence for the formation of internal hydrogen bond between H_{OH} and O_{NO2} (Fig. 6) can be seen from HPLC data (Table 1). Normally, the introduction of nitro group to an alkane led to increase of permittivity and polarity e.g. *n*-hexane and nitromethane have permittivities 1.8819 and 36.562, respectively (Frisch et al. 2009). However, the retention times of Nit peptides at reverse phase are higher than those of Tyr analogues (Table 1). Thus, the formation of internal hydrogen bond between nitro group and hydroxyl group of Nit prevented the interaction with solvent, whereas the Tyr hydroxyl can freely interact with water in mobile phase.

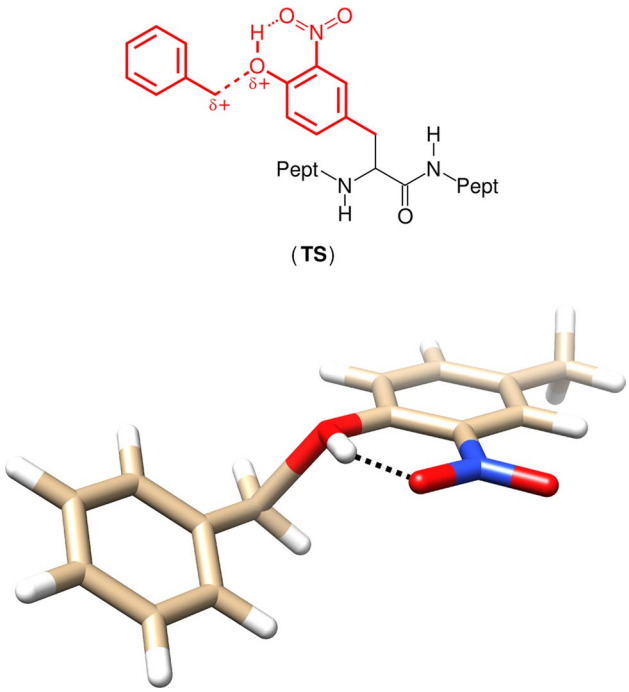

Fig. 5 Stabilization of transition state by formation of hydrogen bond

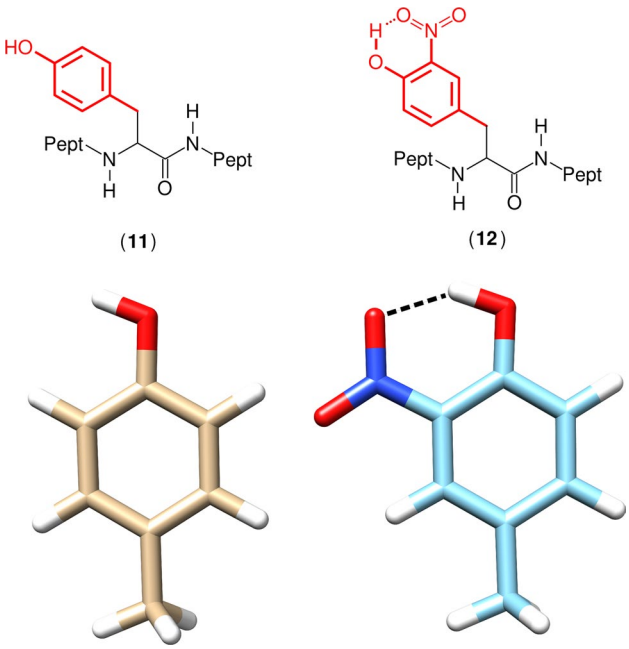

Fig. 6 Internal hydrogen bond is probably responsible for higher hydrophobicity of nitrotyrosine containing peptides at low pH

We have compared the syntheses of neurodegenerative peptides derived from alpha-synuclein using Fmoc–Nit(Bn)–OH (**1**) and Fmoc–Nit–OH (**2**). For syntheses of alpha-synuclein(25–53) containing one Tyr residue, the yields of the peptides prepared with both Nit derivatives were the same (Table 1, **14a** and **14b**). The situation was different for derivatives of alpha-synuclein(118–140). At the *N*-terminus, the unprotected phenolic group can cause less side reactions. Thus, only peptides **16a** and **19a** with ^{125}Nit provided higher yields without benzyl protection. For the combination of ^{125}Nit and ^{136}Nit, the desired peptide resisted to common 4 h deprotection procedure. Complete benzyl removal was achieved after 8 h cleavage (**20b**). For alpha-synuclein(118–140) with all three tyrosines nitrated, the desired peptide **22b** was obtained only with Fmoc–Nit(Bn)–OH. In the absence of benzyl protecting group, the synthesis provided only deletion peptides lacking one Nit residue (**22a**).

Conclusions

We have prepared Fmoc–Nit(Bn)–OH as a suitable synthon for syntheses of nitrotyrosine containing peptides by Fmoc/tBu strategy. In majority of cases, the peptides prepared with Fmoc–Nit(Bn)–OH are available in higher yields than those prepared without protection of phenolic function. Interestingly, in presence of three Nit residues, the peptide can be hardly prepared without phenolic group protection. The assistance of neighboring ortho nitro group led probably to the decrease of activation barrier for the cleavage of benzyl group from phenolic function of nitrotyrosine. Thus, the cleavage of benzyl mostly occurred during the cleavage of peptides from the resin with trifluoroacetic acid and scavengers.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

Human participants and animal statement This paper does not contain any studies with human participants or animals performed by any of the authors. For this type of study formal consent is not required.

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