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ORIGINAL ARTICLE

Solid phase and solution synthesis of NvocLys($CO(CH_2)_5NH$–NBD)OCH_2CN, a trifunctional fluorescent lysine derivative

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Abstract Herein, we describe a general strategy for the facile synthesis of a multifunctional amino acid derivative bearing both fluorescent and photolabile groups such as the lysine derivative NvocLys($CO(CH_2)_5NH$–NBD)OCH_2CN (**1**) that can be used as a biophysical tool for studying protein structure. The synthetic strategy involves functionalization of the amine groups while the amino acid is attached to a solid support, followed by esterification of the carboxylic acid in solution. The solid support protects the caboxylic acid, preventing a side reaction associated with the synthesis in solution and obviating the need for chromatographic purification of several intermediates. This synthetic strategy can be used for the preparation of a variety of amino acid derivatives with unusual α-amine and side chain functionalities.

Keywords Fluorescent amino acid · NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) · Photolabile protecting group · Nvoc (nitroveratryloxycarbonyl) · Lysine · Solid phase synthesis

Abbreviations

Boc	*t*-Butyloxycarbonyl
DIEA	*N*,*N*-Diisopropylethylamine
ESI–MS	Electrospray ionization mass spectrometry
FAB–MS	Fast atom bombardment mass spectrometry
Fmoc	9-Fluorenylmethyloxycarbonyl
HOAt	1-Hydroxy-7-azabenzotriazole
MeIm	*N*-Methylimidazole
MSNT	2,4,6-Mesitylenesulfonyl-3-nitro-1,2,4-triazolide
Mtt	4-Methyltrityl
NBD–C_6	6-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)–amino)hexanoic acid
Nvoc	Nitroveratryloxycarbonyl
PyAOP	7-Azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
Z	Benzyloxycarbonyl

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Introduction

While changes in a protein's sequence have traditionally been introduced by site directed mutagenesis, newer techniques have emerged that do not confine substitutions in proteins to the 20 naturally occurring amino acids, but also allow incorporation of synthetically altered or "unnatural" amino acids. These techniques utilize oligonucleotide-directed mutagenesis to incorporate unnatural amino acids into protein sequences (Anderson and Schultz 2003; Doring et al. 2001; Noren et al. 1989; Wang et al. 2006; Xie et al. 2004). Amino acids with a fluorescent label and/or special functional groups, such as a photolabile protecting group, can be used in the synthesis of fluorescent peptides (Bradshaw et al. 1994) and proteins (Turcatti et al. 1997)

as well as in direct aminoacylation of tRNAs in the "stop codon suppression" technique developed by Noren et al. (1989). This technique has been successfully used in vitro to make proteins with exotic amino acids incorporated into specific positions (Bain et al. 1989; Nowak et al. 1995; Turcatti et al. 1996) including incorporation of 2,3-diaminopropionic acid (Dap) bearing the fluorescent 3-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)– (NBD) group into neurokinin-2 (NK2) receptors to probe its structure and ligand interactions (Turcatti et al. 1997).

Robertson et al. (1991) synthesized active cyanomethyl esters of a variety of amino acids for aminoacylation of tRNAs. These amino acids utilized nitroveratryloxycarbonyl (Nvoc) as the protecting group for the α-amine because the photolytic deprotection of Nvoc is less damaging to the aminoacyl tRNA than the chemical methods such as acidolysis and catalytic hydrogenation employed to remove amine protecting groups such as Boc and Z, respectively. The solution synthesis of derivatives of trifunctional amino acids such as aspartic acid for aminoacylation of tRNAs also required protection of the α-carboxylic acid and necessitated chromatographic purification of intermediates and the final products.

We were interested in developing a facile method to synthesize various amino acid derivatives with functional groups of interests which did not involve the purification of intermediates that can lead to a decrease in overall yield. Specifically, we wanted a synthetic route to prepare amino acid derivatives that can be easily loaded onto a tRNA. We chose the synthesis of a fluorescently labeled lysine derivative (Fig. 1), NvocLys($CO(CH_2)_5NH$–NBD)OCH_2CN (**1**), containing the fluorescent NBD group that can be attached to a tRNA and subsequently incorporated into a protein. The synthesis of a lysine derivative with three different functional groups attached, namely to the α-amine, ε-amine, and α-carboxylic acid, posed a challenge in choosing an appropriate protection–deprotection strategy for the synthesis.

Initially the synthesis of **1** was attempted in solution starting from Lys(Boc)OH following the literature procedure of reacting Nvoc–Cl with the free α-amine in a basic aqueous solution (Noren et al. 1989). This approach suffered from the formation of the dimer NvocLys(Boc)–Lys(Boc)OH as a major side product along with the desired NvocLys(Boc)OH. Formation of dipeptides during the synthesis of Fmoc–amino acid derivatives using Fmoc–Cl under basic conditions has been reported; this side reaction can be minimized by using Fmoc–succinimidyl carbonate (Fmoc–OSu) instead of Fmoc–Cl (Sigler et al. 1983). Since the separation of the two products by silica gel chromatography proved to be difficult, the Boc protecting group was removed from the ε-amine side chain by treatment with TFA, the resulting mixture of NvocLysOH and

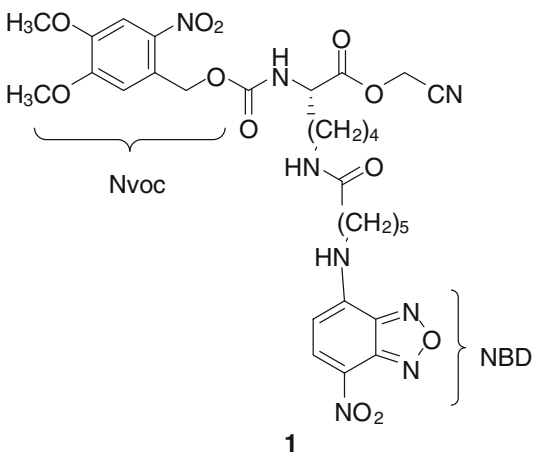

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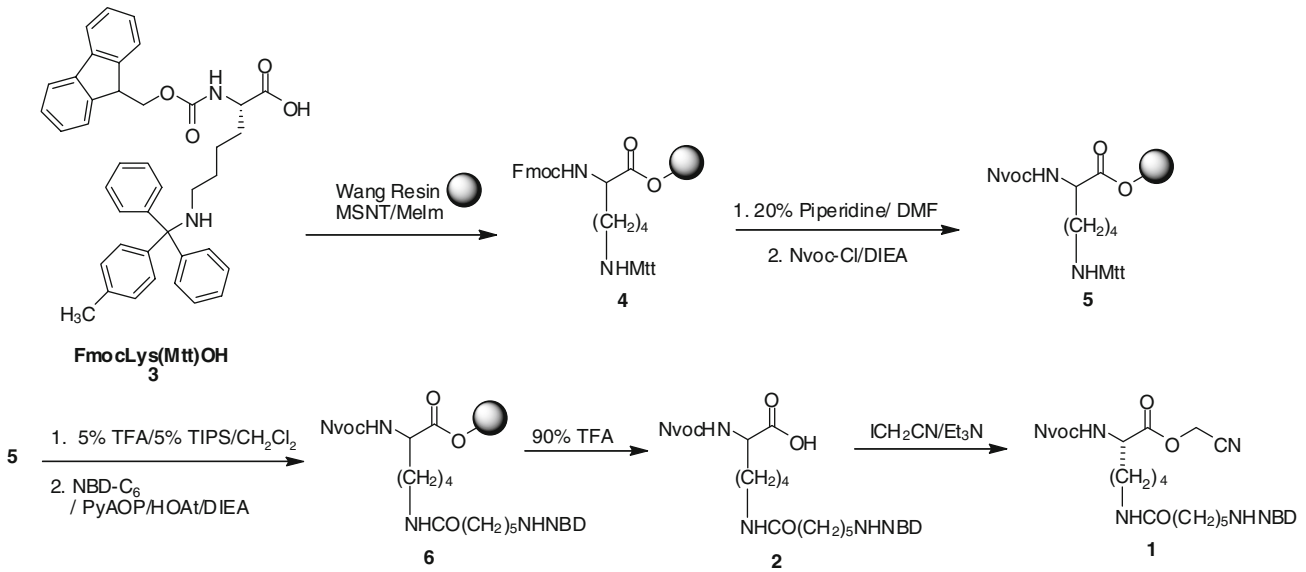
Fig. 1 N^{α}-Nitroveratryloxycarbonylamino-N^{ε}-6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-L-lysine cyanomethyl ester (NvocLys($CO(CH_2)_5NH$–NBD)OCH_2CN, **1**)

NvocLys-LysOH was separated by reversed phase HPLC and the products identified by mass spectrometry.[1] This analysis indicated that the side reaction in the previous step yielded the dimer in almost equal amounts to the desired monomer. These derivatives also proved to be difficult to handle because of their extremely hygroscopic nature. While this side reaction can be avoided by utilizing Nvoc–OSu, which can be synthesized by reacting Nvoc–Cl with *N*-hydroxysuccinimide under Schotten–Baumann conditions, an attractive alternative was synthesizing **1** using a solid support, which would avoid dimer formation and the purification of hygroscopic intermediates. Anchoring the amino acid through its α-carboxylic acid to a solid support protects the α-carboxyl group during the derivatization of other functional groups allowing functionalization of the α-amine and the side chain. Following the modifications to these functional groups, the amino acid derivative can be removed from the solid support.

We wanted the cyanomethyl ester of the fluorescently labeled amino acid for loading onto a tRNA. A resin with Kenner's acylsulfonamide "safety-catch" linker (Kenner et al. 1971) that could directly give an ester of NvocLys($CO(CH_2)_5NH$–NBD)OH (**2**) upon cleavage from the solid support could not be used since the suitable nucleophile, glycolonitrile ($HOCH_2CN$), was available only as a 55% aqueous solution and the water in the glycolonitrile appeared to compete in the cleavage reaction.

This observation led us to develop a synthetic strategy for the desired amino acid using a combination of solid phase and solution synthesis. For this, we used the

[1] NvocLysOH: HPLC (5–40% B over 23 min, A = 0.1% TFA/H_2O, B = 0.1% TFA/MeCN) t_R = 15.3 min. FAB–MS *m/z* for $C_{16}H_{23}N_3O_8$ $[M+H]^+$ calculated 386.1, found 386.2; NvocLys–LysOH: HPLC t_R = 19.2 min FAB–MS *m/z* for $C_{22}H_{35}N_5O_9$ $[M+H]^+$ calculated 514.5, found 514.3.



Scheme 1 Solid phase and solution synthesis of NvocLys($CO(CH_2)_5NH$–NBD)OCH_2CN (**1**)

conventional Wang resin to functionalize the α-amine and side chain of the amino acid, followed by esterification in solution. The use of the Wang resin avoided problems associated with the synthetic strategy using the "safety-catch linker" as well as the side reaction encountered when the synthesis was carried out entirely in solution. The high loading capacity of the Wang resin also provides high yields of the functionalized amino acid. The functional group interconversions could be carried out using nonpolar solvents that are conducive to the swelling properties of the resin. After functionalization of the amines, TFA cleavage gave the free carboxylic acid, which could then be converted to the cyanomethyl ester in solution under suitable reaction conditions (Scheme 1). Here, we report the use of a solid support to facilitate the synthesis of **1**.

Materials and methods

Fmoc-L-Lys(Mtt)OH and MSNT were purchased from Calbiochem–Novabiochem Corp. (San Diego, CA), nitroveratryl chloroformate was purchased from Fluka Chemical Corp. (Milwaukee, WI), and NBD–C_6 (NBD–$NH(CH_2)_5COOH$) was purchased from Molecular Probes, Inc. (Eugene, OR). The Wang resin was obtained from Polymer Laboratories (Amherst, MA). All other reagents and solvents were purchased from Aldrich Chemical Co. or Fischer Scientific and used as received.

TLC was performed on Whatman silica gel plates (Whatman Ltd., Florham Park, NJ). Preparative thin layer chromatography of NvocLys($CO(CH_2)_5NH$–NBD)OH was carried out using Whatman silica gel plates (20 × 20 cm, 250 μm layer). The HPLC analysis of intermediates was carried out on a Beckman System Gold fitted with a programmable solvent module 126 and a diode array detector model 168. HPLC analysis was performed with a binary solvent system consisting of aqueous 0.1% TFA (solvent A) and MeCN containing 0.1% TFA (solvent B) on a Vydac 218-TP column (4.6 × 250 mm) with a Vydac guard cartridge and a linear gradient (5–40%) over 23 min at a 1 mL/min flow rate. The intermediates and final products were analyzed by electrospray ionization mass spectrometry (ESI–MS) on an LCT mass spectrometer (Waters Inc., Milford, MA) and/or by fast atom bombardment mass spectrometry (FAB–MS) on a Kratos MS50RF mass spectrometer at Oregon State University. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 500 MHz spectrometer with dual C/H cryoprobe (CPDUL) using tetramethylsilane (TMS) as the internal standard.

Synthesis of NvocLys($CO(CH_2)_5NH$–NBD)OH (2)

FmocLys(Mtt)OH, **3** (1.4 g, 2.2 mmol), 2,4,6-mesitylenesulfonyl-3-nitro-1,2,4-triazolide (MSNT, 0.65 g, 2.2 mmol) and *N*-methylimidazole (104 μL, 1.3 mmol) dissolved in DMF (5 mL) were added to the Wang resin (1.1 mmol/g, 75–150 μm, 1 g) pre-wetted with CH_2Cl_2/DMF (1:1, 5 mL) and the mixture agitated for 24 h. The resin was filtered and washed thoroughly with DMF and CH_2Cl_2 and dried; Fmoc quantitative analysis (Fields et al. 1991) showed 90% loading (0.59 mmol/g resin). The resin was mixed with a solution of acetyl imidazole (435 mg, 3.9 mmol) and DIEA (25 μL, 0.2 mmol) in DMF and CH_2Cl_2 (1:1, 15 mL) overnight to cap any unreacted hydroxyl groups on the resin. The resin was washed

thoroughly with DMF and CH_2Cl_2 and dried. The resulting resin **4** was treated with 20% piperidine in DMF (20 mL) for 20 min and washed with DMF and CH_2Cl_2. The resin was swollen in DMF/CH_2Cl_2 mixture (1:1, 10 mL) for 10 min. Nvoc–Cl (728 mg, 2.64 mmol) and DIEA (340 μL, 2.64 mmol) in DMF/CH_2Cl_2 (1:1, 5 mL) were added and the mixture was shaken for 12 h. The resin **5** was washed with CH_2Cl_2 and DMF until all the unreacted Nvoc–Cl was removed, as indicated by a clear filtrate. A small aliquot of the resin (5 mg) was cleaved with 2 mL cleavage mixture (90% TFA, 5% TIPS, 2.5% CH_2Cl_2, 2.5% H_2O) for analysis. The resin aliquot was filtered and diluted with 10% acetic acid (15 mL) and the filtrate was extracted with ether (3 × 5 mL). The aqueous layer was collected, lyophilized, and analyzed by mass spectrometry—NvocLysOH: ESI–MS *m/z* for $C_{16}H_{23}N_3O_8$ $[M+H]^+$ calculated 386.15, found 386.1.

The resin **5** bearing NvocLys(Mtt) was mixed with 5% TFA and 5% TIPS in CH_2Cl_2 (20 mL) for 1 h and then washed thoroughly with DMF and CH_2Cl_2. NBD–C_6 (400 mg, 1.36 mmol), PyAOP (708 mg, 1.36 mmol), and HOAt (185 mg, 1.36 mmol) were dissolved in DMF/CH_2Cl_2 (1:1, 4 mL) and mixed with DIEA (474 μL, 2.71 mmol) and resin bearing NvocLys for 5 min when another 4 mL of DMF/CH_2Cl_2 was added. The mixture was shaken at 37°C for 24 h. The resin **6** was washed with DMF and CH_2Cl_2 until the filtrate was clear. The resin **6** was then suspended in cleavage reagent (90% TFA in CH_2Cl_2, 15 mL) for 4 h, and filtered. The filtrate was diluted with 10% acetic acid, and the aqueous layer was extracted with ether. The aqueous layer was lyophilized to give the crude NvocLys($CO(CH_2)_5$NH–NBD)OH (**2**) as a dark reddish-yellow oily residue. Crude **2** was purified using preparative thin layer chromatography $CHCl_3$:MeOH (6:0.2). Pure **2** was obtained as a yellow amorphous powder (344 mg, 47% overall yield).

NvocLys($CO(CH_2)_5$NH–NBD)OH (**2**)

R_f ($CHCl_3$:MeOH 6:0.1) 0.61; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.5 (d, J = 8.4 Hz, 1H, –CONH–), 7.8 (m, 1H, Ph), 7.7 (s, 1H, Ph), 7.5 (m, 1H, Ph), 7.2 (s, 1H, Ph), 6.4 (d, J = 8.6 Hz, 1H, –NH–CH–CO–), 5.4–5.2 (m, 2H, Ph–CH_2–O–), 3.9 (m, 3H, –OCH_3), 3.8 (m, 3H, –OCH_3), 3.47 (m, 2H, –CH_2–), 3.02 (m, 2H, –CH_2–), 2.86–2.85 (m, 4H, –CH_2–), 1.7–1.5 (m, 4H, –CH_2–), 1.3–1.1 (m, 4H, –CH_2–), 1.09 (m, 2H, –CH_2–). ^{13}C NMR (500 MHz DMSO-d_6) δ 174.2, 171.7, 155.5, 153.3, 147.5, 145.1, 138.7, 137.8, 128.4, 120.3, 118.5, 116.1, 109.8, 107.9, 99.0, 62.6–62.2, 56.3–56.0, 54.4, 43.2, 35.2, 30.9, 28.8, 27.4, 26.0, 24.9, 22.9. ESI–MS *m/z* for $C_{28}H_{35}N_7O_{12}$ $[M-H]^-$ calculated 660.2, found 660.1, $[M+Na]^+$ calculated 684.3, found 684.2.

Synthesis of NvocLys($CO(CH_2)_5$NH–NBD)OCH_2CN (1)

Pure **2** (0.13 g, 0.2 mmol) was dissolved in anhydrous MeCN (4 mL) and Et_3N (20 μL, 0.13 mmol) added. The mixture was stirred for 10 min followed by the addition of ICH_2CN (30 μL, 0.4 mmol) and stirred under N_2 for 6 h. The progress of the reaction was monitored by TLC ($CHCl_3$:MeOH, 4:1), which showed substantial unreacted starting material. Additional ICH_2CN (30 μL) was added and the reaction stirred for an additional 18 h at which time all of the starting material had reacted. $CHCl_3$ was added and the organic solution was washed with saturated Na_2CO_3 and dried over anhydrous $MgSO_4$. The mixture was filtered and the filtrate evaporated under the reduced pressure to give a dark reddish brown residue (40 mg, 30% yield). The crude product was purified on a silica gel column using a gradient of $CHCl_3$:MeOH (50:0.1–50:2), and the solvent was evaporated to give **1** as an orange colored viscous oil (15 mg, 11% yield).

NvocLys($CO(CH_2)_5$NH–NBD)OCH_2CN (**1**)

R_f ($CHCl_3$:MeOH 5:1) 0.63; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.5 (d, J = 8.5 Hz, 1H, –CONH–), 7.7 (s, 1H, Ph), 7.0 (s, 1H, Ph), 6.76 (m, 1H, Ph), 6.19–6.18 (d, J = 8.5 Hz, 1H, Ph), 5.71 (m, 1H, Ph), 5.59–5.47 (m, 2H, Ph–CH_2–O–), 4.9–4.7 (m, 2H, –CH_2–), 4.4 (m, 1H, –CH–), 4.0 (s, 3H, –OCH_3), 3.9 (m, 3H, –OCH_3), 3.53 (m, 2H, –CH_2–), 2.2 (m, 2H, –CH_2–), 1.94–1.82 (m, 4H, –CH_2–), 1.79–1.73 (m, 4H, –CH_2–), 1.59–1.55 (m, 4H, –CH_2–), 1.46–1.43 (m, 2H, –CH_2–). ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.2, 171.2, 155.7, 153.7, 148.2, 144.3, 144.0, 139.6, 136.6, 127.5, 123.1, 113.9, 110.2, 108.1, 64.1, 56.6–56.4, 53.4, 49.0, 43.6, 38.4, 36.0, 31.1, 29.7, 28.9, 27.8, 26.2, 24.6, 22.7, 22.0. ESI–MS *m/z* for $C_{30}H_{36}N_8O_{12}$ $[M+H]^+$ calculated 701.6, found 701.2; $[M+Na]^+$ calculated 723.4, found 723.2.

Results and discussion

Scheme 1 shows the preparation of **1** using a combination of solid phase and solution synthesis. FmocLys(Mtt)OH was loaded onto the Wang resin using MSNT (2 equiv relative to the resin) and *N*-methylimidazole (MeIm, 1.1 equiv relative to the resin) as the reagents in 1:1 DMF/CH_2Cl_2 (Harth-Fritschy and Cantacuzene 1997). Almost 90% loading was achieved after stirring for 24 h based on quantitative Fmoc analysis. Any unreacted hydroxyl groups on the resin were acetylated using *N*-acetyl imidazole. The Fmoc protecting group on the α-amine was replaced by Nvoc, by first deprotection using 20% piperidine and subsequently reacting the free α-amine with

Nvoc–Cl in the presence of DIEA. The ε-amine Mtt protecting group was removed under mild acidic conditions, and NBD–$NH(CH_2)_5COOH$ was coupled to the resulting free ε-amine using PyAOP, HOAt, and DIEA. The amino acid was then cleaved from the resin using 90% TFA in CH_2Cl_2. After chromatographic purification, pure NvocLys–$(CO(CH_2)_5$(NH–NBD))OH (**2**) was converted to the cyanomethyl ester using iodoacetonitrile and triethylamine in solution in anhydrous acetonitrile at room temperature. The identity and purity of the final product was confirmed by NMR and mass spectrometry. This synthetic protocol involving solid phase synthesis of the Nvoc-protected amino acid followed by its cyanomethylation in solution was also successfully applied to the synthesis of $NvocPheOCH_2CN$[2], starting from Fmoc-protected phenylalanine.

Conclusions

We have shown that the combination of solid phase and solution interconversions can be applied to the facile synthesis of derivatives of multifunctional amino acids such as lysine. Solution phase interconversions of primary amine and side chain groups pose challenges in the choice of protection strategies and can require the purification of intermediates. Solid phase synthesis obviated the need to purify several intermediates and provided protection of the carboxylic acid, greatly simplifying the synthesis. The use of a readily available high load resin also makes this synthetic scheme useful for the facile synthesis of unusual amino acid derivatives such as fluorescently labeled amino acids in reasonable yields. We have further derivatized the carboxylic acid in solution to yield the cyanomethyl ester that can be used to load the amino acid derivative onto a dinucleotide, which can then be used to incorporate the modified amino acid into protein sequences.

This synthetic route is also useful for preparing a variety of other amino acid derivatives with unusual N-substituents without the complication of dimer formation. The side chains of multifunctional amino acids can be conveniently functionalized with biophysically useful labels such as fluorescent groups while the amino acid is still attached to the solid support. Such attachment of the amino acid to a solid support along with orthogonal protection of the side chain functionality affords an extremely useful method of derivatizing amino acids with relative ease.

[2] $NvocPheOCH_2CN$: R_f (hexane:EtOAc 1:1) 0.53; ^1H NMR (300 MHz, DMSO-d_6) δ 8.33 (d, 1H, –CONH–), 7.73 (s, 1H, Ph), 7.33 (m, 5H, Ph), 7.11 (s, 1H, Ph), 5.39 (m, 2H, $–CH_2–$), 5.05 (s, 1H, $–CH_2CN–$), 4.43 (m, 1H, –CH–), 3.90 (s, 6H, $–OCH_3$), 3.12 (dd, 2H, $–CH_2–$); APCI–MS *m/z* for $C_{21}H_{21}O_3N_8$: $[M-H]^-$ calculated 443.4, found 441.9.

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