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Reinvestigating the synthesis and efficacy of small benzimidazole derivatives as presequence protease enhancers

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Abstract

Presequence protease (PreP) is a proteostatic enzyme that plays a key role in the maintenance of mitochondrial health. Defects in PreP stability are associated with neurological disorders in humans, and altered activity of this enzyme modulates the progress of Alzheimer's disease-like pathology in mice. As agonists that boost PreP proteolytic activity represent a promising therapeutic avenue, we sought to determine the structural basis for the action of benzimidazole derivatives (**3c** and **4c**), first reported by Vangavaragu et al. (Eur. J. Med. Chem. 76 (2014) 506–516) that enhance the activity of PreP. However, we found the published procedure for the synthesis of **3c** yielded aldimine **A** instead. We then developed an alternative synthesis and obtained **3c**, termed compound **C,** and an alternative benzimidazole derivative, termed compound **B**. We tested compounds **A**, **B** and **C** for their ability to enhance the activities of human PreP. In contrast to the previous report, we observed that none of the compounds **A**, **B**, or **C** (**3c**) modulated the catalytic activity of human PreP. Here we report our findings on the mis-identification of the reported benzimidazoles and the lack of biological activity of such compounds on human PreP. Thus, PreP modulators for PreP-based therapies remain to be discovered.

Keywords

Presequence Protease Activator; Small Benzimidazole Derivative; Structure Reassignment; Alzheimer's Disease

Introduction

Mitochondria, organelles of endosymbiotic origin, play key roles in diverse cellular functions, e.g., apoptosis and the generation of ATP and many other biosynthetic intermediates [2]. Many mitochondrial proteins (~900–1,500) are nuclear-encoded and have

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[a]. In Vangavaragu *et al's paper*, 1 μg PreP, 0.1 μg substrate V, 0.001 μM to 5 μM compounds in a final volume of 250 μL, and a SpectraMax Gemini fluorometer were used. Under the same enzyme and substrate concentrations, our fluorescence-based assay rapidly reached saturation so that the accurate determination of catalytic rate of human PreP could not be achieved (Figure S1B). We thus used much high substrate-enzyme ratios in order to accurately measure PreP's catalytic rate for the assays in this report.

a presequence that targets them to the mitochondrial matrix or intra-membrane space [3]. After the proper translocation of these proteins by the TOM/TIM translocase complex, a protein's presequence is cleaved off by the mitochondrial processing protease [3]. Such presequences are rich in hydrophobic and positively charged residues and therefore highly toxic to mitochondria. Presequence Protease (PreP) has been shown to be the key enzyme required for maintaining mitochondrial proteostasis, as it cleaves presequences into oligopeptides that can then be recycled [3, 4]. In addition, PreP also degrades toxic peptides that are imported into mitochondria, e.g., amyloid β, a peptide closely linked to the progression of Alzheimer's disease [4–6].

Recently, it has been shown that a mutation, Arg183 to Gln, leads to reduced levels of PreP, and is associated with neuronal disorders such as mental retardation and psychosis in humans [7]. In conjunction with the finding that PreP also negatively regulates the levels of amyloid β in synaptic mitochondria [5, 6], we hypothesized that compounds that enhance PreP activity represent a promising therapeutic avenue for neurological diseases. We have recently solved several crystal structures of human PreP, in the presence and absence of amyloid β, in an effort to elucidate the molecular basis of how PreP uses a sizable catalytic chamber, hydrophobic exosite and catalytic cleft to recognize amyloidogenic peptide substrates of diverse size and sequence [4]. To elucidate the structural basis for small-molecule enhancement of PreP activity, we set out to co-crystalize the small benzimidazole derivatives (**3c** and **4c**, Figure 1), first reported by Vangavaragu et al. as activators of human PreP [1]. However, when we repeated the procedure reported for the preparation of **3c** by the reaction of 4-nitro-*o*-phenylenediamine (**1**) with 5-bromosalicylaldehyde (**2**) we did not obtain the compound reported. Additionally, we have found the structure of compound **4c** was also likely incorrectly reported based on the reported NMR spectrum. Here we reinvestigate the reaction of 4-nitro-*o*-phenylenediamine (**1**) with 5-bromosalicylaldehyde (**2**) and report a successful alternative approach to synthesize **3c**. In further contrast to Vangavaragu et al. paper [1], we observed a lack of activity of these compounds in modulating PreP activity *in vitro*. Thus, small molecule enhancers of PreP remain to be discovered.

Results and Discussion

We followed Vangavaragu *et al*'s procedure [1] for the preparation of compound **3c** by the reaction of aldehyde **2** with diamine **1** in a 2:1 molar ratio in 2,2,2-trifluoroethanol (TFE). We found that if the reaction was carried out overnight at room temperature, we could isolate a product, designated as **A**, in low yield (27% yield). Modification of the reaction under ten times diluted conditions and extension of the reaction time to 46 hours gave **A** in 95% yield (Scheme 1).

We assigned the structure of **A** as the aldimine derivative 2-[(*E*)-[(2-amino-5-nitrophenyl)imino]methyl]-4-bromophenol (Scheme 1) based upon NMR (^{1}H, ^{13}C, DEPT 135, COSY, C-H HMQC, C-H HMBC), MS and HRMS (high resolution exact MS) and confirmed our assignment using DEPT 135, C-H HMQC NMR analysis (Figure 2). Compound **A** contains thirteen carbons (^{13}C NMR) including seven CH carbons (positive peaks in DEPT 135) and six carbons with no bonds to hydrogen atoms (peaks disappeared in

DEPT 135). For a compound with structure of **3c**, a negative peak of CH_2 carbon in DEPT 135 would be expected. Thus, we conclude that the procedure yields a compound with the structure of **A** [8] rather than of **3c.**

We note that compound **A** has ^{1}H and ^{13}C NMR spectra that appear to be identical to the spectra reported in Vangavaragu et al [1] for a compound they assigned as structure **4c**, which reportedly was obtained by the reaction of **1** with **2** in the presence of trimethylsilyl chloride dissolved in water. Vangavaragu *et al.* reported no HRMS or multidimensional NMR analysis for the compound assigned as **4c**, but our observation that compound **A** has the same NMR spectrum suggests that their assignment was incorrect, their synthesis likely yielding compound **A** rather than **4c**.

To synthesize **3c**, we next carried out reactions of aldehyde **2** and diamine **1** under different conditions. Reaction in methanol (ACS grade, 99.9%) at 70 ºC for 2 hours gave compound **A** in 45% yield. However, when the reaction was carried out under argon in anhydrous methanol at 70 ºC for 6 hours, we isolated products **B** (56% yield) and **C** (17% yield, identical to **3c**) as shown in Scheme 1. We assigned the structure of **B** by NMR (^{1}H, ^{13}C, COSY, C-H HMQC, C-H HMBC), HRMS and comparison to authentic samples prepared by reported methods [9, 10]. We assigned the structure of compound **C** based on NMR (^{1}H, ^{13}C, DEPT 135, COSY, C-H HMQC, C-H HMBC, NOESY), MS and HRMS. The chemical shifts: δ 5.39 (s, 2H) ppm in ^{1}H NMR and δ 44.0 (CH_2) ppm in ^{13}C NMR clearly indicate the presence of one methylene group (Figure 3a & 3b). In addition, based on the DEPT 135 spectra, there are nine CH carbons (positive peaks), one CH_2 carbon (one negative peak) and ten carbons without bonds to hydrogen atoms (peaks disappeared) (Figure 3c). In NOESY spectra of this compound, we observed NOEs between methylene group (δ 5.39 ppm) with the following four peaks [δ 10.25 (s), 8.50 (d, J = 2.0 Hz), 7.43 (d, J = 2.4 Hz) and 6.80 (d, J = 2.4 Hz). Together, these analytical data support the assigned structure for compound **C**.

The structure we assigned to compound **C** is identical to the structure assigned to **3c** by Vangavaragu et al. However, in contrast, the NMR data reported by Vangavaragu *et al.* for compounds they assigned as **3c** and **4c** (Figure 1) show no diagnostic peaks for a methylene group [1]. Moreover, the authors reported near identical ^{1}H and ^{13}C NMR data for the compounds assigned as **3c** and **4c** [1]. These spectra match those of compound **A** and thus cannot be used to distinguish between these three compounds. Since we obtained compound **A** by following a slightly modified Vangavaragu *et al.*'s procedure for the synthesis of **3c**, we conclude that the procedures reported by Vangavaragu *et al.* to give **3c** and **4c** likely produce compound **A** instead.

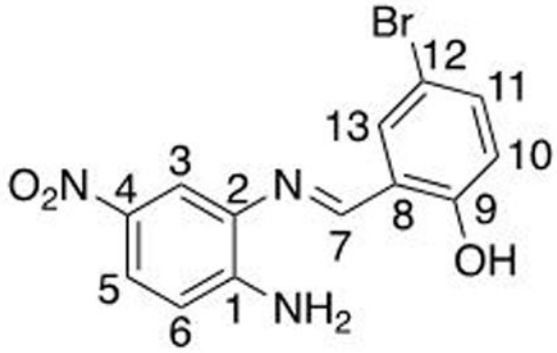
Nevertheless, having successfully synthesized compounds **A**, **B**, and **C**, we then tested their ability to modulate the activity of recombinant, purified human PreP using the fluorogenic substrate V under conditions that were similar to those reported by Vangavaragu et al where the fluorescent signal increased linearly within the observed time interval (Figure S1A) [1]. Surprisingly, we did not observe any modulatory effect of these compounds over a wide range of compound concentrations (0.0001–10 μM), which were reported to activate human PreP based on the report in Vangavaragu *et al* [1] (Figure 4)[a]. We also performed the assays using the exact conditions previously reported (1 μg PreP, 0.1 μg substrate V, and 0.001 μM

to 5 μM compounds in a final volume of 250 μL). However, the reaction fell out of the linear range quickly due to the limited available substrate, which prevented accurate catalytic rate measurements (Figure S1B). When reduced PreP to 0.1 μg to achieve the linear increase of fluorescence signal during the time of measurement, we observed no enhancement of human PreP's catalytic activity by compound **A** or **C** (Figure S1C, S2, Table S1).

Conclusion

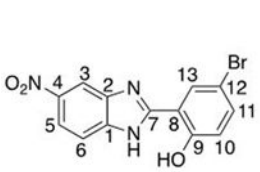
The reaction of 4-nitro-*o*-phenylenediamine (**1**) with 5-bromosalicylaldehyde (**2**) was investigated under three different conditions. Three products **A, B, C** were obtained and the structures were fully confirmed. Unfortunately, these compounds show no ability to activate PreP. Future work is required to identify and characterize efficacious small-molecule enhancers for PreP, which may prove useful in the treatment of human neurological conditions such as Alzheimer's disease.

Experimental methods:

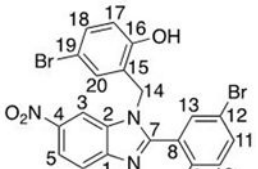


A

2-[(*E*)-[(2-Amino-5-nitrophenyl)imino]methyl]-4-bromophenol (A): A mixture of 5-bromosalicylaldehyde (201 mg, 1.0 mmol) with 4-nitro-*o*-phenylenediamine (77 mg, 0.50 mmol) in trifluoroethanol (5.0 mL) was stirred at room temperature for 46 hours. The solid was filtered, rinsed with cold ethanol and ether to give a yellow solid **A**: 130 mg. The mother liquid was evaporated by rotary evaporator and the residue was loaded on silica gel, purified by silica gel chromatography, eluting with 20% ethyl acetate in hexane to give additional **A**: 30 mg. The total yield of **A** is 95% yield. ^{1}H NMR (DMSO-d_6) δ 11.59 (s, 1H, 9-OH), 8.93 (s, 1H, CH=N, 7-H), 8.11 (d, 1H, *J* = 2.0 Hz, 13-H), 7.96–7.92 (m, 2H, 3-H, 5-H), 7.54 (dd, 1H, *J* = 8.8, 2.0 Hz, 11-H), 6.95 (d, 1H, *J* = 8.8 Hz, 10-H), 6.81–6.76 (m, 3H, 6-H, 1-NH_2); ^{13}C NMR (DMSO-d_6) δ 159.2 (CH=N, 7-CH), 158.4 (9-C), 150.4 (1-C), 136.0 (4-C), 135.5 (11-CH), 133.6 (2-C), 132.6 (13-CH), 124.5 (5-CH), 122.6 (8-C), 118.9 (10-CH), 114.2 (3-CH), 113.2 (6-CH), 110.5 (12-C) ppm. MS (ES-APCI) MH^+: 336.0, 338.0; HRMS (TOF, ESI/APCI) calcd for $C_{13}H_{11}BrN_3O_3$ [MH^+] 335.9984, found 335.9977.



B



C

4-Bromo-2-(5-nitro-1*H*-benzimidazol-2-yl)phenol (B) and 4-Bromo-2-[1-[(5-bromo-2-hydroxyphenyl)methyl]-6-nitro-1*H*-benzimidazol-2-yl]-phenol (C): Under argon, a mixture of 5-bromosalicylaldehyde (201 mg, 1.0 mmol) with 4-nitro-*o*-phenylenediamine (77 mg, 0.50 mmol) in anhydrous methanol was heated to reflux in a 70 ºC oil-bath for 6 hours. After it was cooled down, the solvent was removed and the residue was loaded on silica gel. The reaction mixture was purified by silica gel chromatography, eluting with 20–25% ethyl acetate in hexane to give two products: the first eluted product **B**: 93 mg (56% yield) and the second eluted product **C**: 45 mg (17% yield) as yellow powder. **B**: ^{1}H NMR (DMSO-d_6) δ 12.89 (brs, 2H, NH, OH), 8.48 (s, 1H, 3-H), 8.25 (s, 1H, 12-H), 8.12 (d, 1H, *J* = 8.0 Hz, 5H), 7.77 (d, 1H, *J* = 8.4 Hz, 6-H), 7.53 (d, 1H, *J* = 7.6 Hz, 11-H), 7.02 (d, 1H, *J* = 8.4 Hz); ^{13}C NMR (DMSO-d_6) δ 156.8 (9-C), 153.9 (7-C), 143.0 (4-C), 134.9 (11-CH), 129.6 (13-C), 119.5 (10-CH), 118.5 (5-CH), 114.7 (8-C), 110.5 (12-C) ppm. MS (ES-API) MH^+: 334.0, 336.0; HRMS (TOF, ESI) calcd for $C_{13}H_9BrN_3O_3$ [MH^+] 333.9827, found 333.9831. **C**: **C:** ^{1}H NMR (DMSO-d_6) δ 10.93 (brs, 1H, 9-OH), 10.25 (s, 1H, 15-OH), 8.50 (d, 1H, *J* = 2.0 Hz, 3-H), 8.12 (dd, 1H, *J* = 8.8, 2.0 Hz, 5-H), 7.83 (d, 1H, *J* = 8.8 Hz, 6-H), 7.56 (dd, 1H, *J* = 8.8, 2.4 Hz, 11-H), 7.43 (d, 1H, *J* = 2.4 Hz, 13-H), 7.19 (dd, 1H, *J* = 8.4, 2.4 Hz, 18-H), 7.01 (d, 1H, *J* = 8.8 Hz, 10-H), 6.80 (d, 1H, *J* = 2.4 Hz, 20-H), 6.68 (d, 1H, *J* = 8.8 Hz, 17-H), 5.39 (s, 2H, 14-CH_2); ^{13}C NMR (DMSO-d_6) δ 156.2 (7-C), 155.2 (9-C), 154.8 (16-C), 147.2 (1-C), 142.9 (4-C), 134.9 (11-CH), 134.8 (2-C), 133.7 (13-CH), 132.0 (18-CH), 131.7 (20-CH), 124.5 (15-C), 119.7 (6-CH), 119.3 (8-C), 118.6 (10-CH), 117.9 (5-CH), 117.5 (17-CH), 110.5 (12-C), 110.0 (19-C), 108.5 (3-CH), 44.0 (14-CH_2) ppm. MS (ES-API) MH^+: 518.0, 520.0, 522.0; HRMS (TOF, ESI) calcd for $C_{20}H_{14}Br_2N_3O_4$ [MH^+] 517.9351, found 517.9345.

Expression and purification of hPreP and enzymatic assay.

Human PreP was expressed and purified as described previously [1]. Briefly, N-terminal His_6-tagged PreP was sub-cloned into an *E. coli* expression vector, pProEx, and was expressed in Rosetta (DE3) *E. coli* at 25 ºC with 300μM IPTG induction for 16 hours. Proteins were then purified via Ni-NTA affinity, Source Q anion exchange, and Superdex 200 gel filtration column chromatography. Purified samples were flash-frozen in liquid nitrogen and stored at −80 ºC. The enzymatic activity of hPreP was quantified by monitoring the cleavage of substrate V (7-methoxycoumarin-4-yl-acetyl-RPPGFSAFK-2,4-dinitrophenyl, R&D Systems) [1], under conditions similar to those reported by Vangavaragu et al [1][a]. Specifically, the reaction was carried out in the presence of 0.1 μg (or 1 μg) hPreP in 20 mM HEPES, pH 8.0 with 10 mM $MgCl_2$ mixed with the indicated amount of substrate V (0.1 or 1.4 μg) and various concentrations of compounds **A**, **B**, or **C** (1×10^{-4}–10 μM) in a final volume of 200 μl or 250 μl at room temperature (24–26 ºC) or 37 ºC. The

hydrolysis of substrate V was measured every 30 seconds for 10 minutes using a fluorometer (Synergy Neo HST Plate Reader) with excitation and emission wavelengths set at 320 nm and 405 nm, respectively. Under the condition with 0.1 μg PreP, the substrate turnover was linear so that specific activity could be accurately determined (Figure S1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We thank Josh Kurutz for checking the NMR spectra and chemical structural assignments, John King and Jordan Mancl for helpful discussion and critical comments on the manuscript, and Prof. Geoffrey Greene for his help and support on this research. This work was supported by the National Institutes of Health R01 GM121964.

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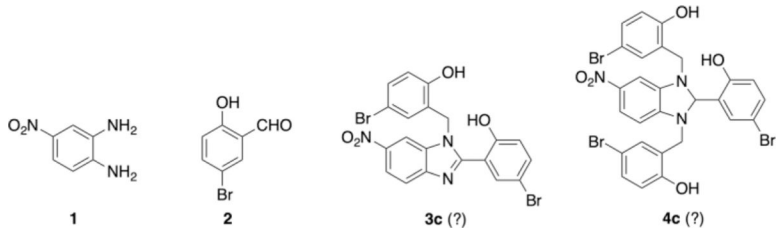


Figure 1.
Structures of benzimidazole derivatives **3c** and **4c** reported [1].

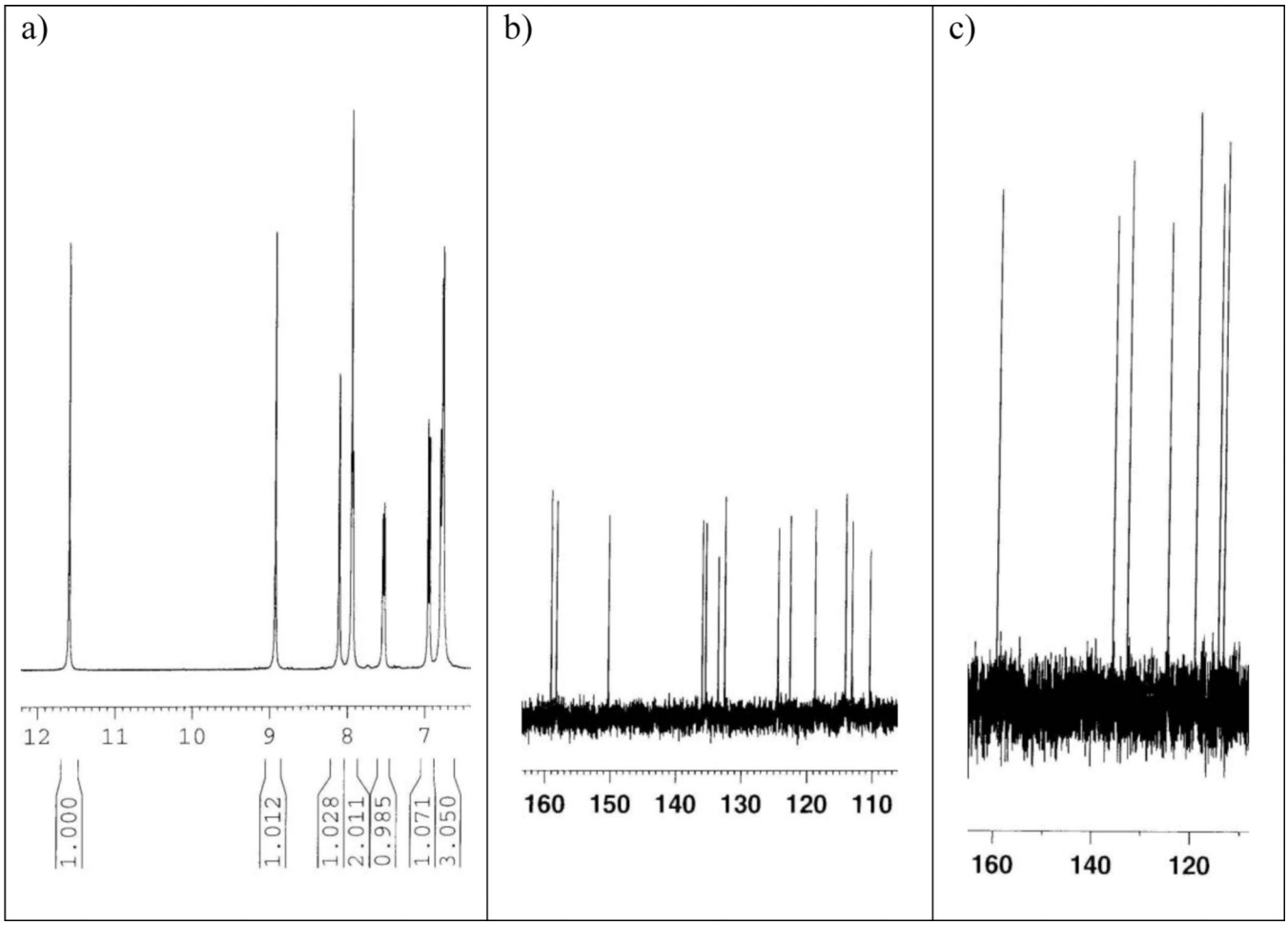


Figure 2.

^{1}H, ^{13}C and DEPT NMR spectra for compound **A**. a) ^{1}H NMR of **A**. b) ^{13}C NMR of **A.** c) DEPT 135 of **A**. Detailed analytical data for compound **A** can be found in supplementary materials (Pages S3–S10).

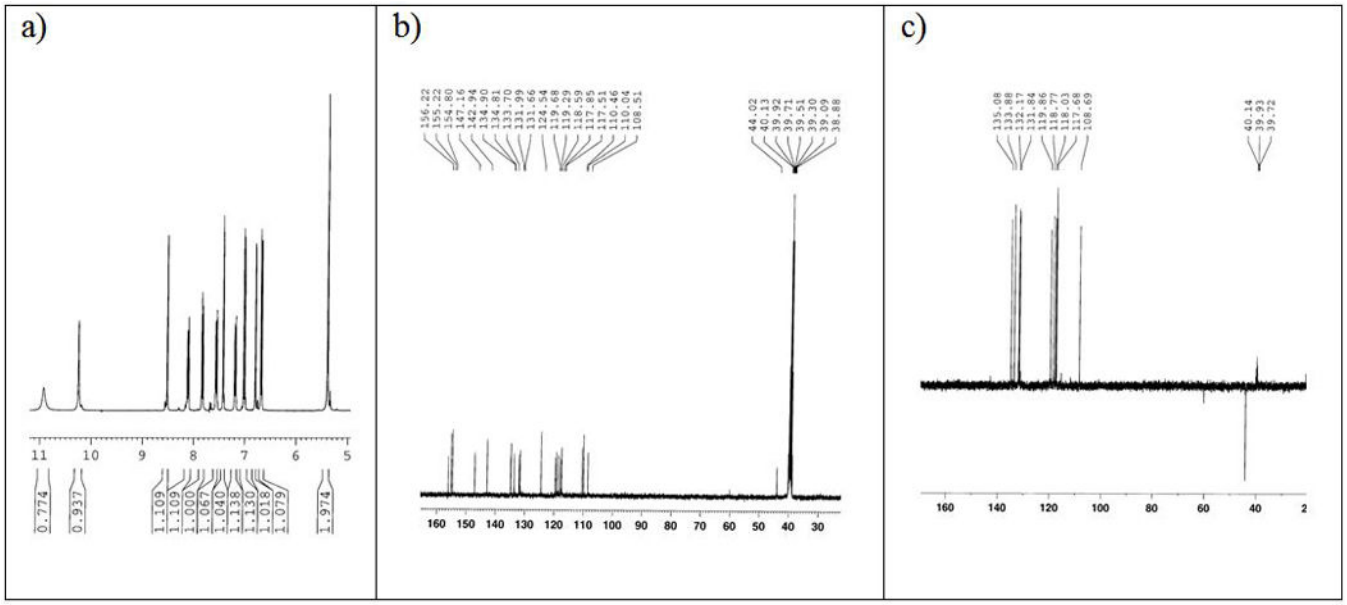


Figure 3.

^{1}H, ^{13}C and DEPT NMR spectra for compound **C**. a) ^{1}H NMR of **C**. b) ^{13}C NMR of **C.** c) DEPT 135 of **C.** Detailed analytical data for compound **C** can be found in supplementary material (Pages S17–S25).

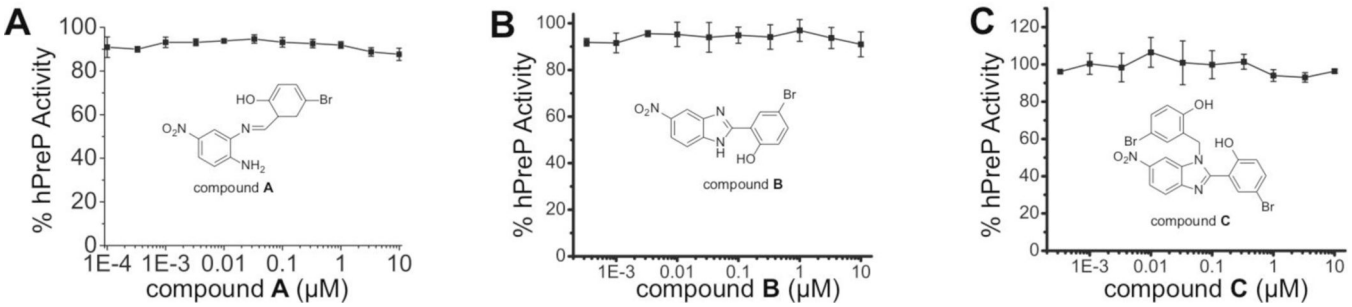
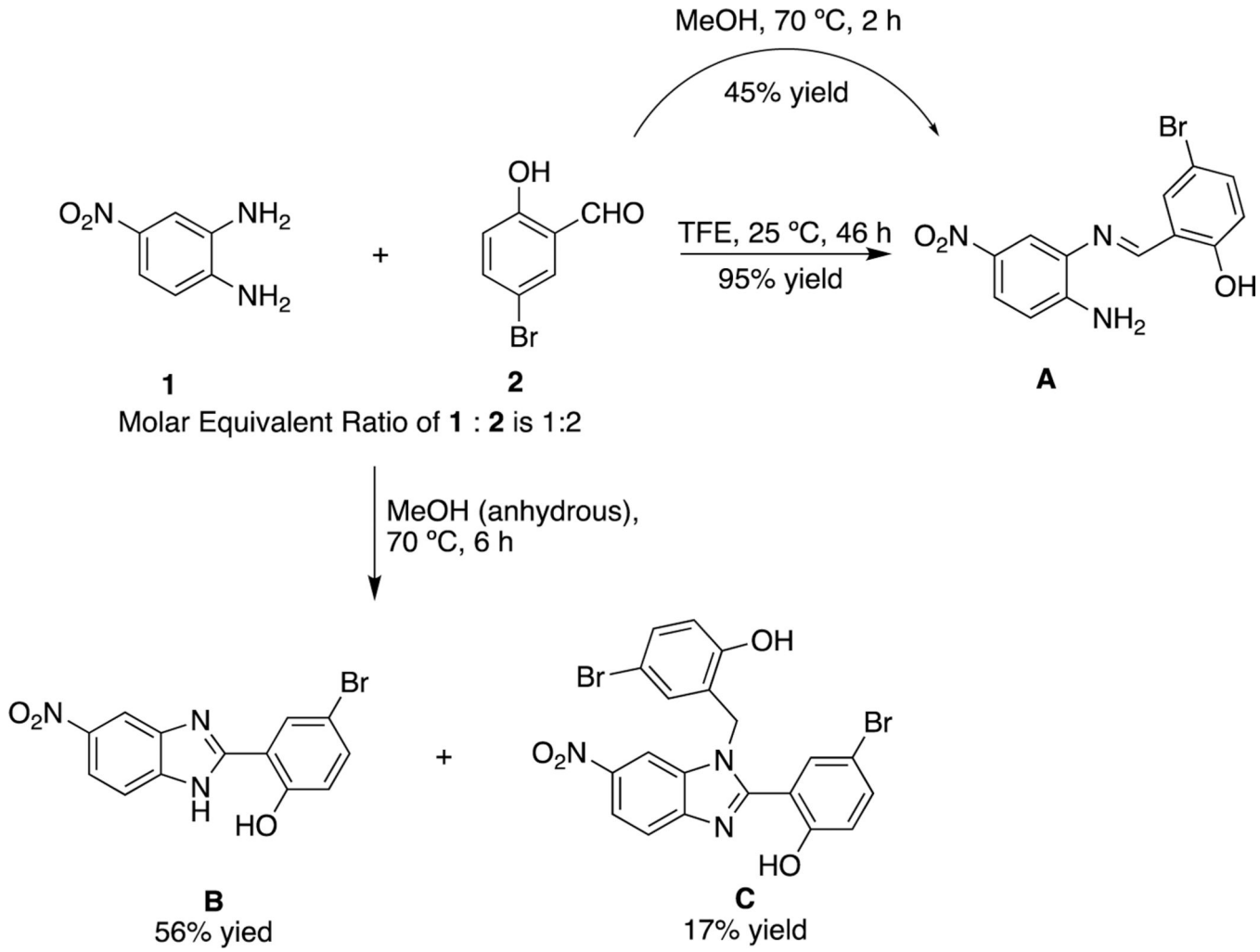


Figure 4.

The lack of effect of compounds **A**, **B**, and **C** on hPreP. Results are shown as the Substrate V degradation rate at room temperature and averaged over four independent experiments. Data represent mean +/− SD.



Scheme 1.

Reactions of 4-nitro-*o*-phenylenediamine (**1**) with 5-bromosalicylaldehyde (**2**) under three different conditions. Detailed analytical data for these compounds can be found in supplemental material (Pages S3–S25).