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Synthesis of *N*,*N*-dimethyl-2,4-dinitro-5-fluorobenzylamine and its reactions with amino acids and peptides

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Abstract—A practical synthesis is described for *N*,*N*-dimethyl-2,4-dinitro-5-fluorobenzylamine (DMDNFB) and its -d_6 analog as an alternative Sanger's reagent (DNFB), for purposes of amino acid derivatization detectable by positive mode electrospray ionization mass spectrometry. DMDNFB is comparable to DNFB in its efficiency to derivatize amino acids and peptides. Various DMDNP (d_0/d_6) derivatives of (modified) lysine were synthesized to evaluate the potential use of isotope-edited LC-ESI-MS as a tool for structural definition of the posttranslational modification of protein-based lysines.

1. Introduction

The determination of amino acids by high performance liquid chromatography (HPLC) has been dominated by pre or post-column derivatization methods to improve separation arising from greater compatibility with reversed-phase columns and to improve detection by using highly chromophoric or fluorophoric groups.[1–4] Typical amino acid reagents where the derivatization chemistry is well understood include *o*-phthalaldehyde (OPA),[5] 5-dimethylamino-1-naphthalenesulfonyl (dansyl) chloride,[6] 2,4-dinitrofluorobenzene (DNFB, Sanger's reagent),[7,8] phenyl isothiocyanate (PITC),[9] and phenylthiohydantoin (PTH)[10] amino acid derivatives. In addition, coupling of HPLC with mass spectrometry (MS) has been widely explored for analyzing posttranslational modification of protein-derived peptides or amino acids to facilitate structural identification on the basis of mass, especially for resolving co-eluting species due to their different *m*/*z* ratios.[11–14] However, chemical derivatization to permit simultaneous mass and spectral detection of modified amino acids by LC-ESI-MS has not been widely explored.[15]

Recently, labeling of α-amino groups of peptides by a 1:1 mixture of DNFB and its 3,5,6-trideuterio analog was coupled with HPLC electrospray ionization (ESI) MS to differentiate cross-linked peptides arising from post-translational protein modifications.[16] According to this method, the posttranslationally modified protein was first reductively methylated to remove free amino groups, then proteolyzed, and finally the freed α-amino groups were dinitrophenylated. Mono and bis-2,4-dinitrophenyl (DNP) derivatives (cross-linked peptides exhibit two α-amino groups) were separated by phenyl chromatography, and cross-linked peptides in the bis-DNP fraction were individually and unambiguously identified by LC-ESI-MS as 1:2:1 *m*/*z* *m*/*m*+3/*m*+6 triplets in the mass spectrum resulting from the binomial distribution of isotopic label in the bis-DNP derivative.

In cases where posttranslational modifications are rare and spread out over different sequence positions of the same amino acid, detection of the modification would be aided by complete proteolysis to the amino acid stage, thereby pooling the modified amino acid. However, although DNFB-derivatized peptides are readily discerned under ESI positive mode detection conditions because larger peptides are inevitably protonatable, our initial studies on DNFB-labeled amino acids indicated a poor response for most simple amino acids and dipeptides. This was understandable in that 2,4-dinitroarylation of the amino group abolishes the only site with significant proton affinity in these small molecules.

The purpose of the present study was to develop a modified version of Sanger's reagent containing a protonatable site so that the derivatized (modified) amino acid could be readily discerned under ESI positive mode detection, regardless of the nature of the amino acid or modification. Thus, a practical route was devised for synthesis of *N*,*N*-dimethyl-2,4-dinitro-5-fluorobenzylamine (DMDNFB, **1a**) and its -d_6 analog (**1b**). The characteristic UV spectrum would permit verification and quantitation of the peptide/protein

Keywords: Sanger's reagent; Amino acid derivatives; Peptide derivatives; Mass spectrometry.
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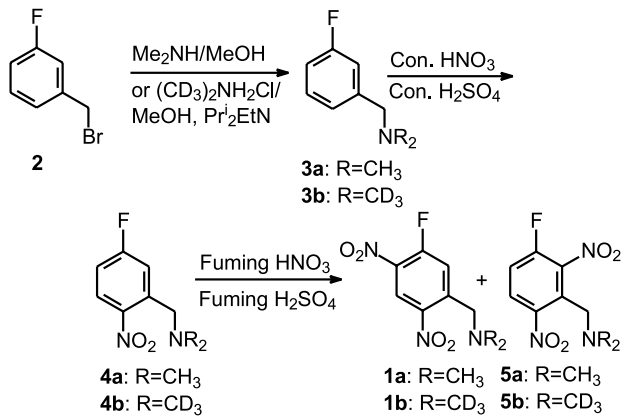
derivatization. When derivatizing a mixture of (modified) peptides or amino acids by a 1:1 mixture of the d_0 and d_6 reagent, the derivatives of interest would appear as either $m/(m+6)$ 1:1 doublets for simple modifications or $m/(m+6)/(m+12)$ 1:2:1 triplets for cross-links in the ESI-MS analysis.

2. Results and discussion

2.1. Preparation of *N*,*N*-dimethyl-2,4-dinitro-5-fluorobenzylamine (DMDNFB, 1a) and its -d_6 analog 1b

There are two alternative strategies to prepare dinitrobenzylamine derivatives: reaction of the corresponding dinitrobenzyl halide with amine,[17] or nitration of benzylamine with fuming sulfuric acid and fuming nitric acid.[18] The former approach is not suitable for preparation of **1a** because treating the intermediate 2,4-dinitro-5-fluorobenzylbromide with dimethylamine would result in reaction at either benzylic or aryl fluoride positions (or both). Thus, compounds **1a** and **1b** were prepared using the second approach (Scheme 1).



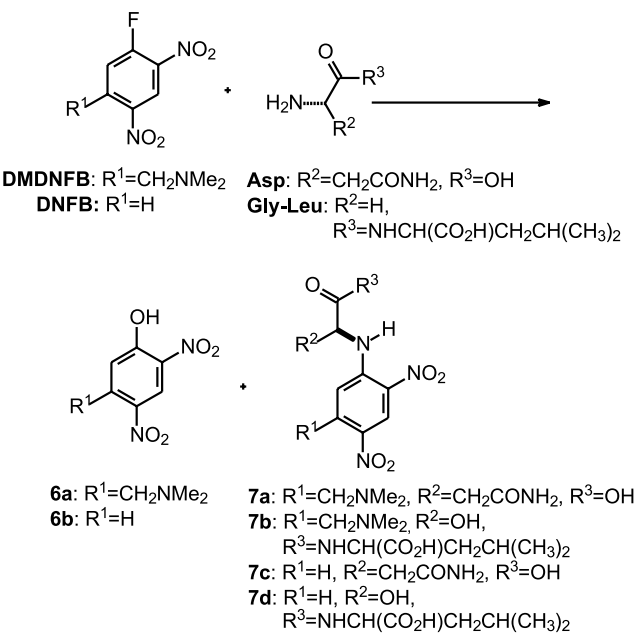
Scheme 1.

Heating 3-fluorobenzylbromide (**2**) with excess dimethylamine in methanol in a sealed tube gave *N*,*N*-dimethyl-3-fluoro-benzylamine (**3a**) in a yield of 85%. Similarly, heating **2** with d_6-dimethylamine hydrochloride in methanol in the presence of diisopropylethylamine (DIPEA) in a sealed tube for 4 days readily afforded the deuterium analog **3b** in a yield of 81%. The nitration was carried out in a stepwise manner. Heating **3a** with concentrated sulfuric acid and nitric acid give a single product in quantitative yield, assigned as *N*,*N*-dimethyl-2-nitro-5-fluorobenzylamine (**4a**) on the basis of ^{19}F couplings[19] observed in the APT ^{13}C NMR spectrum. Compound **4a** was further treated with fuming sulfuric acid and fuming nitric acid to afford **1a** in 72% yield, along with *N*,*N*-dimethyl-2,6-dinitro-3-fluoro-benzylamine (**5a**) in 11% yield. Compounds **1a** and **5a** could be easily separated by silica gel chromatography. We found that the ratio of fuming sulfuric acid to fuming nitric acid was critical for the introduction of the second nitro group. If the ratio is less than 3:1, the yield of **4a** was very low. In the same manner, DMDNFB-d_6 **1b** was prepared starting from **3b** and a 1:1 mixture of DMDNFB-d_0 and DMDNFB-d_6 **1b** was prepared starting from a 1:1 mixture of **3a** and **3b**.

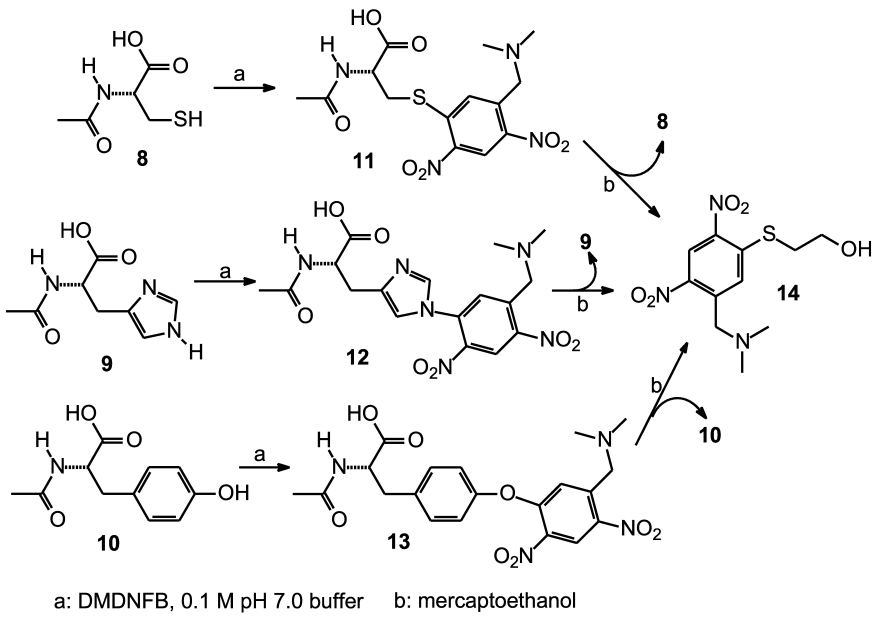
2.2. Reaction of DMDNFB and DNFB with asparagine and glycyl-L-leucine

To evaluate the reactivity of DMDNFB with the α-amino groups of amino acids and peptides under neutral conditions, the reaction of DMDNFB (40 μM) with asparagine (400 μM), the amino acid with the lowest α-amino pK_a, was carried out in pH 7.0, 0.1 M phosphate buffer. UV–Vis spectrometric monitoring indicated that the reaction followed pseudo first order kinetics in the first 2 h, yielding a second order rate constant of 1.76×10^{-3} min^{-1} M^{-1} after factoring out the [Asp] from k_{obs}. Under the same condition, the rate constant for the reaction of DNFB with asparagine was 1.67×10^{-4} min^{-1} M^{-1}. These results demonstrated that DMDNFB is about 10 times more reactive than DNFB toward the α-amino group of asparagine under this condition.

Reactions of DMDNFB (**1a**, free base) and DNFB with asparagine and glycyl-L-leucine were also carried out in an NMR tube and monitored by NMR spectrometry (Scheme 2). The reaction of 20 μmol each of DMDNFB and asparagine in 0.5 mL of a 1:1 mixture of 0.1 M, pH 7.0 phosphate buffer in D_2O and DMF-d_7 at room temperature was complete in 3 h and afforded a more than 90% yield of N^{α}-(5-(dimethylaminomethyl)-2,4-dinitrophenyl)asparagine (**7a**, N^{α}-DMDNP-Asp) and less than 10% of the hydrolysis product N^{α}-(5-(dimethylaminomethyl)-2,4-dinitrophenol (**6a**, DMDNPOH). However, the reaction of DNFB with asparagine under the same reaction condition was relatively slow and afforded an about 50% yield of N^{α}-(2,4-dinitrophenyl)asparagine (**7c**, N^{α}-DNP-Asp) in 4 h. Under the same conditions, the reaction of DMDNFB with Gly–Leu was complete in 1 h and afforded N^{α}-DMDNP-Gly–Leu (**7b**) almost quantitatively. In contrast to the results using pH 7.0 phosphate buffer, when the modification of asparagine (20 μmol) with either DMDNFB or DNFB (20 μM) was conducted in a 1:1 mixture of 5% $NaHCO_3$ in D_2O and DMF-d_7 (0.5 mL), there was no deficit



Scheme 2.



Scheme 3.

in the reaction with DNFB. Thus, the reaction with DMDNFB was complete in 3 h at room temperature and afforded a 81% yield of **7a** and a 18% yield of **6a**, whereas the reaction of DNFB was complete in 3 h and afforded **7c** almost quantitatively. These results demonstrated that DMDNFB is a reasonable alternative reagent to DNFB for labeling α-amino group of amino acids or peptides.

2.3. Reaction of side-chain functional groups of amino acids with DMDNFB and thiolysis of their products with mercaptoethanol

To develop DMDNFB as an alternative N-terminal labeling reagent, the reaction of DMDNFB and amino acids or peptides should lead to N^{α}-DMDNP amino acid or peptide derivatives as final products. However the thiol group of cysteine, the imidazole group of histidine, the phenol group of tyrosine, and the ϵ-amino group of lysine should also react with DMDNFB, as with DNFB. To investigate the reactivity of the first three functional groups toward DMDNFB, the reaction of either N^{α}-acetyl-L-cysteine **8**, N^{α}-acetyl-L-histidine **9**, or N^{α}-acetyl-L-tyrosine **10** (40 mM) with DMDNFB (40 mM) was carried out in 0.1 M pH 7.0 sodium phosphate buffer at room temperature (Scheme 3) and monitored by TLC. The reaction of **8** with DMDNFB was complete in 30 min and the product was N^{α}-acetyl-*S*-DMDNP-L-cysteine **11**. The reaction of **10** with DMDNFB transpired more slowly, and DMDNFB was consumed in 24 h. N^{α}-acetyl-*O*-DMDNP-L-tyrosine **13** was obtained after a column chromatographic purification. However, the ^{1}H NMR spectrum of the residue obtained following workup of the reaction of **9** with DMDNFB for 24 h indicated that only about half of the N^{α}-acetyl-L-histidine was converted to N^{α}-acetyl-N^{τ}-DMDNP-L-histidine **12**. During the purification of **12**, it suffered hydrolytic decomposition to N^{α}-acetyl -L-histidine and DMDNPOH (**6a**) so that we could not obtain a pure sample of **12**.

In the case of amino acid derivatization by Sanger's reagent, it is reported that *S*-DNP-L-cysteine, *O*-DNP-L-tyrosine and N^{τ}-DNP-L-histidine can be reverted to the free amino acids by mild thiolysis using mercaptoethanol.[20] Thus it was expected that mercaptoethanol would revert the DMDNP derivatives **11**–**13** to their precursors **8**–**10**. Thus, mercaptoethanol was added to the above reaction mixtures in situ, and TLC monitoring of these reactions demonstrated **12** and **13** were easily reverted to their corresponding precursors in 1 h. The latter reversion could also be followed by UV–Vis spectrophotometry (Fig. 1). Although **11** was not completely reverted to N^{α}-acetyl-L-cysteine by mercaptoethanol in 1 h at pH 7, the reaction proceeded to completion in 1 h at pH 8. The ϵ-amino group of lysine would be expected to be irreversibly derivatized by DMDNFB along with the α-amino group, so reductive methylation or some other protection strategy would be needed prior to enzymatic hydrolysis if monoderivatization was desired. Overall, by a combination of lysine protection, controlled derivatization, and thiolysis, DMDNP derivatized amino acids or peptides can be limited to the α-amino group.

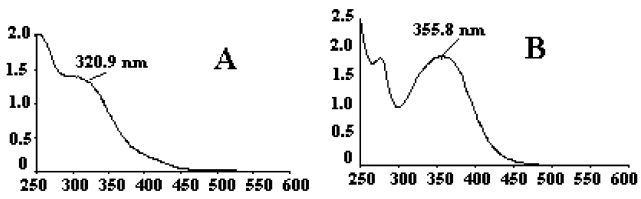
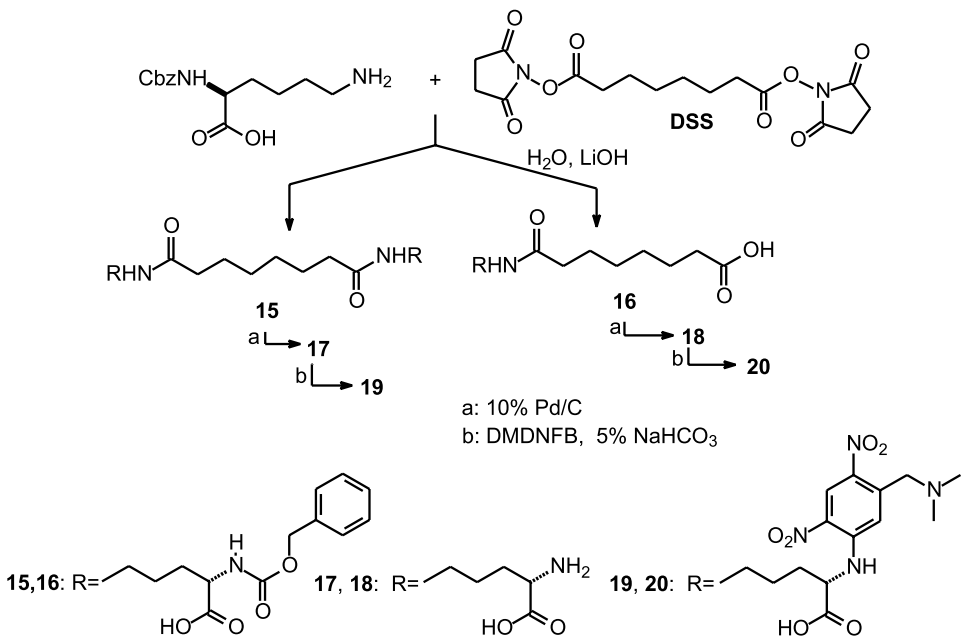


Figure 1. UV–Vis spectrum of (A) N^{α}-acetyl-*O*-DMDNP-tyrosine (0.15 mM) and (B) after treatment with mercaptoethanol for 1 h in 0.1 M pH 7.0 sodium phosphate.

2.4. Synthesis of lysine derivatives of DSS and succinyl chloride and their reactions with DMDNFB studied by isotope-edited ESI-MS

Lysine residues in proteins are major targets for modification by sugars and lipoxidation-derived reactive aldehydes during conditions of physiological oxidative stress.

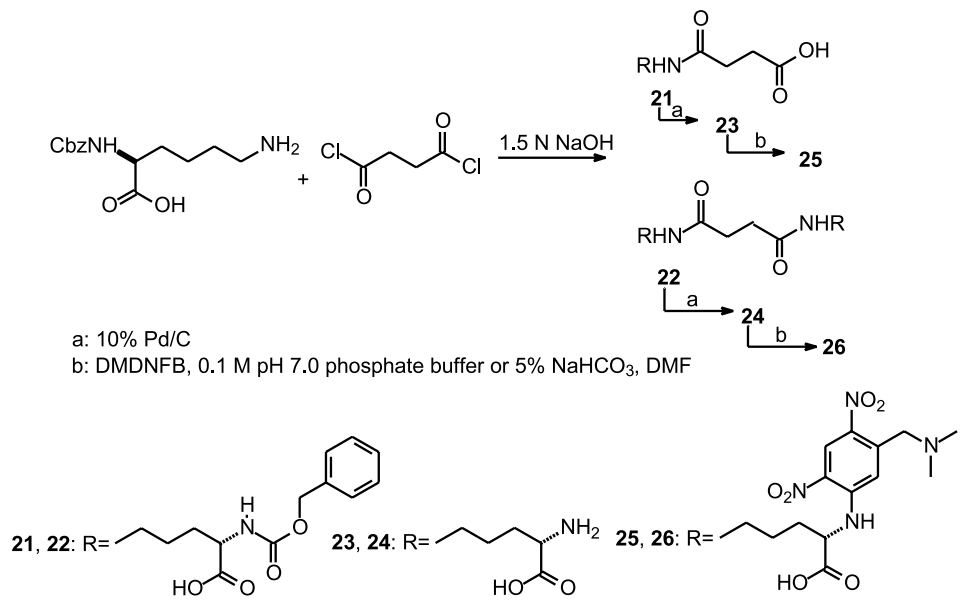


Scheme 4.

To evaluate the reactivity of the α-amino group toward DMDNFB of some modified lysines (with and without cross-linking) that would be generated by hydrolysis of a modified protein sample, we used disuccinimidyl suberate (DSS), a common lysine cross-linking reagent, that would react to give both a cross-link modification, the bis amide **17**, and, under partial hydrolysis conditions, a non-cross-link modification, the mono amide **18** (Scheme 4). Thus, treatment of N^{α}-Cbz-lysine with DSS in a ratio of 3:1 yielded the bis amide **15**, and treatment of N^{α}-Cbz-lysine with DSS in a ratio of 1:3 followed by hydrolysis using 1 M aqueous LiOH yielded the mono amide **16**. The Cbz group was removed from **15** and **16** by hydrogenolysis to give compounds **17** and **18**, respectively.[21]

Although the reaction of **17** or **18** with DMDNFB in a 1:1 mixture of pH 7.0, 0.1 M phosphate buffer and DMF resulted mainly in the hydrolytic by-product DMDNPOH, DMDNP derivatives **19** and **20** were generated, albeit in low yield, when the reactions were carried out in a 1:2 mixture of 5% $NaHCO_3$ and DMF. Thus, using a 1:1 mixture of DMDNFB and DMDNFB-d_6, ESI-MS analysis following work-up as described in the experimental section revealed 1:2:1 triplets with *m*/*z* 877.5/883.6/889.5 (singly charged ion) and *m*/*z* 439.5/442.6/446.1 (doubly charged ion) for the reaction of **17**, as expected for bis-DMDNP derivative **19**, and a 1:1 doublet with *m*/*z* 526.5/532.5 (singly charged ion) for the reaction of **18**, as expected for mono-DMDNP derivative **20**.

One possible explanation for the low yield of dinitrophenylation of compounds **17** and **18** is that the hydrophobic



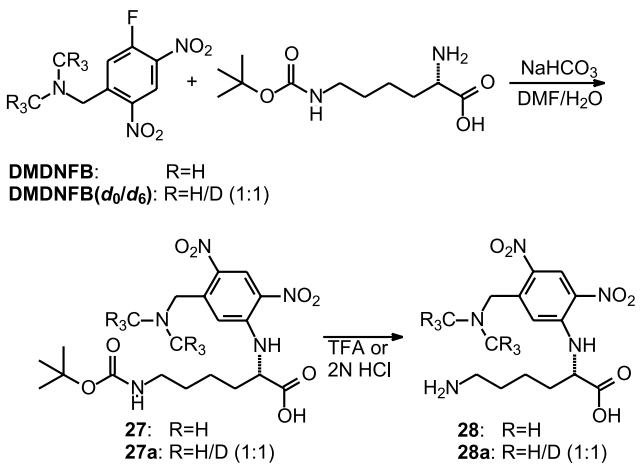
Scheme 5.

environment of the tether is lowering the reactivity of the α-amino group. To test this possibility, it was desirable to use a less hydrophobic tether, and we decided on succinyl (two methylenes in contrast to six methylenes for suberyl). Treatment of N^{α}-Cbz-lysine with succinyl chloride in a ratio of 1:1 in a solution of 1.5 N NaOH afforded a 2:1 mixture of monomer **21** and dimer **22**, which was subjected to hydrogenolysis (Pd/C) to remove the Cbz group to yield mixture of **23** and **24** (Scheme 5). The monomer **23** was washed off the catalyst with methanol and the dimer **24** was washed off the catalyst using 0.1 N HCl. Individual NMR tube scale reactions of monomer **23** and dimer **24** with DMDNFB in a 1:1 mixture of pH 7.0, 0.1 M sodium phosphate buffer in D_2O and DMF-d_7 yielded mono-DMDNP derivative **25** in a yield of 35% and bis-DMDNP derivative **26** in a yield of 30%, whereas the yields increased to 68 and 76%, respectively, using a 1:2 mixture of 5% $NaHCO_3$ in D_2O and DMF-d_7. Based on this result, compounds **25** and **26** were synthesized on a preparative scale in a 1:2 mixture of 5% $NaHCO_3$ and DMF. Attempts to separate a mixture of **25** and **26** on a phenyl superose column used to separate mono and bis-2,4-dinitrophenyl peptide derivatives[16] were unsuccessful. Apparently, the presence of the *N*,*N*-dimethylaminomethyl substituent interferes with the differential affinity of the mono vs. bis-2,4-dinitrophenyl compounds for the phenyl media, at least in the case of **25** and **26**.

2.5. Preparation of N^{α}-(2,4-dinitro-5-(dimethylaminomethyl)phenyl)-L-lysine (N^{α}-DMDNP-lysine, 28) and a 1:1 mixture of N^{α}-DMDNP-lysine and N^{α}-DMDNP-d_6-lysine (28a)

N^{α}-DMDNP-lysine (**28**) was prepared as shown in Scheme 6. Treatment of N^{ϵ}-*t*-Boc-lysine with 1.5 equiv. of DMDNFB yielded N^{ϵ}-*t*-Boc-N^{α}-DMDNP-lysine **27**. Although treatment of **27** with TFA in methylene chloride[22] did not remove the Boc group, it was removed by either heating in TFA at reflux or in 2 N HCl at room temperature, to afford N^{α}-DMDNP-lysine **28**. Also the 1:1 mixture of N^{α}-DMDNP-lysine and N^{α}-DMDNP-d_6-lysine **28a** was prepared using a 1:1 mixture of DMDNFB and DMDNFB-d_6.

The latter mixture will be a convenient lysine surrogate for the use of isotope-edited LC-ESI-MS (positive mode) to monitor the reactions of protein-based lysines with the reactive aldehydes present during physiological oxidative stress. The three advantages are first that the HPLC chromatogram (UV–Vis at 360 nm) will reveal how many species in the incubation mixture contain the lysine moiety. Second, the isotopic pattern in the mass spectrum of each peak will tell how many lysine moieties are contained in the species: compounds containing one N^{α}-DMDNP-lysine would exhibit a *m*/*z* *m*/*m*+6 1:1 doublet in the mass spectrum and compounds containing two N^{α}-DMDNP-lysines would exhibit a 1:2:1 *m*/*z* *m*/*m*+6/*m*+12 triplet in the mass spectrum. Thus it will be easy to distinguish non-cross-link from cross-link modifications and to tell their relative importance. Third, the presence of the *N*,*N*-dimethylaminomethyl basic center will ensure robust ion current signals in the ESI positive mode.



Scheme 6.

The ESI positive mode mass spectrum of the 1:1 d_0:d_6 N^{α}-DMDNP-lysine mixture (**28a**) exhibited a 1:1 *m*/*z* 370.40/376.40 doublet for the expected singly charged (protonated) ions, but also an unexpected 1:2:1 *m*/*z* 739.1/745.2/751.2 triplet, corresponding to a singly protonated non-covalent dimer of the reagent. This latter observation suggests caution in simply interpreting the observation of any *m*/*z* *m*/*m*+6/*m*+12 triplet in terms of a covalent cross-link. Thus, an observed triplet at *m*/*z* 2*x*+1/2*x*+7/2*x*+13 will signal a covalent cross-link only when there is no corresponding *m*/*z* *x*+1/*x*+7 doublet also observed.

3. Conclusions

In this paper, *N*,*N*-dimethyl-2,4-dinitro-5-fluorobenzylamine (DMDNFB, **1a**) and *N*,*N*-dimethyl-d_6-2,4-dinitro-5-fluorobenzylamine (**1b**) were synthesized. Parallel UV–Vis and NMR spectrometric studies on aminolysis of 2,4-dinitrofluorobenzene (DNFB) and DMDNFB by amino acids or peptides demonstrated that DMDNFB was at least as reactive as DNFB under neutral conditions and could form N^{α}-derivatives of amino acids and peptides with comparable efficiency. As with DNFB, DMDNFB also modifies the nucleophilic side-chains of Cys, Tyr, and His, but these adducts can be selectively reversed using mercaptoethanol. A variety of lysine-based derivatives of DMDNFB-d_0 and -d_6 were then prepared to illustrate the potential use of this reagent and isotope edited ESI mass spectrometry to investigate the lysine-based post-translational modification of proteins.

4. Experimental

4.1. General

^{1}H NMR (300 or 200 MHz) and ^{13}C NMR (75.1 or 50.1 MHz) spectra were recorded on Varian Gemini 300 or 200 instruments In all cases, tetramethylsilane or the solvent peak served as internal standard for reporting chemical shifts, which are expressed as parts per million downfield from TMS (δ scale) In the ^{13}C NMR line listings, attached proton test (APT) designations are given as (+) or (−) following the chemical shift Some carboxamide ^{13}C signals were not observed. High-resolution mass spectra

(HRMS) were obtained at 20 eV on a Kratos MS-25A instrument. TLC was performed on glass plates precoated with silica gel $60F_{254}$. Compounds on the developed plate were visualized by short-wavelength UV light (λ=254 nm), by placing the plate in a chamber filled with iodine vapor, or by spraying with ammonium phosphomolybdic acid solution or ninhydrin solution. All preparative column chromatography was performed using 32–63 μm silica gel under nitrogen pressure (flash chromatography). Purity of compounds was assessed by TLC and the lack of detectable extraneous signals in the ^{1}H NMR spectra. The water that was used for all studies was purified by a Millipore system. UV–Vis spectra were obtained using a Perkin–Elmer Lambda 20 spectrophotometer.

3-Fluorobenzylbromide, 2 M dimethylamine in methanol, dimethylamine-d_6 hydrochloride, succinyl chloride, 10% Pd–C, N^{α}-Cbz-lysine, N^{ϵ}-*t*-Boc-lysine, and 2,4-dinitrofluorobenzene (DNFB) were purchased from Aldrich Chemical Company. DSS was purchased from Pierce.

4.2. HPLC-ESI-MS-MS analyses

The reversed phase HPLC with electrospray ionization (ESI) mass spectrometry analysis of DMDNP and DNP amino acid derivatives was performed with a HP1100 equipped with either a Vydac Low TFA C_{18} column (eluent A was 95% H_2O, 5% acetonitrile, and 0.02% TFA and eluent B was 5% H_2O, 95% acetonitrile, and 0.02% TFA; the flow rate was 0.3 mL/min) or a Phenomenex Aqua C_{18} column (eluent A was 100% H_2O, 0.1% formic acid, and 0.02% TFA and eluent B was 90% acetonitrile, 0.1% formic acid, and 0.02% TFA; the flow rate was 0.5 mL/min), where the gradient was 100% A to 100% B over 60 min. The eluent was monitored at 214 nm as channel A and 350 nm as channel B and the UV–Vis spectrum of each peak was obtained from 200 to 600 nm. Electrospray ionization mass spectrometry was performed using a Finnegan LCQ^{deca} (San Jose, CA) in the positive mode using nitrogen as sheath (90 bars) and auxiliary gas (20 bars). The heated capillary temperature was 250 °C, the electrospray voltage was 5.2 kV, and the capillary voltage was set to −4 V. Three scan events were used: (i) 200–1000 *m/z* full scan MS, (ii) data-dependent zoom scan on the most intense ion from the MS full spectrum, and (iii) data-dependent full scan MS/MS on the most intense ion from the MS full spectrum. The MS/MS collision energy was set at 35 V.

4.3. Synthetic compounds

4.3.1. *N*,*N*-Dimethyl-3-fluorobenzylamine (3a).[23] A sealed tube charged with a methanolic solution of dimethylamine (2 M, 3 mL, 6 mmol) and 3-fluorobenzylbromide (378 mg, 2 mmol) was heated at reflux for 24 h. The reaction mixture was cooled and methanol was evaporated. The residue was dissolved in water (8 mL) and adjusted to pH 12 with 1 N NaOH, and the solution was extracted with ether (3×20 mL). The organic layer was combined and dried over anhydrous Na_2SO_4. The ether was evaporated to give **3a** as a colorless oil in a yield of 84.7% (260 mg): ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 6H), 3.41 (s, 2H), 6.94 (td, 1H, *J*=8.5, 2.3 Hz), 7.02–7.08 (2H), 7.26 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 45.4 (−), 63.9 (+), 113.9 (−, d, *J*=21 Hz), 115.8 (−, d, *J*=21 Hz), 124.5 (−, d, *J*=1.7 Hz), 129.7 (−, d, *J*=8.0 Hz), 141.7 (+, d, *J*=6.8 Hz), 163.0 (+, d, *J*=244 Hz).

4.3.2. *N*,*N*-Dimethyl-3-fluorobenzylamine-d_6 (3b). A sealed tube charged with dimethylamine-d_6 hydrochloride (175 mg, 2 mmol) in methanol (2 mL), diisopropylethylamine (0.70 mL, 4 mmol) and 3-fluorobenzylbromide (189 mg, 1 mmol) was heated to reflux for 96 h. The reaction mixture was cooled and the methanol was evaporated. The residue was dissolved in water (4 mL) and adjusted to pH 12 with 1 N NaOH. Then the aqueous solution was extracted with ether (3×10 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give **3b** as a colorless oil (128 mg, 80.5%): ^{1}H NMR ($CDCl_3$) δ 3.41 (s, 2H), 6.94 (td, 1H, *J*=8.5, 2.3 Hz), 7.02–7.08 (2H), 7.23 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 63.8 (−), 114.0 (+, d, *J*=21 Hz), 115.8 (+, d, *J*=21 Hz), 124.6 (+, d, *J*=1.7 Hz), 129.7 (+, d, *J*=8.0 Hz), 141.7 (−, d, *J*=6.8 Hz), 163.0 (−, d, *J*=243.8 Hz); EI HRMS *m/z* calcd for $C_9H_6D_6FN$ M^+ 159.1330, found 159.1330.

4.3.3. *N*,*N*-Dimethyl-2-nitro-5-fluorobenzylamine (4a). Compound **3a** (100 mg, 0.65 mmol) was added to a mixture of sulfuric acid (98%, 0.3 mL) and nitric acid (69%, 0.1 mL) at 50 (C. The mixture was stirred and kept at 100 °C for 2 h, then cooled and poured into ice water (20 mL). The aqueous solution was adjusted to pH 10 with 1 N NaOH and extracted with ethyl acetate (3×30 mL). The organic layer was combined and dried over anhydrous Na_2SO_4 followed by filtration. Ethyl acetate was evaporated in vacuo to give **4a** quantitatively as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 6H), 3.75 (s, 2H), 7.06 (m, 1H), 7.48 (dd, 1H, *J*=9.6, 2.7 Hz), 7.98 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 45.7 (−), 60.2 (+), 114.6 (−, d, *J*=23.3 Hz), 117.5 (−, d, *J*=24.5 Hz), 127.3 (−, d, *J*=9.7 Hz), 139.2 (+, d, *J*=8.4 Hz), 145.2 (+), 165.0 (+, d, *J*=253.5 Hz); EI HRMS *m/z* calcd for $C_9H_{11}FN_2O_2$ (M^+) 198.0805, found 198.0815.

4.3.4. *N*,*N*-Dimethyl-d_6-2-nitro-5-fluorobenzylamine (4b). According to the procedure for the synthesis of **4a**, compound **4b** (pale yellow oil) was prepared quantitatively from **3b**: ^{1}H NMR ($CDCl_3$) δ 3.72 (s, 2H), 7.03 (m, 1H), 7.45 (dd, 1H, *J*=9.6, 2.7 Hz), 7.94 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 45.0 (+, m), 60.0 (+), 114.6 (−, d, *J*=23.3 Hz), 117.5 (−, d, *J*=24.5 Hz), 127.4 (−, d, *J*=9.7 Hz), 139.2 (+, d, *J*=8.4 Hz), 145.2 (+), 164.9 (+, d, *J*=253.5 Hz); EI HRMS *m/z* calcd for $C_9H_5D_6FN_2O_2$ M^+ 204.1181, found 204.1180.

4.3.5. *N*,*N*-Dimethyl-2,4-dinitro-5-fluorobenzylamine (1a). Fuming nitric acid (1 mL) was added to a solution of **4a** (500 mg, 1.9 mmol) in fuming sulfuric acid (6 mL) and the mixture was heated to 100 °C for 2 h. The reaction mixture was cooled and poured into ice (100 g). The aqueous solution was neutralized to pH 7.0 with 1 N NaOH and extracted with ethyl acetate (5×100 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated in vacuo to give a mixture of *N*,*N*-dimethyl-2,4-dinitro-5-fluorobenzylamine **1a** and *N*,*N*-dimethyl-2,6-dinitro-5-fluorobenzylamine **5a** in a ratio of 6:1 based on the integration in the ^{1}H NMR spectrum of the crude product. The mixture was subjected to silica gel chromatography,

eluting with ethyl acetate–hexane (v/v, 4:5) to give **1a** and **5a** as yellow oils in yields of 72.3 and 11.3%, respectively. **1a**: ^{1}H NMR ($CDCl_3$, free base) δ 2.30 (s, 6H), 3.83 (s, 2H), 7.87 (d, 1H, J=11.0 Hz), 9.73 (d, 1H, J=6.7 Hz); ^{13}C NMR ($CDCl_3$) δ 45.7 (−), 60.0 (+), 120.8 (+, d, J=19.3 Hz), 123.7 (−, d, J=1.2 Hz), 144.0 (+), 145.6 (+, d, J=7.6 Hz), 154.5 (+, d, J=19.6 Hz), 157.5 (+, d, J=216.8 Hz); EI HRMS m/z calcd for $C_9H_{10}FN_3O_4$ M^+ 243.0655, found 243.0649. The HCl salt of **1a** was prepared by passing HCl gas through a solution of **1a** in ether: mp 152–154 °C; ^{1}H NMR (methanol-d_4) δ 3.09 (s, 6H), 4.80 (s, 2H), 8.13 (d, 1H, J=10.6 Hz), 9.08 (d, 1H, J=6.9 Hz); ^{13}C NMR (methanol-d_4) δ 44.6 (−), 58.9 (+), 126.2 (−), 126.7 (−, d, J=24.4 Hz), 134.4 (+, d, J=10.1 Hz), 139.3 (+, d, J=9.2 Hz), 146.0 (+, d, J=5.8 Hz), 158.7 (+, d, J=221.0 Hz). **5a**: ^{1}H NMR ($CDCl_3$) 2.16 (s, 6H), 3.71 (s, 2H), 7.34 (t, J=8.2, 9.0 Hz) 7.90 (dd, J=9.0, 4.6 Hz); ^{13}C NMR ($CDCl_3$) δ 45.2 (−), 54.9 (+), 116.7 (d, J=21.0 Hz), 127.4 (−, d, J=9.0 Hz), 131.1 (+), 140.5 (+, d, J=8.3 Hz), 146.6 (+, d, J=2.7 Hz), 155.0 (+, d, J=263.4 Hz); EI HRMS m/z calcd for $C_9H_{10}FN_3O_4$ M^+ 243.0655, found 243.0649. According to the procedure for synthesis of **1a**, the 1:1 mixture of **1a** and *N*,*N*-dimethyl-d_6-2,4-dinitro-5-fluorobenzylamine (**1b**) was prepared using a 1:1 mixture of **4a** and **4b** in a yield of 70.8%.

4.3.6. N^{α}-(2,4-Dinitro-5-(dimethylaminomethyl)-phenyl)glycyl-L-leucine (7b). A solution of **1a** (100 mg) in DMF (2 mL) was added to a suspension of glycyl-L-leucine (50 mg, 0.266 mmol) and sodium bicarbonate (100 mg) in water (1 mL) and the mixture was stirred at room temperature for 1 h. The solution was diluted with water (10 mL) and extracted with ethyl acetate (2×10 mL). Water was evaporated to give a crude product, which was subjected to preparative TLC eluted with methanol–ethyl acetate (1:1, v/v) to give **7b** as a yellow powder in a yield of 95.4%: mp >215 °C (dec); ^{1}H NMR (methanol-d_4) δ 0.91 (d, 6H, J=5.9 Hz), 1.60–1.71 (3H), 2.30 (s, 6H), 3.85 (s, 2H), 4.24 (s, 2H), 4.39 (m, 1H) 7.11(s, 1H), 8.95 (s, 1H); ^{13}C NMR (methanol-d_4) δ 22.2 (+), 23.8 (+), 26.3 (+), 43.3 (+), 46.0 (−), 46.9 (+), 55.0 (−), 62.0 (+), 117.6 (+), 126.6 (+), 131.1 (−), 138.1 (−), 143.7 (−), 148.0 (−), 169.5 (−), 179.8 (−); FAB HRMS m/z calcd for $C_{17}H_{26}N_5O_7$ $(M+H)^+$ 412.1832 found 412.1840.

4.3.7. N^{α}-(2,4-Dinitro-5-(dimethylaminomethyl)-phenyl)-L-asparagine (7a). According to the procedure for the synthesis of **7b**, **7a** was obtained from L-asparagine as a yellow powder in a yield of 85%: mp >200 °C (dec); ^{1}H NMR (methanol-d_4) δ 2.25 (s, 1H), 2.78 (dd, 1H, J=13.9, 6.8 Hz), 2.87 (dd, 1H, J=13.9, 4.6 Hz), 3.81 (s, 2H), 4.53 (t, 1H), 7.17 (s, 1H), 8.94 (s, 1H); ^{13}C NMR (methanol-d_4) δ 39.5 (−), 46.0 (−), 57.1 (−), 60.2 (+), 116.2 (−), 125.3 (−), 131.1 (+), 137.3 (+), 143.3 (+), 148.1 (+), 173.2 (+), 175.8 (+); FAB HRMS m/z calcd for $C_{13}H_{18}N_5O_7$ $(M+H)^+$ 356.1206, found 356.1212.

4.3.8. N^{α}-(2,4-Dinitrophenyl)glycyl-L-leucine (7d). A solution of DNFB (19 μL, 0.15 mmol) in ethanol (0.8 mL) was added to a suspension of glycyl-L-leucine (18.8 mg, 0.1 mmol) and sodium bicarbonate (33.6 mg) in water (0.4 mL) and the mixture was stirred at room temperature for 2 h. Ethanol was evaporated and the aqueous residue was diluted to 4 mL with water and extracted with ether (3×2 mL). The aqueous layer was acidified to pH 2 with 2 N HCl and extracted with ethyl acetate (3×2 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to give **7d** as a yellow powder in a yield of 91.8%: mp >240 °C (dec); ^{1}H NMR (methanol-d_4) δ 0.92 (d, 6H, J=5.9 Hz), 1.53–1.69 (3H), 4.22 (s, 2H), 4.37 (td, 1H, J=3.3, 10.0 Hz), 7.02 (d, 1H, J=9.6 Hz), 8.28 (dd, 1H, J=2.7, 9.6 Hz), 9.04 (d, 1H, J=2.6 Hz); ^{13}C NMR (methanol-d_4) δ 22.0 (−), 23.8 (−), 26.3 (−), 43.1 (+), 46.9 (+), 55.0 (−), 116.3 (−), 124.5 (−), 131.0 (−), 132.2 (+), 137.5 (+), 149.3 (+), 164.3 (+), 169.4 (+); FAB HRMS m/z calcd for $C_{14}H_{19}N_4O_7$ $(M+H)^+$ 355.1254 found 355.1263.

4.3.9. N^{α}-Acetyl-*S*-(2,4-dinitro-5-(dimethylaminomethyl)phenyl)-L-cysteine (11). According to the procedure for synthesis of **7b**, **11** was obtained from N^{α}-acetyl-L-cysteine as a yellow powder in 100% yield: ^{1}H NMR (methanol-d_4) δ 2.00 (s, 3H), 2.97 (s, 6H), 3.50 (dd, 1H, J=14.1, 6.6 Hz), 3.65 (dd, 1H, J=14.2, 6.3 Hz), 4.52 (t, 1H, J=6.5 Hz), 4.63 (s, 2H), 8.29 (s, 1H), 9.00 (s, 1H); ^{13}C NMR (methanol-d_4) δ 22.7 (−), 35.4 (+), 44.7 (−), 53.4 (−), 59.3 (+), 125.0 (−), 131.9 (+), 134.2 (−), 145.9 (+), 146.7 (+), 147.1 (+), 173.3 (+), 175.0 (+); FAB HRMS m/z calcd for $C_{14}H_{19}N_4O_7S$ $(M+H)^+$ 387.0974, found 387.0975.

4.3.10. N^{α}-Acetyl-*O*-(2,4-dinitro-5-(dimethylaminomethyl)phenyl)-L-tyrosine (13). According to the procedure for the synthesis of **7b**, **13** was obtained from N^{α}-acetyl-L-tyrosine as a yellow powder in 89.3% yield: ^{1}H NMR (HCl salt in methanol-d_4) δ 1.92 (s, 3H), 2.70 (s, 6H), 2.96 (dd, 1H, J=14.1, 5.7 Hz), 3.21 (dd, 1H, J=13.8, 5.0 Hz), 4.35 (s, 2H), 4.58 (m, 1H), 7.14 (d, 2H, J=8.4 Hz), 7.38 (d, 2H, J=8.4 Hz), 7.89 (s, 1H), 8.87 (s, 1H); ^{13}C NMR (HCl salt in methanol-d_4) δ 22.7 (−), 38.5 (+), 44.7 (−), 55.4 (−), 69.7 (+), 121.4 (−), 124.2 (−), 125.5 (−), 132.8 (−), 135.9 (+), 137.7 (+), 141.0 (+), 143.6 (+), 154.2 (+), 156.2 (+), 172.9 (+), 175.3 (+); FAB HRMS m/z calcd for $C_{20}H_{23}N_4O_8$ $(M+H)^+$ 447.1516, found 447.1513.

4.3.11. *N*,*N*-Dimethyl-2,4-dinitro-5-((2-hydroxyethyl)thio)benzylamine (14). According to the procedure for the synthesis of **7b**, **14** was obtained from mercaptoethanol as a yellow resin in 100% yield: ^{1}H NMR ($CDCl_3$) δ 2.31 (s, 6H), 2.51 (b, 1H), 3.31 (t, 2H, J=6.18 Hz), 3.87 (s, 2H), 3.99 (t, 2H, J=6.24 Hz), 7.97 (s, 1H), 8.85 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 35.11 (+), 45.78 (−), 59.93 (+), 60.16 (+), 123.26 (−), 128.16 (−), 140.25 (+), 143.72 (+), 144.35 (+); EI HRMS m/z calcd for $C_{11}H_{15}N_3O_5S$ M^+ 301.0732, found 301.0726.

4.3.12. 2-Amino-6-(7-(5-amino-5-carboxypentylaminocarbonyl)heptanoylamino)hexanoic acid (17). Disuccinimidyl suberate (DSS) (50 mg, 0.135 mmol) and N^{α}-Cbz-lysine (151.2 mg, 0.54 mmol) were suspended in DMF (5 mL) and the mixture was stirred at room temperature for 1 h. The reaction mixture was subjected to flash chromatography eluted first with acetone, then with a mixture of acetone and acetic acid (v/v 95:5). The solvent was evaporated and the residue was dissolved in water (20 mL). The aqueous solution was extracted with ethyl acetate (3×10 mL). The combined organic layer was dried

over anhydrous Na_2SO_4 and evaporated to give 2-(benzyloxycarbonylamino)-6-(7-(5-(benzyloxycarbonylamino)-5-carboxypentyl-aminocarbonyl)heptanoylamino)hexanoic acid (**15**) as a white powder in a yield of 73.7% (70 mg): ^{1}H NMR (methanol-d_4) δ 0.91–1.60 (18H),1.88 (m, 2H), 2.15 (t, 4H, J=7.1 Hz), 3.14 (t, 4H, J=6.7 Hz), 4.13–4.20 (m, 2H), 5.05 (s, 4H), 7.30–7.40 (10H); ^{13}C NMR (methanol-d_4) δ 24.4 (+), 26.8 (+), 27.0 (+), 30.0 (+), 32.5 (+), 37.2 (+), 40.2 (+), 55.4 (−), 67.7 (+), 128.9 (−), 129.1 (−), 129.6 (−), 138.3 (+), 158.7 (+), 172.1 (+), 176.3 (+). A solution of **15** (50 mg) in 4.4% methanolic formic acid (2.5 mL) was added to a suspension of Pd–C (10%, 50 mg) in 2.5 mL of 4.4% methanolic formic acid. The mixture was stirred at room temperature for 3 h, then filtered, and the catalyst was washed with methanol (3×5 mL) followed by 1 N HCl (3×5 mL). The filtrate was evaporated to give **17** as a white powder in a yield of 92.9%: ^{1}H NMR (D_2O) δ 1.17–1.27 (8H), 1.37–1.49 (12H), 2.14 (t, 4H, J=7.0 Hz), 3.07–3.16 (6H); ^{13}C NMR (D_2O) δ 22.5 (−), 26.1 (−), 28.6 (−), 28.7 (−), 30.2 (−), 36.6 (−), 39.6 (−), 53.7 (+), 172.9 (−), 178.0 (−); FAB HRMS m/z calcd for $C_{20}H_{38}N_4O_6Na$ 453.2689 $(M+Na)^+$, found 453.2655.

4.3.13. 2-Amino-6-(7-carboxyheptanoylamino)hexanoic acid (18). Disuccinimidyl suberate (DSS) (100 mg, 0.27 mmol) and N^{α}-Cbz-lysine (37.5 mg, 0.135 mmol) was suspended in DMF (5 mL) and the mixture was stirred at room temperature for 1 h. The DMF was removed under high vacuum and the residue was subjected to flash chromatography eluted first with acetone, then with acetone–acetic acid (95:5, v:v). The solvent was removed and the residue was dissolved in a mixture of tetrahydrofuran (THF, 5 mL) and 1 M aqueous LiOH solution (1.7 mL) was added. The reaction mixture was stirred at room temperature for 2 h and then adjusted to pH 3.0 with 2 N HCl. THF was evaporated, the residue was dissolved in water (20 mL), and the aqueous solution was extracted with ethyl acetate (3×10 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, and evaporated to give 2-(benzyloxycarbonylamino)-6-(7-carboxyheptanoylamino)-hexanoic acid (**16**) as a white powder in a yield of 95.6% (56.3 mg): ^{1}H NMR (methanol-d_4) δ 1.32–1.36 (4H), 1.47 (m. 2H), 1.58–1.61 (6H), 2.00 (m, 2H), 2.27 (t, 2H, J=7.4 Hz), 2.41 (t, 2H, J=7.2 Hz), 3.23 (t, 2H, J=6.5 Hz), 3.83–3.90 (m, 1H), 5.05 (s, 2H), 7.32 (s, 5H); ^{13}C NMR (methanol-d_4) δ 24.3 (+), 26.3 (+), 27.0 (+), 30.0 (+), 30.1 (+), 33.5 (+), 35.7 (+), 37.1 (+), 40.3 (+), 57.0 (−), 67.5 (+), 128.8 (−), 129.0 (−), 129.5 (−), 138.4 (+), 158.5 (+), 176.2 (+), 176.2 (+), 176.3 (+). A solution of compound **16** (100 mg) in 4.4% methanolic formic acid (5 mL) was added to a suspension of Pd–C (10%, 50 mg) in 5 mL of 4.4% methanolic formic acid. The mixture was stirred at room temperature for 3 h, then filtered, and the catalyst was washed with methanol (3×5 mL). The solvent was evaporated to give **18** as a white powder in a yield of 94.8%: ^{1}H NMR (D_2O) δ 1.34 (4H), 1.47 (m, 2H), 1.58–1.61 (6H), 2.00 (m, 2H), 2.27 (t, 2H, J=7.4 Hz), 2.41 (t, 2H, J=7.2 Hz), 3.23 (t, 2H, J=6.5 Hz), 4.13 (t, 1H, J=7.2 Hz); ^{13}C NMR (D_2O) δ 21.7 (+), 24.2 (+), 25.3 (+), 27.8 (+), 27.8 (+), 27.9 (+), 29.5 (+), 33.9 (+), 35.8 (+), 38.9 (+), 53.0 (−), 177.1 (+), 177.2 (+), 179.3 (+); FAB HRMS m/z calcd for $C_{14}H_{27}N_2O_5$ $(M+H)^+$ 303.1920, found 303.1899.

4.3.14. 6-(3-Carboxylpropionylamino)-2-(5-(dimethylaminomethyl)-2,4-dinitrophenyl-amino)hexanoic acid (25). A solution of succinyl chloride (775 mg, 5 mmol) in acetone (0.5 mL) was added dropwise to a solution of N^{α}-Cbz-lysine (2.1 g, 7.5 mmol) in 5 mL of 1.5 N aqueous NaOH, with maintenance of the pH in the range of 10–11 by periodic addition of 1.5 N NaOH. The mixture was stirred at room temperature for 30 min, acidified with 2 N HCl to pH 2, and extracted with ethyl acetate (50+15+15 mL). The organic layer was combined and dried, and the solvent was removed in vacuo to give 2.4 g of a residue. The ^{1}H NMR spectrum showed the presence of two compounds **21** and **22** in a ratio of 2:1. A solution of the crude mixture (100 mg) in 4.4% methanolic formic acid (5 mL) was added to a suspension of Pd–C (10%, 50 mg) in 5 mL of 4.4% methanolic formic acid. The mixture was stirred at room temperature for 3 h, then filtered, and the catalyst was washed with methanol (3×5 mL). The solvent was evaporated to give 2-amino-6-(3-carboxypropionyl-amino)hexanoic acid (**23**) as a white powder: ^{1}H NMR (D_2O) δ 1.31–1.55 (4H), 1.83 (m, 2H), 2.48–2.59 (4H), 3.16 (t, 2H, J=5.9 Hz), 3.70 (t, 1H, J=6.2 Hz); ^{13}C NMR (D_2O) 21.7 (+), 28.0 (+), 29.9 (+), 30.1 (+), 30.8 (+), 38.9 (+), 54.7 (−), 174.6 (+), 175.0 (+), 177.9 (+). A solution of DMDNFB (24.3 mg, 0.1 mmol) in DMF (0.25 mL) was added to a suspension of **23** (14.7 mg, 0.05 mmol) and $NaHCO_3$ (100 mg) in water (130 μL). The mixture was stirred at room temperature for 2 h and extracted with ethyl acetate (3×5 mL). The aqueous layer was acidified with 2 N HCl (1 mL) and then evaporated to dryness. The residue was washed with water (0.3 mL) to give **25** as a yellow powder: ^{1}H NMR (methanol-d_4) δ 1.43–1.56 (4H), 1.94 (m, 2H), 2.28 (s, 6H), 2.40 (s, 4H), 3.14 (t, 2H, J=6.6 Hz), 3.81 (s, 2H), 4.18 (t, 1H, J=5.43 Hz), 7.14 (s, 1H), 8.95 (s, 1H); ^{13}C NMR (methanol-d_4) δ 24.0 (+), 30.4 (+), 33.4 (+), 34.1 (+), 35.0 (+), 40.3 (+), 46.0 (−), 59.6 (−), 62.1 (+), 117.7 (−), 127.0 (−), 130.5 (+), 137.3 (+), 143.8 (+), 146.1 (+), 147.6 (+), 175.9 (+), 177.5 (+); FAB HRMS m/z calcd for $C_{19}H_{28}N_5O_9$ $(M+H)^+$ 470.1887 found 470.1873.

4.3.15. 6-(3-(5-Carboxy-5-(5-(dimethylaminomethyl)-2,4-dinitrophenylamino)pentyl-carbamoyl)propionyl-amino)-2-(5-(dimethylaminomethyl)-2,4-dinitrophenyl-amino)hexanoic acid (26). Following methanol washing of the catalyst in the preparation of **23**, washing with water and evaporation afforded 2-amino-6-(3-(5-amino-5-carboxy-pentylaminocarbonyl)propionylamino)-hexanoic acid (**24**) as a white powder: ^{1}H NMR (D_2O) δ 1.33–1.55 (8H), 1.81–1.86 (4H), 2.47 (s, 4H), 3.15 (t, 4H, J=7.0 Hz), 3.70 (t, 2H, J=5.9 Hz); ^{13}C NMR (D_2O) 21.7 (+), 28.0 (+), 30.1 (+), 31.4 (+), 38.9 (+), 54.7 (−), 174.6 (+). A solution of DMDNFB (38.9 mg, 0.16 mmol) in DMF (0.4 mL) was added to a suspension of **24** (17.8 mg, 0.04 mmol) and $NaHCO_3$ (100 mg) in water (200 μL). The mixture was stirred at room temperature for 2 h and extracted with ethyl acetate (3×5 mL). The aqueous layer was acidified with 2 N HCl (1 mL) and then water was evaporated. The residue was washed with water (0.3 mL) to give **26** as a yellow powder: ^{1}H NMR (methanol-d_4) δ 1.43–1.51 (8H), 1.97 (m, 4H), 2.28 (s, 12H), 2.39 (s, 4H), 3.14 (t, 4H, J=6.6 Hz), 3.80 (s, 4H), 4.18 (t, 2H, J=5.4 Hz), 7.13 (s, 2H), 8.91 (s, 2H); ^{13}C NMR (methanol-d_4) δ 23.8 (+), 30.2 (+), 32.7 (+), 33.3 (+), 40.2 (+), 46.0 (−), 59.5 (−), 62.1 (+), 117.7 (−),

127.0 (−), 130.4 (+), 137.2 (+), 143.8 (+), 147.6 (+), 174.6 (+), 177.4 (+); FAB HRMS calcd for $C_{34}H_{49}N_{10}O_{14}$ 821.3430, found 821.3519.

4.3.16. N^{ϵ}-*t*-Boc-N^{α}-(2,4-dinitro-5-(dimethylaminomethyl)phenyl)-L-lysine (27). According to the procedure for the synthesis of **7b**, **27** was obtained from N^{ϵ}-*t*-Boc-L-lysine as a yellow powder in a yield of 92.5%: mp >180 °C (dec); ^{1}H NMR (methanol-d_4) δ 1.38 (s, 9H), 1.43–1.47 (4H), 1.95 (m, 2H), 2.30 (s, 6H), 3.00 (t, 2H, J=4.9 Hz), 3.81 (s, 2H), 4.17(t, 1H, J=8.4 Hz), 7.14 (s, 1H), 8.91 (s, 1H); ^{13}C NMR (methanol-d_4) δ 23.8 (+), 28.8 (−), 30.9 (+), 33.4 (+), 41.2 (+), 46.1 (−), 59.5 (−), 62.1 (+), 79.8 (+), 117.7 (−), 127.0 (−), 130.4 (+), 137.1 (+), 143.7 (+), 147.5 (+), 158.5 (+), 177.5 (+). HRMS FAB *m/z* calcd for $C_{20}H_{32}N_5O_8$ $(M+H)^+$ 470.2251, found 470.2247. According to the procedure for synthesis of **7b**, the 1:1 mixture (**27a**) of **27** and N^{ϵ}-*t*-Boc-N^{α}-(2,4-dinitro-5-(dimethyl-d_6-aminomethyl)phenyl)-L-lysine was prepared using a 1:1 mixture of **1a** and **1b** and N^{ϵ}-*t*-Boc-L-lysine in a yield of 89.6%.

4.3.17. N^{α}-(2,4-Dinitro-5-(dimethylaminomethyl)phenyl)-L-lysine (28). Compound **27** (70.5 mg, 0.15 mmol) was dissolved in TFA (10 mL) and heated at reflux for 24 h. TFA was evaporated to give **28** quantitatively as a yellow powder. ^{1}H NMR (methanol-d_4) δ 1.48 (m, 2H), 1.59 (m, 2H), 1.99 (m, 2H), 2.31 (s, 6H), 2.91 (t, 2H, J=7.6 Hz), 3.83 (s, 2H), 4.21 (t, 1H, J=5.0 Hz), 7.13 (s, 1H), 8.90 (s, 1H); ^{13}C NMR (methanol-d_4) δ 23.2 (+), 28.5 (+), 32.8 (+), 40.6 (+), 45.5 (−), 59.1 (−), 61.5 (+), 119.1 (−), 127.3 (−), 130.9 (+), 136.8 (+), 140.6 (+), 147.4 (+), 176.9 (+); HRMS FAB *m/z* calcd for $C_{15}H_{24}N_5O_6$ $(M+H)^+$ 370.1727, found 370.1709. According to the procedure for the synthesis of **28**, the 1:1 mixture (**28a**) of **28** and N^{α}-(2,4-dinitro-5-(dimethylaminomethylene-d_6)-phenyl)-L-lysine was prepared from **27a** in a yield of 97.2%.

4.4. UV–Vis spectrometric monitoring of incubations of either DNFB or DMDNFB with either asparagine or glycyl-L-leucine

A solution of either DNFB or **1a** (DMDNFB) (2 mM) was prepared separately in acetone. A solution of either glycyl-L-leucine or asparagine (400 μM) was prepared separately in 0.1 M phosphate buffer, and the pH was adjusted to 7.0 by 1 N NaOH. Following autozeroing of a cuvette containing 2.94 mL of the latter at 25 °C, 0.06 mL of the acetone solution of either DNFB or DMDNFB was added, and the cuvette was shaken. The reaction was followed by monitoring the formation of the aminolysis product, scanning from 300 to 500 nm, at 20 min intervals for DNFB and 5 min intervals for DMDNFB. The infinity absorbance was determined from the solution of the authentic samples obtained through independent synthesis as described earlier.

4.5. ^{1}H NMR spectrometric monitoring of incubations of either DNFB or DMDNFB with either amino acids or peptides

A solution of either DMDNFB or DNFB (80 mM, 250 μL) in DMF-d_7 was added to a solution of either an amino acid, a peptide or a lysine derivative (80 mM of free amine groups, 250 μL) in either 0.1 M pH 7.0 phosphate buffer or 5% $NaHCO_3$ in D_2O in a NMR tube. The ^{1}H NMR spectrum was taken at the beginning, 10, 30 min, 1, 2, 3, and 24 h.

4.6. Reaction of either N^{α}-acetyl-L-histidine,-L-cysteine, or -L-tyrosine with DMDNFB and thiolysis of their products (mercaptoethanol)

A solution of DMDNFB (40 mM, 0.1 mL) in DMF was added to a solution of either N^{α}-acetyl-L-histidine, -L-cysteine, or -L-tyrosine (40 mM, 0.1 mL) in 0.1 M phosphate buffer at pH 7.0 and the reaction mixture was monitored with thin layer chromatography (TLC). After all the DMDNFB was consumed, mercaptoethanol (1 μL) was added to the reaction mixture, and the reaction was monitored by TLC to see whether the initial products (N^{τ}-DMDNP-N^{α}-acetyl-L-histidine, *S*-DMDNP-N^{α}-acetyl-L-cysteine, or *O*-DMDNP-N^{α}-acetyl-L-tyrosine) were converted to their corresponding precursors. Two of the three initial products were confirmed by comparison to the authentic samples **11** and **13** described above.

4.7. ESI-MS analysis of incubations of 17 or 18 with a 1:1 mixture of DMDNFB-d_0 and DMDNFB-d_6

A solution of DMDNFB (2.43 mg, 0.01 mmol) in DMF (25 μL) was added to a suspension of **17** (1.75 mg, 0.005 mmol) and $NaHCO_3$ (10 mg) in water (13 μL). A solution of DMDNFB (3.89 mg, 0.016 mmol) in DMF (40 μL) was added to a suspension of **18** (2.0 mg, 0.004 mmol) and $NaHCO_3$ (10 mg) in water (20 μL). The two mixtures was stirred at room temperature for 2 h and extracted with ethyl acetate (3×0.5 mL). The aqueous layers were acidified with 2 N HCl (0.1 mL) and then the mixtures were subjected to ESI-MS analysis.

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References and notes

1. Hancock, W. S.; Harding, D. R. K. *CRC Handb HPLC Sep. Amino Acids, Pept., Proteins* **1984**, *1*, 235–262.
2. Lingeman, H.; Underberg, W. J. M. *Detection oriented derivatization techniques in liquid chromatography. Chromatography science series*; Marcel Dekker: New York, 1990; Vol. 48.
3. Riley, C. M.; Stobaugh, J. F.; Lunte, S. M. *Progr. Pharmaceut. Biomed. Anal.* **1994**, *1*, 41–89.
4. Fekkes, D. *J. Chromatogr. B Biomed. Appl.* **1996**, *682*, 3–22.
5. Lindroth, P.; Mopper, K. *Anal. Chem.* **1979**, *51*, 1667–1674.
6. Hsu, K. T.; Currie, B. L. *J. Chromatogr.* **1978**, *166*, 555–561.

7. Sanger, F. *Biochem. J.* **1945**, *39*, 507–515.
8. De Bruin, S. H.; Bucci, E. *J. Biol. Chem.* **1971**, *246*, 5228–5233.
9. Heinrikson, R. L.; Meredith, S. C. *Anal. Biochem.* **1984**, *136*, 65–74.
10. Iskandaranl, Z.; Pietrzyk, D. *J. Anal. Chem.* **1981**, *53*, 489–495.
11. Gutsche, B.; Diem, S.; Herderich, M. *Adv. Exp. Med. Biol.* **1999**, *467*, 757–767.
12. Barr, D. B.; Ashley, D. L. *J. Anal. Toxicol.* **1998**, *22*, 96–104.
13. Palma, M. S.; Itagaki, Y.; Fujita, T.; Hisada, M.; Naoki, H.; Nakajima, T. *Nat. Toxins* **1997**, *5*, 47–57.
14. Knebel, N. G.; Sharp, S. R.; Madigan, *J. Chromatogr. B Biomed. Appl.* **1995**, *673*, 213–222.
15. Johnson, D. W. *Rapid. Commun. Mass. Spectrom.* **2001**, *15*, 2198–2205.
16. Chen, X.; Chen, Y. H.; Anderson, V. E. *Anal. Biochem.* **1999**, *273*, 192–203.
17. Mahmood, S.; Iqbal, M. Z.; Qamar, N. *J. Chem. Soc. Pak.* **1992**, *14*, 80–82.
18. Boyer, J. H.; Pagoria, P. F.; Pillai, T. P.; Pace, M. D.; Stec, D. *J. Chem. Res. Synop.* **1986**, 416–417.
19. Adcock, W.; Dewar, M. J. S.; Gupta, B. D. *J. Amer. Chem. Soc.* **1973**, *95*, 7353–7358.
20. Shaltiel, S. *Biochem. Biophys. Res. Commun.* **1967**, *29*, 178–183.
21. ElAmin, B.; Anantharamaiah, G. M.; Royer, G. P.; Means, G. E. *J. Org. Chem.* **1979**, *44*, 3442–3444.
22. Bartlett, P. A.; Barstow, J. F. *J. Org. Chem.* **1982**, *47*, 3933–3941.
23. Li, S.; Bernstein, E. R.; Seeman, J. I. *J. Phys. Chem.* **1992**, *96*, 8808–8813.