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Expected and unexpected products from reacting Sanger's reagent with *p*-phenylenediamine

Michael John Plater and William TA Harrison

Abstract

p-Phenylenediamine reacts with Sanger's reagent in hot ethanol to give the expected mono- and di-substitution products, but in ethanol at room temperature, it gave exclusively 2-nitro-5-fluorophenyl-*p*-phenylenediamine, where a hydrogen atom is displaced by attack at an activated, unsubstituted position. The reactions of *p*-phenylenediamine and aniline with Sanger's reagent were compared in the cheap, 'green' solvent ethanol.

Keywords

2,4-Dinitrofluorobenzene, aniline, glycine, *p*-phenylenediamine, Sanger's reagent

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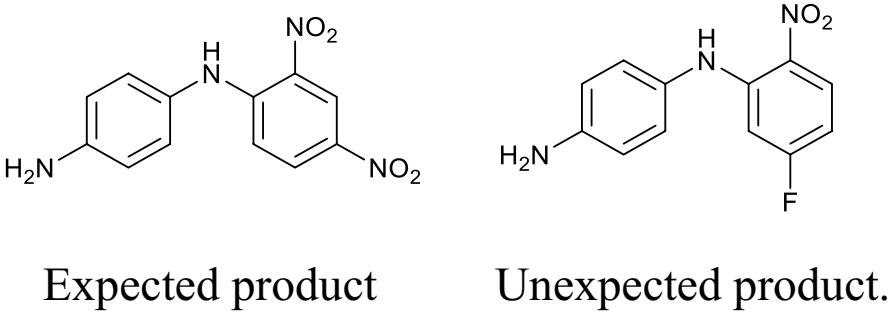
An unexpected product forms from reacting Sanger's reagent with *p*-phenylenediamine



Expected product Unexpected product.

Introduction

Sanger's reagent, 2,4-Dinitrofluorobenzene (DNFB) **1**, is used for the chromatographic detection and quantification of amino acids, peptides and proteins (Figure 1).[1] Its effectiveness is based on the reaction of the reagent with free alpha and epsilon amino groups to form stable, yellow dinitrophenyl derivatives.[1] It allows the terminal amino acid of a peptide chain to be determined.[1]

Selective cleavage of the labelled peptide chain allowed the amino sequence of insulin to be determined by examining the overlap of peptide sequences.[1] DNFB **1** was a better reagent than 2,4-dinitrochlorobenzene because it reacted at room temperature and did not need heating.[1] The other reagent studied in this paper, *p*-phenylenediamine **6**, is commonly used as a hair dye precursor to structure **7** by oxidation with H_2O_2/aqNH_3.[2–12] (Figure 2). 2,4-Dinitrofluorobenzene **1** and *p*-phenylenediamine **6** were interesting reagents to react together with a view to making isomers of trimer **7**, that might expand the range of hair dyes, and to explore the reactivity of DNFB **1**.

Discussion

p-Phenylenediamine **6** was reacted with DNFB **1** in a 1:2 ratio at 50 °C in EtOH for 24 h (Figure 3).

The main product **8**, from a bis adduct formation reaction, was difficult to purify by chromatography owing to its poor solubility and pigmentary properties. This accounts for the low yield. A quantity of the half-coupled product **9**

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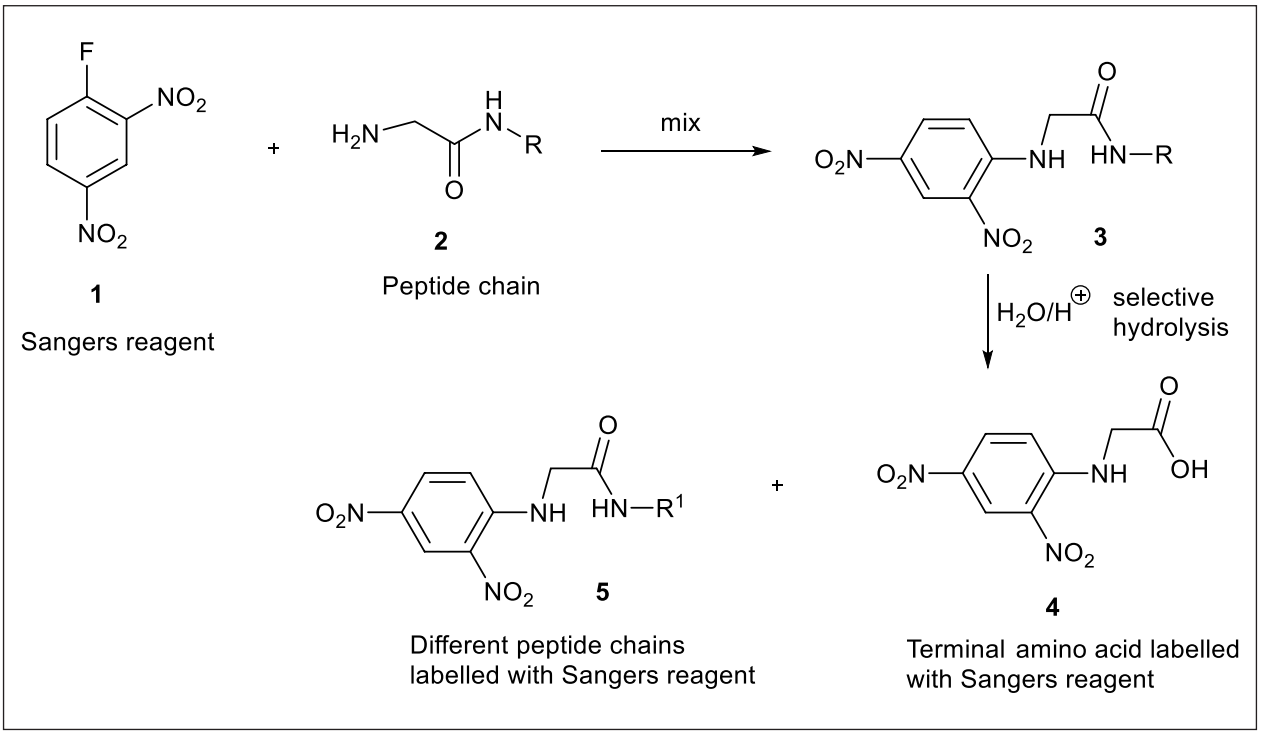

Figure 1. The formation of a terminal amino acid and peptide chains labelled with Sanger's reagent **1**.[1] Compound **5** forms when a peptide bond along the chain hydrolyses.

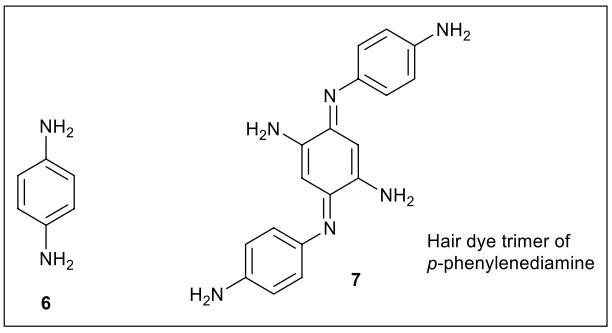

Figure 2. *p*-Phenylenediamine **6** and its hair dye trimer **7**.

was also formed. A front-running compound **10**[13] was also formed in variable but significant quantities and was characterised by its proton and carbon NMR spectra. This known compound forms here by the displacement of fluorine with ethanol in the hot solution. In hot isopropanol, the isopropyl analogue of compound **10** did not form, so the product was mainly compound **8** (89%) contaminated with a small amount of compound **9**. Isopropanol is more hindered than ethanol so the formation of a substitution product from it and DNFB **1** is suppressed.

Compound **8** crystallises in the monoclinic space group $P2_1/n$ with half a molecule in the asymmetric unit with the complete molecule generated by crystallographic inversion symmetry (Figure 4). The dihedral angle between the central and pendant aromatic rings is 39.94 (7)° and the N2/O1/O2 and N3/O3/O4 nitro groups are twisted from the latter ring by 4.84 (10) and 17.48 (7)°, respectively. An intramolecular N – H···O hydrogen bond occurs [H···O = 1.934 (19) Å, N – H···O = 138.0 (16)°] and the same group also participates in a much weaker intermolecular N – H···O interaction.

An experiment with *p*-phenylenediamine **6** and two equivalents of DNFB **1** at room temperature in EtOH in the presence of two equivalents of Et_3N was conducted to minimise or avoid the reaction of EtOH with DNFB **1**. An unexpected product **11** was formed in high yield (Figure 5). Compound **11** was not isolated previously from a column along with compounds **8-10** (Figure 4) but the column was complex in yellow colour and the failure to isolate compound **11** does not prove that none of it formed as a minor product.

Compound **11** crystallises in the monoclinic space group $P2_1/c$ with two molecules in the asymmetric unit (Figure 6). In the N1 molecule the dihedral angle between the aromatic rings is 54.29 (16)° and the equivalent angle in the N4 molecule is 51.22 (15)°. The nitro group is close to coplanar with its attached ring in both molecules [dihedral angles of 5.23 (7) and 8.46 (8) for the N1 and N4 molecules, respectively]. Each molecule features an intramolecular N – H···O hydrogen bond [H···O = 1.94 Å, N – H···O = 133° for the N1 molecule and H···O = 1.93 Å, N – H···O = 133° for the N4 molecule]. In the crystal, hydrogen-bonded (010) layers arise from N – H···O hydrogen bonds formed by the N2 and N5 terminal amine groups as well as weak N – H···F links arising from the N1 and N4 secondary amine groups.

Only a mono coupled product formed so the second primary amine is less reactive. A fluorine atom was clearly present from the NMR spectra as it couples with hydrogen and carbon. The primary amine of *p*-phenylenediamine **6** has reacted at an unsubstituted position of DFNB **1** and

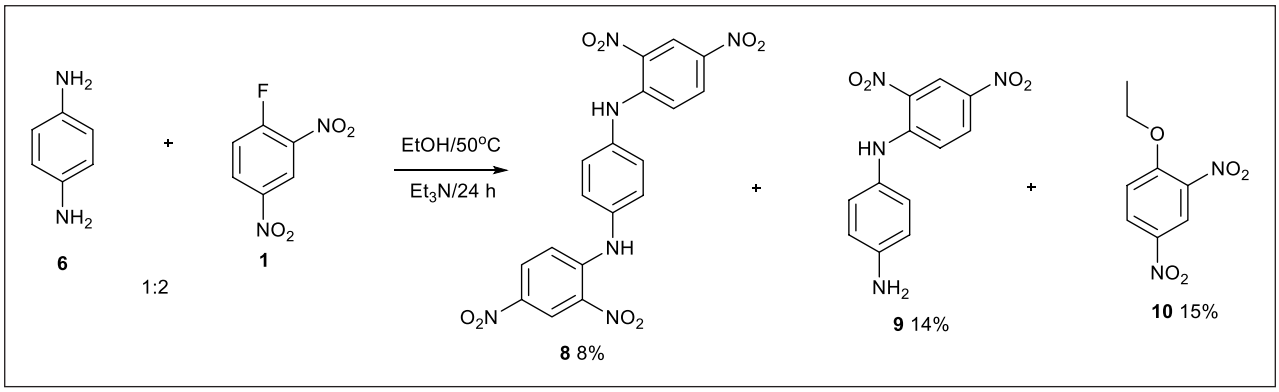

Figure 3. The products **8-10** from reacting *p*-phenylenediamine **6** with DNFB **1**.

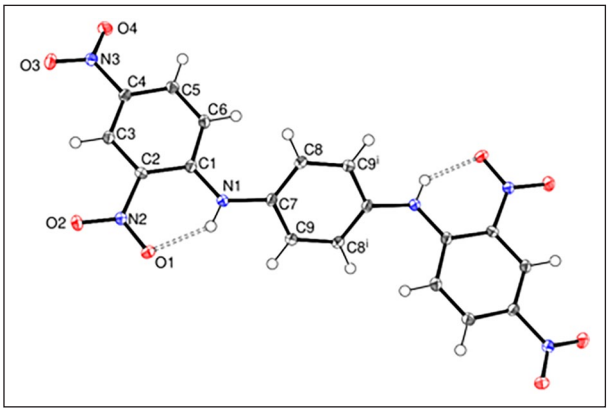

Figure 4. The molecular structure of compound **8** showing 50% displacement ellipsoids and hydrogen bonds as double dashed lines. Symmetry code: (i) 1–*x*, –*y*, 1–*z*.

eliminated a nitro group as nitrous acid with Et_3N. An addition–elimination mechanism is drawn in Figure 7.

This is an unusual way for the reaction proceed. Et_3N is present when the reaction was done at a low and high temperature (Figure 7) so is probably not changing the reaction pathway. *p*-Phenylenediamine behaves like a stronger nucleophile, owing to repulsion between the nitrogen lone pairs, a higher energy HOMO, and can drive the alternative reaction pathway. The *para* nitro group is eliminated rather than the *ortho* nitro group which is more sterically crowded. Both the fluorine substituted site and the unsubstituted site are activated by two nitro groups. The product is still bright yellow so it would be detectable if any of it formed with an amino acid or peptide chain.

One possible explanation for the experimental outcome might be the strength of the carbon–fluorine bond which is very strong. Related addition–elimination reactions are also known for 1,3-dinitrobenzene[14,15] under oxidative conditions and the nitration of furan in acetic anhydride[16,17] which is the least aromatic of the π-excessive heterocycles. A nitro group is not eliminated though. The displacement of hydrogen, rather than a halogen, has previously been called vicarious nucleophilic substitution where the leaving group is attached to the nucleophile.[18,19]

DNFB **1** was reacted with aniline **14** to compare with the products formed from *p*-phenylenediamine **6** (Figure 8). The expected and known substitution product **15** was formed[20] in which the fluorine atom has been displaced. No other products were formed in significant amounts. The extra equivalent of aniline binds to the HF rather than using Et_3N to do this.

The asymmetric unit of **15** (space group $P2_1/n$) consists of one molecule (Figure 9) in which the dihedral angle between the aromatic rings is 50.32 (5)°, in agreement with the crystal structure of a sample of 15 which has been reported (Cambridge Structural Database reference code TAMHEX).[21] It was prepared here under controlled, comparable conditions for a comparison with structure **11**.

The amino acids glycine **16** and (R)-phenylglycine **17** (Figure 10) were reacted with Sanger's reagent **1** under comparable conditions to see if any unexpected addition product formed (Figures 5 and 7).[22,23] Only the expected displacement product was formed with these two amino acids (thin-layer chromatography (TLC) 50% DCM:50% MeOH and 400 MHz NMR) (Figures 11 and 12).

Conclusion

p-Phenylenediamine **6** reacts with DNFB **1** in hot ethanol to give the expected products **8** and **9**, but in ethanol at room temperature, an unexpected product **11** forms. The expected products form by displacement of fluorine and the unexpected product forms by the displacement of hydrogen from an unsubstituted position. Both sites of attack are activated by two nitro groups. The fluorine is strongly electron withdrawing but the carbon–fluorine bond is very strong, which might influence the outcome. A mechanism is drawn which rationalises how the reaction can proceed. It is an addition–elimination mechanism in which the negatively charged complex must protonate first before the nitro group is eliminated. Either nitro group could eliminate depending upon where the intermediate anion protonates. The energy barrier leading to the more stable product is higher as it requires a higher temperature to form. The reactions of *p*-phenylenediamine **6** and

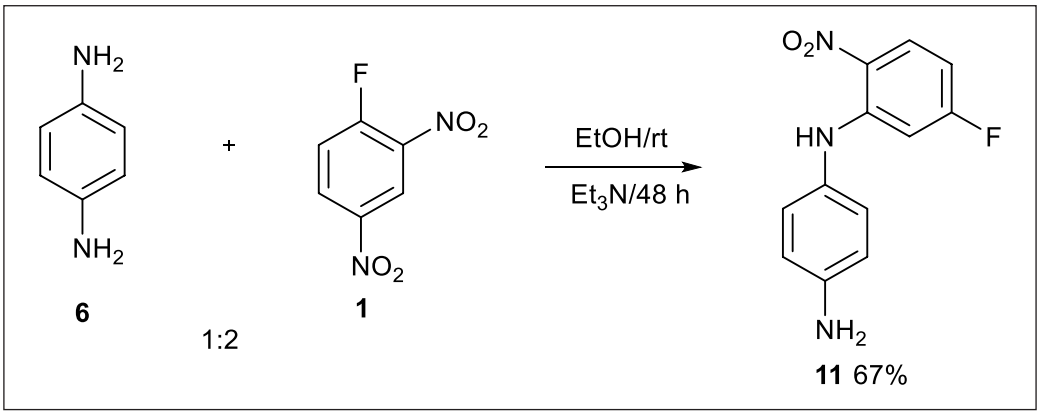

Figure 5. Formation of unexpected compound **11**.

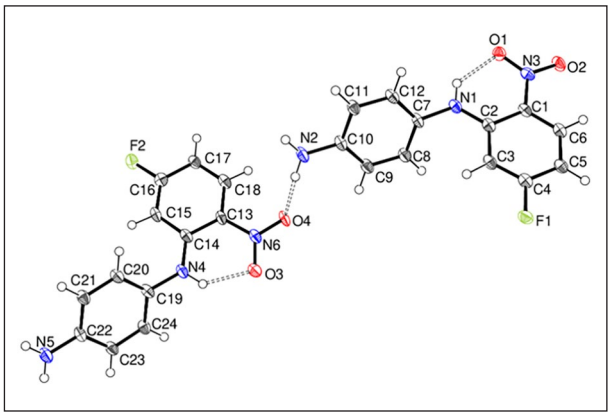

Figure 6. The molecular structure of **11** showing 50% displacement ellipsoids. Hydrogen bonds are indicated by double-dashed lines.

aniline **14** with DNFB **1** were compared and showed that aniline **14** gave the expected product. The product is still bright yellow so would act as a label if this reaction had occurred in the Sanger's method. However, preliminary studies have shown that amino acids react with DNFB **1** entirely as expected. Compound **11** is unknown by a Reaxy's search.

Experimental

Infrared spectra were recorded on a diamond-attenuated total reflection (ATR) Fourier transform (FTIR) spectrometer. Ultraviolet (UV) spectra were recorded using a Perkin Elmer Lambda 25 UV-Vis spectrometer with EtOH as the solvent. The term sh means shoulder. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 400 and 100.5 MHz, respectively, using a Varian 400 spectrometer. Chemical shifts, δ, are given in ppm and using a solvent peak as an internal reference. Coupling constants, *J*, are given in Hz. High-resolution mass spectra were obtained at the University of Wales, Swansea, using an Atmospheric Solids Analysis Probe (ASAP) (positive mode) instrument: Xevo G2-S ASAP. Melting points were determined on a Kofler hot-stage microscope.

2,4-Dinitroethoxybenzene 10, bis(2,4-dinitrophenyl)-p-phenylenediamine 8, 2,4-dinitrophenyl-p-phenylenediamine 9

p-Phenylenediamine (500 mg, 4.6 mmol) and Et_3N (0.94 g, 9.3 mmol) in EtOH (50 mL) were mixed with 2,4-dinitrofluorobenzene **1** (1.72 g, 9.2 mmol) and heated at 50 °C for 24 h.[24] After cooling the mixture was diluted with water (200 mL), extracted with DCM (100 mL), dried over $MgSO_4$ and concentrated in vacuo. TLC analysis with DCM showed that two yellow products were present and a spot running just ahead of the starting material 2,4-dinitrofluorobenzene **1** which was the first *title compound* **10** (146 mg, 15%) m.p. 86–87 °C δ_H (400 MHz; $CDCl_3$) 1.57 (3H, t, J = 8.0), 4.34 (2H, q, J = 8.0), 7.22 (1H, d, J = 8.0, B part of AB system), 8.44 (1H, d, J = 8.0, A part of AB system) and 8.74 (1H, s); δ_C (100.1 MHz; $CDCl_3$) 14.2, 66.8, 114.3, 121.6, 129.1, 138.9, 140.0 and 156.6; Elution with DCM gave the second *title compound* **8** (163 mg, 8%) as bright red crystals, m.p. > 225 °C (from dichloromethane:light petroleum ether). λ_{max} (EtOH)/nm 220 (log ε 3.5) and 340(3.2); ν_{max} (diamond)(cm^{-1}) 3282w, 3088w, 1613m, 1584m, 1501s, 1421m, 1318m, 1275m, 1216m, 937m, 858m, 823s, 739s, 631s and 495s; δ_H (400 MHz; D_7DMF) 7.45 (2H, d, J = 8.0, B part of AB system), 7.69 (4H, s), 8.35 (2H, d, J = 8.0, A part of AB system), 9.03 (2H, s) and 10.34 (2H, s, br); δ_C (100.1 MHz; $CDCl_3$) 117.4, 123.5, 126.9, 129.9, 131.9, 136.5, 137.2 and 146.9; *m/z* (Orbitrap ASAP) 441.0793 (M^+ + H, 100%) $C_{18}H_{12}N_6O_8H$ requires 441.0795. Elution with DCM gave the third *title compound* **9** (178 mg, 14 %) as crystals, m.p. 184–185 °C (from dichloromethane:light petroleum ether). λ_{max} (EtOH)/nm 237 (log ε 3.7) and 356 (3.5); ν_{max} (diamond)(cm^{-1}) 3423w, 3349w, 3266w, 1614s, 1577s, 1511s, 1486s, 1413s, 1329s, 1300s, 1250vs, 1220vs, 1142s, 1122s, 1059s, 917s, 837s, 746s, 699s, 653s, 515s and 495s; δ_H (400 MHz; $CDCl_3$) 3.88 (2H, s, br), 6.78 (2H, d, J = 8.0, B part of AB system), 7.04 (1H, d, J = 8.0, B part of AB system), 7.08 (2H, d, J = 8.0, A part of AB system), 8.15 (1H, d, J = 8.0, A part of AB system), 9.19 (1H, s) and 9.84 (1H, s, br); δ_C (100.1 MHz; $CDCl_3$) 116.0, 116.1, 124.2, 126.9, 127.4, 129.7, 130.5, 136.8, 146.3 and 148.3; *m/z* (Orbitrap ASAP) 275.0786 (M^+ + H, 100%) $C_{12}H_{10}N_4O_4H$ requires 275.078

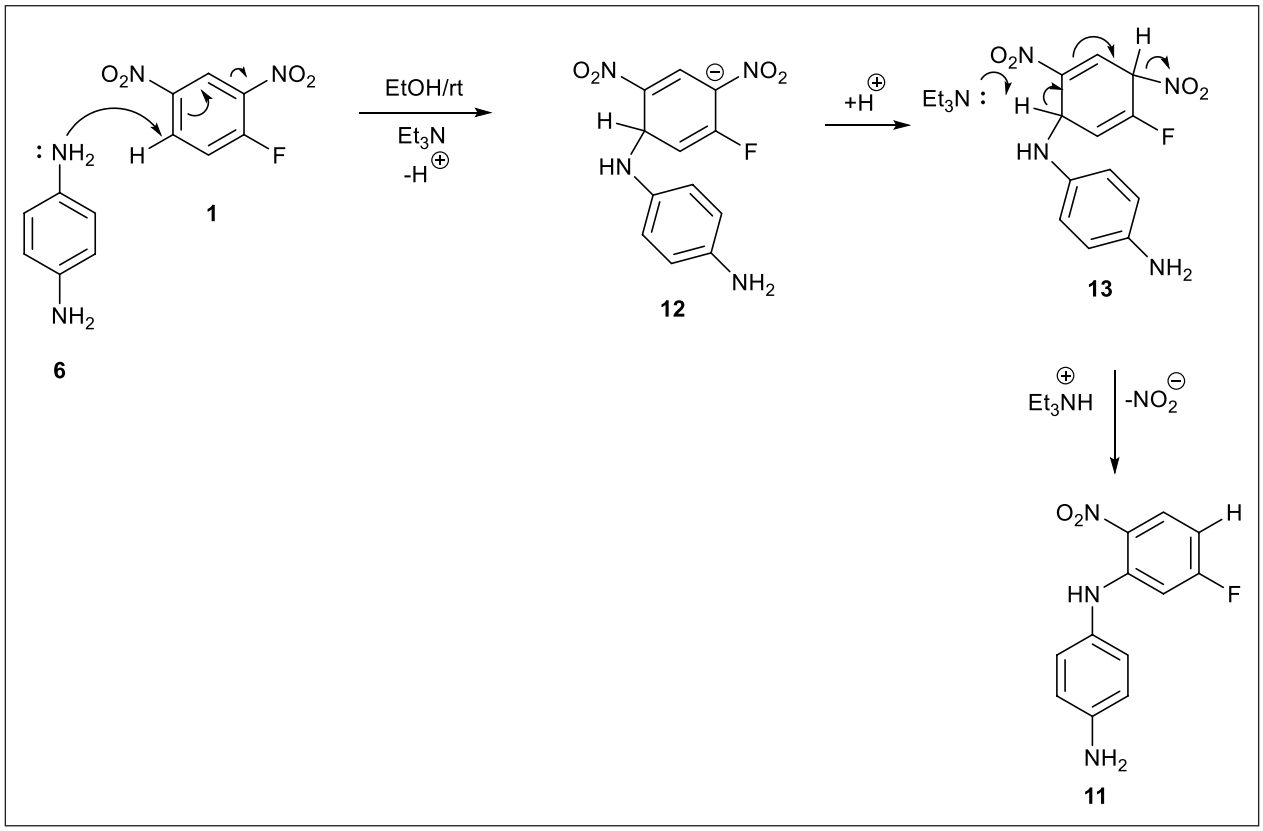

Figure 7. A mechanism for the formation of unexpected product **11**.

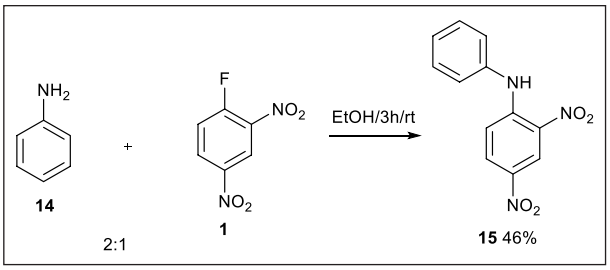

Figure 8. The reaction of aniline **14** with DNFB **1** to form **15**.

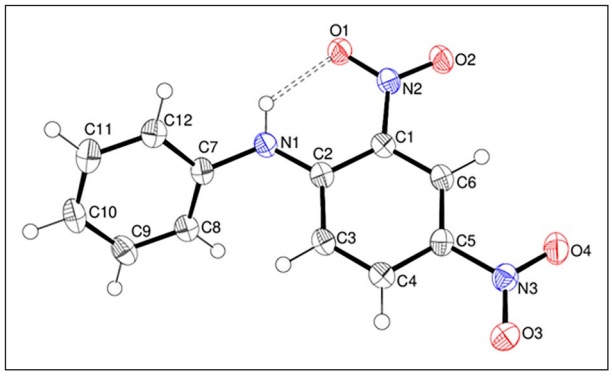

Figure 9. The molecular structure of compound **15** showing 50% displacement ellipsoids.

The reaction was repeated with *p*-phenylenediamine **6** (100 mg, 0.93 mmol) and Et_3N (187 mg, 1.85 mmol) in ^{i}PrOH (30 mL) with 2,4-dinitrofluorobenzene **1** (345 mg,

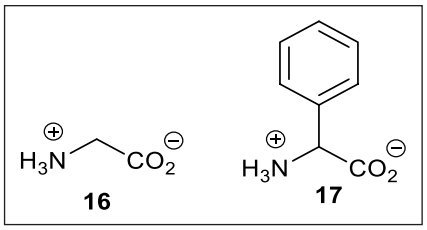

Figure 10. Glycine and (R)-phenylglycine.

1.85 mmol) and heated at 70 ºC for 20 h. After cooling the mixture was diluted with water (180 mL), left standing for 1 h, filtered with a sinter No 4 and washed with water (10 mL). This gave the second *title compound* **8** as a crude product which was air dried (363 mg, 89%). This product is contaminated with a small amount of 2,4-dinitro-*p*-phenylenediamine **9**, which is hard to remove because of the poor solubility of the product. This was identified by TLC and comparison to a standard.

2-Nitro-5-fluorophenyl-p-*phenylenediamine* *11*

p-Phenylenediamine **6** (200 mg, 1.85 mmol) and Et_3N (0.374 g, 3.7 mmol) in EtOH (50 mL) were mixed with 2,4-dinitrofluorobenzene **1** (0.689 g, 3.7 mmol) and stirred in EtOH (50 mL) at room temperature for 48 h. The mixture was diluted with water (200 mL) and filtered. The precipitate was dissolved in DCM (200 mL), dried over $MgSO_4$, filtered and evaporated to give a crude product (380 mg,

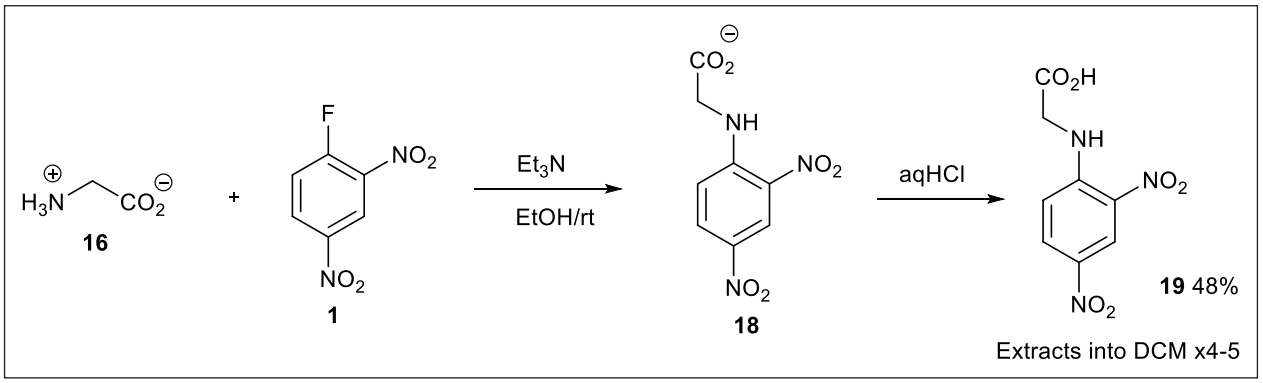

Figure 11. A protocol for reacting amino acids with Sanger's reagent **1** under controlled comparable conditions, used in this work, followed by chromatography.[1]

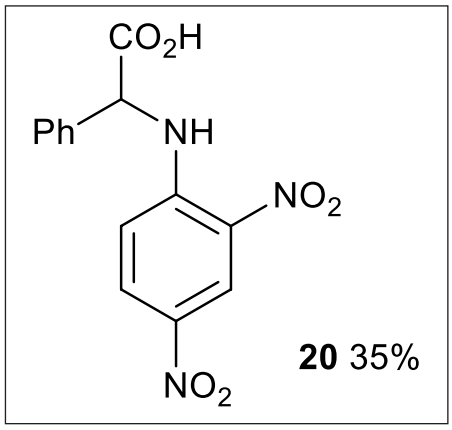

Figure 12. Compound formed from reacting Sanger's reagent with phenylglycine.

83%). This was purified by chromatography on silica. Ether eluted the *title compound* (308 mg, 67 %) as red/brown crystals, m.p. 167–168 °C (from dichloromethane:light petroleum ether). λ_{max} (EtOH)/nm 220 (log ε 4.0), 277(sh) (3.7) and 412 (3.4); ν_{max} (diamond)(cm^{-1}) 3434w, 3358w, 3321w, 1621s, 1576s, 1496s, 1456s, 1399s, 1250s, 1189s, 1126s, 1078s, 991s, 851s, 827s, 749s, 588s and 570s; δ_H (400 MHz; $CDCl_3$) 3.82 (2H, s), 6.58 (1H, dtd, J = 12.0 and 3.0), 6.60 (1H, dd, J = 12.0 and 3.0), 6.76 (2H, d, J = 8.0, B part of AB system), 7.06 (2H, d, J = 8.0, A part of AB system), 8.25 (1H, dd, J = 12.0 and 8.0) and 9.49 (1H, s, br); δ_C (100.1 MHz; $CDCl_3$) 101.2 (d, F-C-C, J = 20.0), 105.2 (F-C-C, J = 20.0), 116.0, 127.5, 128.3, 128.4, 129.7 (d, C-C-C-F, J = 10.0), 145.3, 147.0 and 167.1 (d, C-F, J = 27.0); *m/z* (Orbitrap ASAP) 248.0839 (M^+ + H, 100%) $C_{12}H_{10}N_3O_2F$+H requires 248.0835

2,4-Dinitrophenylaniline 15

2,4-Dinitrofluorobenzene **1** (200 mg, 1.1 mmol) in EtOH (15 mL) was mixed with aniline (200 mg, 2.2 mmol) for 3 h at room temperature.[25] The precipitate was filtered and air dried to give the *title compound* (130 mg, 46 %) as orange/red crystals, m.p. 158–159 °C (from dichloromethane:light petroleum ether). λ_{max} (EtOH)/nm 227 (log ε 4.0) and 351(4.1); ν_{max} (diamond)(cm^{-1}) 3315w, 1616s, 1595s, 1580s, 1516s, 1494s, 1419m, 1321s, 1251s, 1222s, 1144s, 1121s, 1058s, 921s, 741vs, 683s, 625s, 508s and 492s; δ_H (400 MHz; $CDCl_3$) 7.19 (1H, d, J = 8.0), 7.34 (2H, d, J = 8.0), 7.41 (1H, t, J = 8.0 and 8.0), 7.53 (2H, t, J = 8.0 and 8.0), 8.19 (1H, dd, J = 8.0 and 2.0), 9.19 (1H, d, J = 2.0) and 10.0 (1H, s, br); δ_C (100.1 MHz; $CDCl_3$) 116.1, 124.1, 125.6, 127.7, 129.9, 130.3, 131.1, 136.7, 137.5 and 147.1; *m/z* (Orbitrap ASAP) 260.0676 (M^+ + H, 100%) $C_{12}H_9N_3O_4H$ requires 260.0671

Amino acids with DNFB 1

General procedure. 2,4-Dinitrofluorobenzene **1** (200 mg, 1.0 mmol), an amino acid (1 mmol) and Et_3N (218 mg, 2.0 mmol) in EtOH (20 mL) were mixed and stirred at room temperature for 4 h.[1,22,23] The glycine is a suspension which slowly dissolves. The solution turns yellow. The mixture was diluted with water (180 mL), treated with dilute aq. HCl (approx. 10 mL, 5.0 M) and extracted repeatedly with dichloromethane (50 mL, × 5). The combined extracts were dried over $MgSO_4$, filtered or decanted and evaporate to dryness. The products were purified by chromatography on silica. Ether eluted unused DNFB **1** first. The amino acid adduct, **19** or **20**, was eluted with Et_2O/MeOH (50:50). They were identified and their purity confirmed by their carbon 13 spectrum. DNFB Glycine adduct **19** (115 mg, 48 %); δ_C (100.1 MHz; $CDCl_3$) 47.5, ($\underline{C}H_2$)116.7, 123.6, 129.5, 130.4, 134.8, 147.5 and 170.6 ($\underline{C}O_2H$) DNFB (R)-Phenylglycine adduct **20** (111 mg, 35%); δ_C (100.1 MHz; $CDCl_3$) 60.6 ($\underline{C}H_2$), 116.5, 123.8, 127.2, 128.2, 129.1, 130.3, 130.6, 135.8, 138.1, 146.6 and 171.5 ($\underline{C}O_2H$).

Crystal structure determinations

The crystal structure of compound **8** (red prism, 0.38 × 0.10 × 0.05 mm, recrystallised from dichloromethane:light petroleum ether), compound **11** (red plate, 0.18 × 0.13 × 0.05 mm, recrystallised from dichloromethane:light petroleum ether) and compound **15** (orange rod 0.32 × 0.05 × 0.03 mm, recrystallized from dichloromethane:light petroleum ether) were established using intensity data collected on a Rigaku CCD diffractometer (Cu Kα radiation, λ = 1.54178 Å) at 100 K. The structures were routinely solved by dual-space methods using SHELXT[26] and the structural models were

completed and optimised by refinement against $|F|^2$ with SHELXL-2018.[27] For compounds **8** and **15**, the N-bound hydrogen atoms were found in difference maps and their positions were freely refined. The N-bound H atoms in compound **11** (N – H = 0.88 Å) and the C-bound H atoms in all structures were placed geometrically (C – H = 0.95 Å) and refined as riding atoms. The constraint $U_{iso}(H) = 1.2U_{eq}$(carrier) was applied in all cases. Full details of the structures and refinements are available in the deposited cifs. The crystal of compound **12** was found to be non-merohedrally twinned and data quality is poor, resulting in rather high *R*-factors, but the structure has been unambiguously determined.

Crystal data for compound **8** ($C_{18}H_{12}N_6O_8$): M_r = 440.34, monoclinic, space group $P2_1/n$ (No. 14), a = 6.8428 (2) Å, b = 7.7513 (3) Å, c = 16.1753 (5) Å, β = 92.032 (3)°, V = 857.41 (5) Å^3, Z = 2, T = 100 K, μ = 1.189 mm^{-1}, ρ_{calc} = 1.706 g cm^{-3}, 8260 reflections measured (10.94 ⩽ 2θ ⩽ 148.7°), 1708 unique (R_{int} = 0.025), $R(F)$ = 0.033 [1616 reflections with $I > 2\sigma(I)$], $wR(F^2)$ = 0.087 (all data), $\Delta\rho_{min, max}$ (e Å^{-3}) = −0.25, +0.30, CCDC deposition number 2268861.

Crystal data for compound **11** ($C_{12}H_{10}FN_3O_2$): M_r = 247.23, monoclinic, space group $P2_1/c$ (No. 14), a = 21.8046 (9) Å, b = 12.6354 (6) Å, c = 7.9040 (4) Å, β = 90.469 (4)°, V = 2177.56 (18) Å^3, Z = 8, T = 100 K, μ = 0.996 mm^{-1}, ρ_{calc} = 1.508 g cm^{-3}, 4405 reflections measured (4.05 ⩽ 2θ ⩽ 135.4°), data not merged due to twinning [refined twin components 0.829 (5):0.171 (5)], $R(F)$ = 0.104 [4006 reflections with $I > 2\sigma(I)$], $wR(F^2)$ = 0.308 (all data), $\Delta\rho_{min, max}$ (e Å^{-3}) = −0.56, +1.09, CCDC deposition number 2268862.

Crystal data for compound **15** ($C_{12}H_9N_3O_4$): M_r = 259.22, monoclinic, space group $P2_1/n$ (No. 14), a = 3.77300 (10) Å, b = 11.0278 (4) Å, c = 26.8121 (11) Å, β = 90.420 (4)°, V = 1115.56 (7) Å^3, Z = 4, T = 100 K, μ = 1.011 mm^{-1}, ρ_{calc} = 1.543 g cm^{-3}, 38625 reflections measured (6.59 ⩽ 2θ ⩽ 150.3°), 2275 unique (R_{int} = 0.091), $R(F)$ = 0.057 [2002 reflections with $I > 2\sigma(I)$], $wR(F^2)$ = 0.175 (all data), $\Delta\rho_{min, max}$ (e Å^{-3}) = −0.29, +0.44, CCDC deposition number 2268863.

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Supplemental material

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