Dinitrophenyl peptides. II. Further studies on the preparation and properties of 2,4-dinitrophenyl glycyl peptides

J. H. Loudfoot and Y. M. Chan
Department of Chemistry, University of Manitoba, Winnipeg, Manitoba

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The study of 2,4-dinitrophenyl peptides has been extended to include the preparation and properties of some further dinitrophenyl dipeptides, namely, the glycyl-L-tryptophan, glycyl-L-proline, glycyl-L-phenylalanine, and glycyl-L-glutamic acid derivatives. Electrophoresis gave a good separation of products, and thin-layer chromatography proved to be a good means of purifying and separating the products. The molar absorbancy varies somewhat in the 350–360, 260–270, and 210–235 mμ ranges in aqueous sodium bicarbonate solution, is more consistent in 95% ethanol at 344 mμ, and shows some variation in the 255–265 mμ region in 95% ethanol; the infrared absorption spectra have been obtained. The characterization of two dinitrophenyl amino acyl chlorides by conversion into the amides, anilides, and *p*-toluidides is also reported.

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Since the previous report (1), the preparation of 2,4-dinitrophenyl (DNP) peptides has been extended to permit further study of their properties; in particular, the DNP derivatives of glycyl-L-proline, glycyl-L-tryptophan, glycyl-L-phenylalanine, and glycyl-L-glutamic acid have been prepared. All four derivatives could be obtained from DNP glycyl choride, but this method was not satisfactory for the preparation of DNP glycyl-L-glutamic acid.

When DNP glycyl chloride was used to prepare the DNP peptides, aqueous sodium bicarbonate was found to be satisfactory as the reaction medium, and no racemization occurred.

EXPERIMENTAL

All melting points are uncorrected.

DNP Glycine and DNP L-Alanine

These were prepared by the method of Levy and Chung (2), using the modification reported (1) for the preparation of DNP glycine, and converted into their chlorides as reported (1).

DNP L-Alanyl Chloride

The water-bath temperature during the reflux period had to be kept at 75° to avoid decomposition of the product. The product remaining after distillation was a pale-yellow liquid (the crude yield from 1.02 g of DNP L-alanine was 1.08 g or 99% of theory), which was characterized as its amide derivatives.

DNP Glycine and DNP L-Alanine Amides

The acid chloride (0.002 mole) was dissolved in 10 ml of benzene, and the solution was stirred for 15–20 min with 10 ml of ice-cold concentrated ammonia. The amide precipitated and was separated by filtration under suction. Purification was effected by recrystallization from dilute ethanol (in the case of the DNP L-alanyl derivative, a previous wash with ice-cold water was necessary).

The yield of DNP glycine amide was 0.435 g (90.5% of theory), m.p. 230–231°.

Anal. Calcd. for $C_8H_8N_4O_5$: C, 40.00; H, 3.33; N, 23.33. Found: C, 40.54; H, 3.28; N, 23.38.

The yield of DNP L-alanine amide was 0.45 g (90.2% of theory), m.p. 206–207°, $[\alpha]_D^{22}$ +103.2° (*c*, 0.6 in 95% ethanol) and +87.4° (*c*, 1.4 in acetone).

Anal. Calcd. for $C_9H_{10}N_4O_5$: C, 42.54; H, 3.94; N, 22.04. Found: C, 42.50; H, 4.22; N, 21.96.

DNP Glycyl and DNP L-Alanyl Anilides and p-Toluidides

A solution of aniline or *p*-toluidine (0.006 mole) in benzene (10 ml) was added to a solution of the acid chloride (0.002 mole) in benzene (10 ml), and the mixture was stirred for 30–40 min at room temperature. The precipitated product was separated by filtration under suction and purified by recrystallization from dilute ethanol.

DNP Glycyl Anilide

This compound was obtained in a 94.3% yield (0.595 g), m.p. 261–262°.

Anal. Calcd. for $C_{14}H_{12}N_4O_5$: C, 53.13; H, 3.80; N, 17.72. Found: C, 53.85; H, 3.69; N, 17.72.

DNP Glycyl p-Toluidide

A yield of 92.8% (0.613 g) was obtained, m.p. 263–264°.

Anal. Calcd. for $C_{15}H_{14}N_4O_5$: C, 54.6; H, 4.24; N, 16.95. Found: C, 54.59; H, 4.09; N, 16.75.

DNP L-Alanyl Anilide

The reaction time was 40 min and the derivative was stored in a vacuum desiccator overnight before recrystallization. The yield of pure product was 91.6% (0.600 g), m.p. 203–204°, $[\alpha]_D^{22}$ +103.3° (*c*, 0.4 in 95% ethanol) and +128.0° (*c*, 0.4 in acetone).

TABLE I

Ultraviolet absorption spectra of DNP peptides and of DNP amino acyl amide derivatives

Compound	0.2 *M* sodium bicarbonate solution						95% ethanol			
	Absorption maxima (mμ)			Molar absorbancy (ϵ_{max})			Absorption maxima (mμ)		Molar absorbancy (ϵ_{max})	
DNP Gly	359	264	232	17 010	9 780	9 320	344	256	17 700	10 100
DNP Gly-L-Try	353	267	221	14 650	11 450	20 000	344	256	17 400	9 240
DNP Gly-L-Pro	355	264	213	15 030	8 103	12 020	344	256	17 850	9 400
DNP Gly-L-Phe	359	264	232	16 450	10 270	11 430	344	264	17 580	13 240
DNP Gly-L-Glu	352	265	234	15 520	9 750	8 630	344	264	17 340	9 400
DNP Gly amide							344	256	17 050	7 635
DNP Gly anilide							344	240	19 150	20 000
DNP Gly *p*-toluidide							344	243	19 360	21 200
DNP L-Ala amide							344	256	18 250	8 670
DNP L-Ala anilide							344	240	19 150	20 000
DNP L-Ala *p*-toluidide							344	243	19 360	21 200

TABLE II

Further infrared absorption data for DNP peptides

Compound	Frequency regions (cm^{-1}) of maximum absorption
DNP Gly-L-Try	1 740, 1 575, 1 420, 1 300, 1 281, 1 254, 1 234, 1 194, 948, 916, 880, 816, and 733
DNP Gly-L-Pro	1 730, 1 665, 1 446, 1 295, 1 274, 1 234, 1 200, 1 183, 1 174, 1 091, 1 044, 988, 919, 868, 777, 766, 707, 688, 670, and 654
DNP Gly-L-Phe	1 725, 1 696, 1 665, 1 565, 1 550, 1 425, 1 368, 1 342, 1 282, 1 249, 1 220, 1 205, 1 192, 1 086, 1 070, 1 028, 1 014, 1 005, 988, 980, 962, 925, 918, 910, 900, 868, 818, 768, 707, 680, 673, and 665
DNP Gly-L-Glu	1 743, 1 715, 1 696, 1 683, 1 550, 1 425, 1 368, 1 340, 1 266, 1 238, 1 225, 1 096, 1 014, 952, 820, 767, and 707

TABLE III

Further infrared absorption data for DNP amino acyl amide derivatives

Compound	Frequency regions (cm^{-1}) of maximum absorption
DNP Gly amide	3 460, 3 180, 1 582, 1 534, 1 520, 1 428, 1 341, 1 326, 1 293, 1 275, 1 245, 1 231, 1 140, 1 112, 1 097, 942, 926, 892, 782, 766, 724, and 662
DNP L-Ala amide	3 460, 3 180, 1 680, 1 588, 1 426, 1 407, 1 341, 1 335, 1 307, 1 278, 1 242, 1 178, 1 120, 1 082, 1 050, 952, 938, 918, 815, 765, 744, 718, and 658
DNP Gly anilide	1 555, 1 538, 1 443, 1 325, 1 288, 1 262, 1 245, 1 226, 1 175, 1 136, 1 110, 1 080, 1 028, 952, 927, 908, 812, 770, and 722
DNP Gly *p*-toluidide	1 682, 1 550, 1 538, 1 502, 1 418, 1 341, 1 316, 1 295, 1 271, 1 258, 1 248, 1 228, 1 180, 1 136, 1 118, 1 038, 953, 926, 824, 813, 763, and 662
DNP L-Ala anilide	1 605, 1 554, 1 538, 1 505, 1 485, 1 440, 1 428, 1 338, 1 324, 1 305, 1 298, 1 265, 1 230, 1 202, 1 195, 1 178, 1 120, 1 088, 1 078, 962, 924, 918, 905, 900, 822, 759, and 717
DNP L-Ala *p*-toluidide	1 550, 1 538, 1 513, 1 505, 1 428, 1 402, 1 367, 1 335, 1 320, 1 303, 1 295, 1 275, 1 265, 1 236, 1 190, 1 118, 956, 941, 920, 913, 904, 822, 763, 714, and 658

Anal. Calcd. for $C_{15}H_{14}N_4O_5$: C, 54.57; H, 4.25; N, 16.96. Found: C, 54.12; H, 4.22; N, 16.78.

DNP L-*Alanyl p-Toluidide*

The reaction time and purification procedure were the same as those for the anilide, giving 0.640 g of product (92.4% yield), m.p. 213–214°, $[\alpha]_D^{22}$ +96.0° (*c*, 0.3 in 95% ethanol) and +103.2° (*c*, 2.0 in acetone).

Anal. Calcd. for $C_{16}H_{16}N_4O_5$: C, 55.84; H, 4.65; N, 16.27. Found: C, 55.81; H, 4.37; N, 15.91.

DNP Peptides

Method A, from the Dipeptide

The reaction between 2,4-dinitrofluorobenzene (0.0005–0.001 mole) and the peptide (0.0005–0.001 mole) in aqueous sodium bicarbonate (0.005–0.01 mole) solution was performed as in ref. 1, but the reaction time and method of isolation and purification differed considerably for each compound reported in this paper, and in certain instances the reaction flask had to be wrapped in order to prevent undesirable photochemical side reactions.

Method B, from DNP Glycyl Chloride

A benzene solution (15 ml) of DNP glycyl chloride (0.002 mole) was added, with stirring, to an aqueous sodium bicarbonate (0.02 mole) solution (25 ml) of the amino acid (0.002 mole) in an ice bath, followed by stirring at ice-bath temperature. The addition time and subsequent stirring period at ice-bath temperature varied markedly with the different compounds. In certain cases it was also necessary to cover the reaction flask to prevent photochemical side reactions. The subsequent isolation and purification differed for each product. The identity of the product obtained by each method and its non-identity with DNP glycine were established by mixture melting point determination.

*DNP Glycyl-*L*-tryptophan*

Method A

The reaction time was 2 h. The ethanol was distilled off under vacuum from a water bath under 40°, the residue dissolved in water, and the solution acidified with concentrated HCl to pH 2, thereby causing an orange crystalline solid to precipitate; the mixture was stored overnight in the refrigerator. The crystalline solid was separated by filtration under suction, washed with ice-cold water to remove excess hydrochloric acid, stored for 24 h in a vacuum desiccator, and then heated at 100° to constant weight. The yield of yellow product was 0.286 g (67.0% of theory), m.p. 233–234° (decomp.), $[\alpha]_D^{22}$ +20° (*c*, 0.5 in 95% ethanol) and +26° (*c*, 0.5 in acetone).

Method B

The acid chloride addition and subsequent ice-bath stirring each required a 2 h period. The reaction mixture was transferred to a separatory funnel, where it was allowed to stand for 2 h for complete separation of the benzene and aqueous layers. The lower aqueous layer was removed and acidified with 4 ml of concentrated hydrochloric acid, causing an orange-colored solid to precipitate; the mixture was left overnight in the refrigerator. The solid was separated by filtration under suction, washed with ice-cold water to remove excess hydrochloric acid, dried by storage overnight in a vacuum desiccator, and heated at 100° to constant temperature.

The crude orange product melted below 130°; it was recrystallized by dissolving it in hot ethanol, adding hot water to cause turbidity, and cooling the solution slowly. The mixture was left for several hours in the refrigerator, and the yellow crystals were separated by filtration under suction and dried to constant weight, yield 0.580 g (67.5% of theory), m.p. 233–234°; $[\alpha]_D^{22}$ +20° (*c*, 0.5 in 95% ethanol), +26.5° (*c*, 1.5 in acetone), and −60° (*c*, 0.2 in 4% aqueous sodium bicarbonate).

Anal. Calcd. for $C_{19}H_{17}N_5O_7$: C, 53.40; H, 3.98; N, 16.39; equiv. wt. 427. Found: C, 53.40; H, 3.89; N, 16.00; equiv. wt. 434.

*DNP Glycyl-*L*-proline*

Method A

The preparation was similar to that of glycyl-L-tryptophan by this method, but exclusion of light during the reaction period was necessary. The crude product was recrystallized twice from aqueous ethanol, and then washed with ether to remove impurities, yield 0.206 g (60.9% of theory), m.p. 191.5–192.5°, $[\alpha]_D^{22}$ −71.8° (*c*, 0.2 in 95% ethanol) and −76° (*c*, 0.2 in acetone).

Method B

The preparation was similar to that of DNP glycyl-L-tryptophan by this method, but the reaction required the exclusion of light. Purification of the crude product was effected as in method A, yield 0.324 g (47.8% of theory), m.p. 191–192°; $[\alpha]_D^{22}$ −71.5° (*c*, 1 in 95% ethanol), −76.0° (*c*, 1.6 in acetone), −50.0° (*c*, 0.2 in 4% sodium bicarbonate), and −100° (*c*, 0.2 in glacial acetic acid).

Anal. Calcd. for $C_{13}H_{14}N_4O_7$: C, 46.14; H, 4.14; N, 16.55; equiv. wt. 338.14. Found: C, 45.9; H, 4.08; N, 16.36; equiv. wt. 340.

*DNP Glycyl-*L*-phenylalanine*

Method A

After the reaction, the solution was extracted twice with ether to remove trace impurities before acidification. The crude product was recrystallized twice from aqueous ethanol, yield 0.120 g (61.6% of theory), m.p. 184–185°, $[\alpha]_D^{22}$ +8.3° (*c*, 1.5 in 95% ethanol) and +18.2° (*c*, 1.5 in acetone).

Method B

The procedure was similar to the preparation of the two foregoing compounds, and the crude product was recrystallized twice from aqueous ethanol and washed with a small amount of ether, yield 0.482 g (71.2% of theory), m.p. 184–185°; $[\alpha]_D^{22}$ +8.2° (*c*, 1.5 in 95% ethanol), +18.2° (*c*, 1.5 in acetone), and −63.6° (*c*, 0.3 in sodium bicarbonate).

Anal. Calcd. for $C_{17}H_{16}N_4O_7$: C, 52.60; H, 4.12; N, 14.42; equiv. wt. 388.35. Found: C, 52.4; H, 4.32; N, 13.51; equiv. wt. 400.

*DNP Glycyl-*L*-glutamic Acid*

Method A

The reaction time was 20 h, and the reaction vessel

had to be covered for rigorous exclusion of light; the ethanol was then distilled (water bath below 40°) under a vacuum to concentrate the mixture. The residue was dissolved in a small amount of water, and the aqueous solution was extracted twice with ether to remove a neutral impurity which melted at 84–85°. The aqueous solution was acidified with concentrated HCl to pH 2, causing a yellow crystalline solid to precipitate. The mixture was kept for 2 h in the refrigerator and then filtered under suction; the solid was dried in a vacuum desiccator and heated at 100° to constant weight. The crude product was recrystallized twice from aqueous ethanol, yield 0.092 g (49.5% of theory), m.p. 194–195°, $[\alpha]_D^{24}$ −20.3° (*c*, 1.5 in 4% $NaHCO_3$).

Method B

The addition of acid chloride required 4 h, with ice-bath stirring for a further 4 h. Rigorous exclusion of light from the reaction vessel was necessary. After removal of the benzene layer, the aqueous layer was extracted twice with ether to remove a neutral impurity of m.p. 84–85°. The aqueous solution was acidified with concentrated hydrochloric acid to pH 2. The resulting yellow crystalline solid was separated by filtration under suction and washed with a small amount of ice-cold water to remove excess hydrochloric acid. The product was dried by storing it overnight in a vacuum desiccator and heating it at 100° to constant weight.

The crude product contained large amounts of DNP glycine and a small amount of other impurities; a pure product could not be obtained by recrystallization. A pure product was obtained, however, by preparative thin-layer chromatography. The adsorbent used was Silic AR (TLC-7FG), the layer was 2 mm thick, the sample was applied in a narrow band, and the chromatogram was developed with chloroform – methanol – acetic acid (95:5:1). Five bands were formed; the DNP glycine lies in the fifth band from the bottom of the plate, and the DNP glycyl-L-glutamic acid in the second band from the bottom. The adsorbent containing the DNP peptide was scraped from the plate and extracted twice with ethyl acetate; the solvent was evaporated and the solid recrystallized from aqueous ethanol, recovery 60–61% of the crude product, yield 61% of theory, m.p. 194–195°, $[\alpha]_D^{24}$ −20.3° (*c*, 0.5 in 4% $NaHCO_3$).

Anal. Calcd. for $C_{13}H_{14}N_4O_9$: C, 42.15; H, 3.80; N, 15.13; equiv. wt. 185.14. Found: C, 41.77; H, 4.44; N, 15.01; equiv. wt. 183.75.

Paper Electrophoresis

With the same apparatus, buffer solution, and field strength as used previously (1), and a current of 3–4.5 mA for 16 h, the distances travelled by DNP glycyl-L-tryptophan, DNP glycyl-L-phenylalanine, DNP glycyl-L-proline, DNP glycyl-L-glycine, and DNP glycine (studied for comparison) were 18.0, 20.8, 21.6, 33.5, and 25.0 cm, respectively. The compounds separated well from a mixture, but DNP glycyl-L-phenylalanine and DNP glycyl-L-proline formed an elongated spot because of their similar mobilities. The apparatus was protected from light to prevent photochemical decomposition.

Thin-Layer Chromatography

A Mallinckrodt Chroma-Kit was used, with Silic AR (TLC-GF) as adsorbent and chloroform – methanol – acetic acid (95:5:1) as developing solvent. A clear separation of the four DNP peptides and DNP glycine was achieved from a 0.1–0.4 mμl sample of mixture, and the calculated R_f values were, respectively, 0.10, 0.25, 0.35, 0.38, and 0.60. The separation of DNP glycyl-L-phenylalanine and DNP glycyl-L-proline was clear-cut and much better than that given by electrophoresis, despite their close R_f values.

Ultraviolet Absorption

The spectra were obtained by means of a Beckman DK instrument with cells of 1 cm path length; the sample concentration was 5×10^{-5} *M* in 1.7% sodium bicarbonate (pH 8.43) or in 95% ethanol. The DNP peptides in sodium bicarbonate solution showed maximum absorption in the 350–360 mμ range, as do DNP amino acids (3), and other peaks were found in the 260–270 and 210–240 mμ ranges; solutions in 95% ethanol showed maxima at 344 and 265 mμ. The maxima and corresponding molar absorbancies (ϵ_{max}) are summarized in Table I, including the data for DNP glycine for comparison. The DNP peptides gave relatively more consistent values (ca. 17 550 ± 300) at 344 than at 350–360 mμ.

Infrared Absorption (4)

Nujol mulls were examined in the region 5 000 – 625 cm^{-1} on a model 21 Perkin–Elmer spectrophotometer equipped with sodium chloride optics. The absorption peaks were checked with DNP glycine and DNP alanine; their maxima throughout the entire range were essentially the same as the literature values (5).

All compounds showed maxima in the 3 350, 1 625 – 1 635, 1 590 – 1 600, 1 495 – 1 505, 1 455 – 1 465, 1 155 – 1 160, 1 055 – 1 065, 830–840, and 740–750 cm^{-1} regions; the DNP peptides showed additional peaks in the 1 525 – 1 535, 1 305 – 1 315, 1 135 – 1 140, 1 110 – 1 120, 925–930, and 720–725 cm^{-1} ranges, and the DNP amide derivatives showed additional peaks in the 1 100, 1 710, 1 665, 1 614, 1 515 – 1 525, 980–990, 690–700, and 670 cm^{-1} regions. Other maxima exhibited by the DNP peptides and by the DNP amide derivatives are listed in Tables II and III, respectively.

The 3 350, 1 625, 1 730 – 1 705, 1 600, 1 500 – 1 450 (two peaks), 1 530 – 1 500, and 1 370 – 1 330 cm^{-1} bands are attributed, respectively, to NH stretching vibration, NH bending vibration, a monocarboxylic acid or a carboxyl group hydrogen bonded, a nitro group in a substituted benzene ("quadrant stretching"), a substituted benzene ring ("semicircle stretching"), nitro groups (antisymmetrical NO stretching), and nitro groups.

The 3 180 cm^{-1} band shown by DNP glycine amide and DNP L-alanine amide is regarded as arising from symmetrical NH stretching in unsubstituted amides. The 1 550 cm^{-1} band shown by the anilides and toluidides is interpreted as arising from the CNH vibration of substituted amides (nitrogen

and hydrogen moving in opposite directions relative to carbon, involving both NH bending and C—N stretching); an overtone of this band would account for the weaker band near 3 100 cm^{-1}.

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