Synthesis and Bioactivity of Photoaffinity Labels of the Plant Growth Regulator 1-(3-Chlorophthalimido)cyclohexanecarboxamide (AC 94377)

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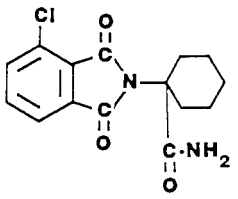
AC 94377 has been shown to mimic the growth-regulating activity of gibberellins. For the investigation of the receptor protein binding site associated with AC 94377 bioactivity, suitable photoaffinity labels are required. Therefore, five photoaffinity-labeled analogues of AC 94377 have been synthesized: 1-(3-chlorophthalimido)cyclohexanecarboxazide (4), 1-(3-azidophthalimido)cyclohexanecarboxamide (8), 1-(3-azido-6-fluorophthalimido)cyclohexanecarboxamide (14), 1-(4-azidophthalimido)cyclohexanecarboxamide (18), and 1-(3-azido-4-methylphthalimido)cyclohexanecarboxamide (24). In the sunflower assay system, plant growth regulating activity was observed with the 3-azido- and 3-azido-4-methyl-substituted phthalimides.

INTRODUCTION

AC 94377 and other substituted phthalimides synthesized by American Cyanamid Co. (1976, 1977) were shown to mimic the plant growth regulating (PGR) activity of gibberellins (Los et al., 1980a; Devlin, 1981). Preliminary



AC 94377

studies with 28 different crop plants treated with AC 94377 showed that there were moderate to very responsive PGR effects with 93% of the treated plants (Los et al., 1980b). Treatment of dormant weed seeds with AC 94377 demonstrated an observable promotion in germination over untreated controls (Metzger, 1983). When dormant weed seeds were buried at 3.8-cm depth, surface application of AC 94377 still caused an enhancement in seed germination (Donald and Hoerauf, 1985). Therefore, the water-insoluble PGR was apparently being leached from the soil surface down to the buried seeds. A series of bioassays conducted with AC 94377 showed no cytokinin or ethylene induction activity (Suttle and Schreiner, 1982); only gibberellin-like activity was observed. By use of the cucumber hypocotyl bioassay, a large number of substituted phthalimides and phthalimide analogues were examined for bioactivity (Yalpani et al., 1989). Evidence was obtained in these studies to suggest that AC 94377 and GA_4 were both competing for the same binding sites.

Selection of the five photoaffinity-labeled structures for synthesis was based upon known chemical and biological data. The acylazido analogue (4) was selected because acyl azides have been employed successfully as photoaffinity labels (Pinney and Katzenellenbogen, 1988). The 3-azido analogue (8) was chosen because its structure most closely resembled that of AC 94377. The 3-azido-6-fluoro analogue (14) was selected because addition of a fluoro group para to the arylazido moiety would afford a more reactive and less selective photoaffinity label (Schuster, 1988). The 4-azido (18) and the 3-azido-4-methyl analogues (24) were chosen because similar phthalimide structures demonstrated biological activity (Yalpani et al., 1989).

EXPERIMENTAL PROCEDURES

Caution must be taken in preparation and handling of the organic azides. All syntheses of azido compounds should be conducted behind an explosion shield even though no problems were encountered in the syntheses described. It is recommended that organic azides be transferred by using nonmetallic spatulas. Heat and strong light should be avoided. Workup and drying of azido products in our syntheses were at temperatures of less than 40 °C to avoid loss of product due to decomposition. In these syntheses no sudden decomposition of azido products was experienced; however, normal laboratory precautions must be undertaken to ensure safety.

Materials. 3-Nitrophthalic acid, 4-methyl-3-nitronaphthalene, and *N,N'*-carbonyldiimidazole were obtained from Aldrich Chemical Co. 3-Fluorophthalic acid, 4-nitrophthalic anhydride, and 1-aminocyclohexanecarboxylic acid were purchased from Lancaster Synthesis Ltd.

Equipment. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AM 400 spectrometer employing the Aspect 3000 processing system. Tetramethylsilane was used as the internal standard. Electron impact mass spectra were taken on a Varian CH-5DF spectrometer. Thin-layer chromatography (TLC) was conducted on 0.25 and 0.50 mm thick plates of Anasil GF (Analabs). Catalytic reductions were accomplished with a Parr Series 3910 low-pressure hydrogenation apparatus.

3-Nitrophthalic Anhydride (1). According to the method of Nicolet and Bender (1961), excess acetic anhydride (190 mL, 2 mol) was added to 211 g (1 mol) of 3-nitrophthalic acid. The mixture was heated at gentle reflux until the phthalic acid was completely dissolved. Reflux was continued for an additional 30 min. The reaction mixture was poured into an evaporating dish in a fume hood, and the excess acetic anhydride was allowed to evaporate under a gentle stream of dry air: yield 95%; MS, m/z (relative intensity) 193 (M^+, 1), 149 (100), 105 (8), 103 (30), 75 (99), 74 (87); ^1H NMR (acetone-d_6) δ 8.34 (t, H, J = 7.9 Hz, Ar H-5), 8.44 (dd, H, J = 7.6, 0.9 Hz, Ar H-6) 8.53 (dd, H, J = 8.0, 0.8 Hz, Ar H-4).

3-Chlorophthalic Anhydride (2). The chlorination reaction was set up by using a water aspirator to place the system under a slightly negative pressure (Shriner et al., 1959). By use of an aspirator, excess Cl_2 was trapped by tap water and disposed down the drain. According to the method of Newman and Scheurer (1956), chlorine gas was bubbled into molten 3-nitrophthalic anhydride (400 g, 2.1 mol) that was being mechanically stirred at approximately 240 °C. During reaction, brown NO_2 gas was evolved. Chlorine was added until NO_2 evolution ceased. Because

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the product, 3-chlorophthalic anhydride, sublimes at the temperature of the reaction, care should be taken to ensure that the product does not plug the exit tube of the reaction flask or cause the mechanical stirrer to bind (if stirring stops, the reaction will overheat). After chlorine addition was complete, the hot mixture was poured into a porcelain pan and allowed to solidify. The product was recrystallized from acetone and washed with diethyl ether: melting point 125–6 °C (lit. mp 124–6 °C); yield 65%; MS, m/z (relative intensity) 182 (M^+, 23), 138 (100), 110 (80), 84 (22), 75 (84), 74 (76), isotopic cluster indicated Cl; ^{1}H NMR (acetone-d_6) δ 8.04 (m, 3 H, Ar H-4,5,6).

1-(3-Chlorophthalimido)cyclohexanecarboxylic Acid (3). Formation of the substituted phthalimide was based on the method of Bose (1973). A 250-mL round-bottom flask was fitted with heating mantle, Dean-Stark moisture trap, and reflux condenser. Into the flask were added 8.2 g (45 mmol) of **2**, 7.2 g (50 mmol) of 1-aminocyclohexanecarboxylic acid, 100 mL of toluene, and 5.0 mL of triethylamine. The reaction was heated at reflux until the theoretical amount of water (0.81 mL) was collected. Time for reaction was approximately 8 h. After reaction, the toluene and triethylamine were removed in vacuo. The brown oil was dissolved in 100 mL of CH_2Cl_2 and filtered. The dichloromethane was removed in vacuo, and the thick oil that remained was treated with 100 mL of 6 N HCl. The product became a solid upon treatment with HCl. The product was filtered, pulverized, and washed with cold water: yield 93%; MS, m/z (relative intensity) 307 (M^+, 6), 289 (6), 262 (23), 194 (19), 182 (100), 164 (55), 126 (79), 110 (42), 108 (33), 81 (92), 75 (57), isotopic clusters indicated one Cl; ^{1}H NMR (acetone-d_6) δ 1.61, 1.98, 2.95 (m, 10 H, cyclohexyl), 7.81 (m, 3 H, Ar H-4,5,6).

1-(3-Chlorophthalimido)cyclohexanecarboxazide (4). Synthesis of **4** was accomplished by converting the carboxyl group to the acid chloride (Kent and McElvain, 1955) and the acid chloride to the acyl azide (Munch-Petersen, 1967). Into a 50-mL flask were placed 3.1 g (10 mmol) of **3** and 10 mL of thionyl chloride. The reaction was heated at reflux for 2 h, and the excess thionyl chloride was removed in vacuo. The acid chloride was stored in a vacuum desiccator to ensure removal of residual thionyl chloride or HCl. To prepare **4**, 1.0 g (15 mmol) of sodium azide was dissolved in 20 mL of water, and the acid chloride was dissolved in 15 mL of dry acetone. The acetone solution of the acid chloride was added with stirring to the solution of sodium azide over a 30-min period at a reaction temperature of 20–25 °C. After addition, the reaction mixture was stirred for an additional 30 min. Then 25 mL of water was added and stirring was continued for 30 min. The light brown precipitate was filtered, washed with cold water, and dried. About 2.8 g of crude product was obtained.

Initial purification was accomplished by dissolving **4** in benzene–dichloromethane (5:1 v/v) and treating with activated carbon. The product was filtered, and the filtrate was passed through a short column of silica gel (60–200 mesh). Final recrystallization was performed in hexane–dichloromethane to yield an ivory-colored product. TLC of **4** in benzene–hexane (9:1 v/v) gave an R_f of 0.58 and an estimated purity of approximately 98%: yield 78%; MS, m/z (relative intensity) 332 (M^+, 1), 288 (8), 275 (12), 262 (85), 261 (64), 194 (16), 182 (100), 164 (44), 138 (37), 110 (28), 81 (44), 69 (54), isotopic clusters indicated one Cl; ^{1}H NMR (acetone-d_6) δ 1.66, 2.00, 2.83 (m, 10 H, cyclohexyl), 7.84 (m, 3 H, Ar H-4,5,6).

1-(3-Nitrophthalimido)cyclohexanecarboxylic Acid (5). For preparation of **5**, the procedure for the synthesis of **3** was employed using 8.7 g (45 mmol) of **1**. Reaction time was about 16 h: yield 87%; MS, m/z (relative intensity) 318 (M^+, 6), 301 (6), 273 (32), 193 (83), 175 (23), 126 (64), 108 (20), 104 (26), 103 (27), 81 (100), 75 (85); ^{1}H NMR (acetone-d_6) δ 1.62, 1.99, 2.94 (m, 10 H, cyclohexyl), 8.12 (t, H, J = 7.5 Hz, Ar H-5), 8.16 (dd, H, J = 7.5, 1.4 Hz, Ar H-6), 8.24 (dd, H, J = 7.6, 1.3 Hz, Ar H-4).

1-(3-Aminophthalimido)cyclohexanecarboxylic Acid (6). Catalytic reduction was based on the procedure of Hartung et al. (1973). Into a 500-mL low-pressure reduction bottle were added 3.2 g (10 mmol) of **5**, 160 mL of benzene, and 40 mL of glacial acetic acid. After dissolution of **5**, 250 mg of 10% Pd/C was added, and reduction was performed at 40 psi of hydrogen. Reduction time for **5** to yield **6** was 3 h. The catalyst was removed by filtration, and the solvent was removed in vacuo. The product was dried in a vacuum desiccator over P_2O_5: yield 97%; MS, m/z (relative intensity) 288 (M^+, 11), 244 (34), 243 (22), 201 (6), 175 (25), 105 (74), 163 (74), 162 (79), 145 (64), 119 (35), 91 (100), 81 (44); ^{1}H NMR (acetone-d_6) δ 1.58, 1.98, 2.97 (m, 10 H, cyclohexyl), 6.99 (dd, H, J = 7.1, 0.6 Hz, Ar H-4), 7.04 (dd, H, J = 8.3, 0.6 Hz, Ar H-6), 7.46 (dd, H, J = 8.3, 7.1 Hz, Ar H-5).

1-(3-Azidophthalimido)cyclohexanecarboxylic Acid (7). Azide synthesis was based on the method of Smith and Boyer (1967). A 300-mL beaker was fitted with magnetic stirrer and thermometer. Then 2.9 g (10 mmol) of **6** and 50 mL of concentrated HCl were added to the beaker and stirred for 1 h at room temperature. The slurry was cooled to approximately 0 °C with an ice–salt bath, and a solution containing 1.38 g (20 mmol) of sodium nitrite in 10 mL of cold water was slowly added with vigorous stirring. The diazonium salt that formed was stirred at 0 to −5 °C for 1 h. With vigorous stirring, a solution of 1.3 g (20 mmol) of sodium azide in 10 mL of cold water was added to the cold diazonium salt over a 30-min period. Care must be taken to add the sodium azide very slowly because a large amount of foaming occurs. The reaction was stirred at 5 °C for 1 h. Then the mixture was poured into approximately 200 g of ice–water and stirred for 30 min. The product was filtered, washed with cold water, dried in vacuo over P_2O_5, and stored in the absence of light: yield 91%; MS, m/z (relative intensity) 314 (M^+, 5), 296 (2), 286 (11), 269 (13), 241 (23), 214 (7), 175 (24), 163 (65), 162 (49), 161 (54), 145 (90), 81 (91), 79 (100); ^{1}H NMR (acetone-d_6) δ 1.61, 2.00, 2.93 (m, 10 H, cyclohexyl), 7.58 (d, H, J = 8.0 Hz, Ar H-4), 7.61 (d, H, J = 7.6 Hz, Ar H-6), 7.85 (t, H, J = 7.8 Hz, Ar H-5).

1-(3-Azidophthalimido)cyclohexanecarboxamide (8). Formation of the acylimidazole adduct was based on the method of Look and White (1970). Into a 100-mL flask equipped with drying tube and magnetic stirrer were added 0.31 g (1 mmol) of **7**, 0.81 g (5 mmol) of *N,N'*-carbonyldiimidazole, and 50 mL of dry dichloromethane. The solution was stirred at room temperature in darkness for 1 h. In a 500-mL three-necked flask was prepared a cold (5 °C) saturated anhydrous ammonia solution in 150 mL of dry dichloromethane–diethyl ether (1:1 v/v). While a temperature of about 5 °C was maintained, the acylimidazole solution of **7** was added to the saturated ammonia solution. Additional ammonia was added to the reaction mixture to ensure saturation of solution. With protection from light and moisture, the reaction was allowed to stir at room temperature overnight. Purification of **8** was accomplished with 0.50 mm thick TLC plates using benzene–acetone (2:1 v/v) as developing solvent: R_f 0.58; yield 90%; MS, m/z (relative intensity) 313 (M^+, 1), 270 (18), 269 (100), 243 (10), 241 (26), 163 (29), 145 (62); ^{1}H NMR (acetone-d_6) δ 1.55, 1.95, 3.02 (m, 10 H, cyclohexyl), 6.41 (s, H, NH), 7.02 (s, H, NH), 7.56 (d, H, J = 8.5 Hz, Ar H-4), 7.59 (d, H, J = 7.3 Hz, Ar H-6), 7.82 (t, H, J = 7.8 Hz, Ar H-5).

3-Fluorophthalic Anhydride (9). The preparation of **9** was accomplished by treatment of 184 g (1 mol) of 3-fluorophthalic acid with acetic anhydride as described for the synthesis of 3-nitrophthalic anhydride (**1**): yield 95%; MS, m/z (relative intensity) 166 (M^+, 48), 122 (100), 94 (96), 74 (8); ^{1}H NMR (acetone-d_6) δ 7.83 (dd, H, J = 16.8, 7.4 Hz, Ar H-6), 7.84 (dd, H, J = 16.8, 7.4 Hz, Ar H-4), 8.15 (td, H, J = 7.4, 4.4 Hz, Ar H-5).

1-(3-Fluorophthalimido)cyclohexanecarboxylic Acid (10). Synthesis of **10** was performed according to the same procedure described for preparation of **3**. For this synthesis, 7.5 g (45 mmol) of **9** was heated at reflux for 16 h: yield 93%; MS, m/z (relative intensity) 291 (M^+, 8), 273 (11), 246 (46), 178 (17), 166 (100), 148 (50), 126 (56), 108 (27), 81 (58); ^{1}H NMR (acetone-d_6) δ 1.59, 2.90, 3.06 (m, 10 H, cyclohexyl), 7.59 (dd, H, J = 15.1, 7.4 Hz, Ar H-6), 7.60 (dd, H, J = 15.1, 7.4 Hz, Ar H-4), 7.93 (td, H, J = 7.7, 4.4 Hz, Ar H-5).

1-(3-Fluoro-6-nitrophthalimido)cyclohexanecarboxylic Acid (11). Nitration reaction was based on the method of Boyer and Buriks (1973). The nitrating reagent was prepared by slowly adding with stirring 5.0 g (49.5 mmol) of potassium nitrate to 20 mL of concentrated sulfuric acid at room temperature. After addition, the flask was stoppered and the reaction was stirred for 1 h at room temperature. In a second flask, 25 mL of concentrated sulfuric acid was added to 2.9 g (10 mmol) of **10**. The flask was stoppered and stirred at room temperature until **10** was completely solubilized. While the temperature was maintained at

25 °C with a water bath, the nitrating reagent was added to the solution of **10** over a 30-min period. Then the flask was loosely stoppered and the reaction was stirred at room temperature for 48 h. After reaction, the mixture was chilled in ice and then slowly added to about 250 g of crushed ice. The product was extracted (3 × 100 mL) with diethyl ether. The combined ether fractions were washed with cold water, and the ether extract was dried over anhydrous sodium sulfate. A crude orange product was isolated from the ether extract. This product was dissolved in a minimum amount of acetone and placed on a column (2 × 15 cm) of silica gel (60–200 mesh). The column was eluted with a solvent of benzene–acetone–acetic acid (100:50:1 v/v). The product eluted from the column just ahead of the orange byproduct. Further purification was by preparative TLC on 0.5-mm plates using benzene–acetone–acetic acid (150:25:1 v/v) as developing solvent: R_f 0.5; yield 28%; MS, m/z (relative intensity) 336 (M^+, 11), 319 (20), 302 (6), 291 (92), 211 (100), 194 (10), 126 (13), 81 (62); ^1H NMR (acetone-d_6) δ 1.59, 2.87, 3.03 (m, 10 H, cyclohexyl), 7.86 (t, H, J = 8.5 Hz, Ar H-4), 8.33 (dd, H, J = 12.8, 4.2 Hz, Ar H-5).

1-(3-Amino-6-fluorophthalimido)cyclohexanecarboxylic Acid (12). Catalytic reduction of 3.4 g (10 mmol) of **11** required 3 h under the same conditions used for preparation of **6**: yield 97%; MS, m/z (relative intensity) 306 (M^+, 25), 262 (37), 261 (27), 193 (21), 181 (100), 180 (99), 163 (53), 138 (16), 137 (15), 136 (16), 135 (12), 109 (77), 81 (77); ^1H NMR (acetone-d_6) δ 1.59, 1.97, 2.96 (m, 10 H, cyclohexyl), 6.02 (s, H, NH), 6.10 (s, H, NH), 7.08 (dd, H, J = 9.1, 3.6 Hz, Ar H-4), 7.24 (t, H, J = 9.0 Hz, Ar H-5).

1-(3-Azido-6-fluorophthalimido)cyclohexanecarboxylic Acid (13). By use of the method for preparation of **7**, 3.1 g (10 mmol) of **12** was diazotized and reacted with sodium azide: yield 89%; MS, m/z (relative intensity) 332 (M^+, 22), 304 (44), 287 (20), 259 (44), 193 (18), 181 (100), 180 (60), 179 (49), 163 (80), 137 (15), 136 (22), 135 (44), 126 (44), 108 (48), 81 (68), 79 (67); ^1H NMR (acetone-d_6) δ 1.60, 1.98, 2.92 (m, 10 H, cyclohexyl), 7.62 (m, 2 H, Ar H-4,5).

1-(3-Azido-6-fluorophthalimido)cyclohexanecarboxamide (14). The conversion of 0.33 g (1 mmol) of **13** into **14** was accomplished by using the method for synthesis of **8**. Purification was by preparative TLC using a developing solvent of benzene–acetone (2:1 v/v): R_f 0.60; yield 82%; MS, m/z (relative intensity) 331 (M^+, 2), 303 (4), 287 (100), 259 (52), 193 (15), 181 (48), 163 (76), 108 (22), 97 (56), 81 (61), 79 (92); ^1H NMR (acetone-d_6) δ 1.53, 1.71, 1.95, 3.02 (m, 10 H, cyclohexyl), 6.44 (s, H, NH), 7.04 (s, H, NH), 7.60 (m, 2 H, Ar H-4,5).

1-(4-Nitrophthalimido)cyclohexanecarboxylic Acid (15). Preparation of **15** was by the same procedure used for synthesis of **3**. Reflux time was 24 h: yield 72%; MS, m/z (relative intensity) 318 (M^+, 4), 300 (4), 273 (26), 205 (6), 193 (15), 175 (11), 126 (32), 108 (11), 103 (30), 81 (100), 75 (52); ^1H NMR (acetone-d_6) δ 1.58, 1.72, 2.99 (m, 10 H, cyclohexyl), 8.14 (dd, H, J = 8.2, 1.5 Hz, Ar H-6), 8.55 (dd, H, J = 1.8, 0.5 Hz, Ar H-3), 8.71 (dd, H, J = 8.1, 2.1 Hz, Ar H-5).

1-(4-Aminophthalimido)cyclohexanecarboxylic Acid (16). Catalytic reduction was by the same procedure used to prepare **6**. Reduction time was 5 h: yield 98%; MS, m/z (relative intensity) 288 (M^+, 6), 244 (82), 243 (33), 201 (6), 187 (6), 176 (46), 163 (94), 162 (100), 145 (83), 120 (56), 119 (60), 91 (71), 81 (63), 79 (27); ^1H NMR (acetone-d_6) δ 1.70, 1.96, 2.95 (m, 10 H, cyclohexyl), 6.95 (dd, H, J = 8.2, 2.1 Hz, Ar H-5), 7.01 (d, H, J = 2.0 Hz, Ar H-3), 7.48 (d, H, J = 8.2 Hz, Ar H-6).

1-(4-Azidophthalimido)cyclohexanecarboxylic Acid (17). Reaction conditions were the same as those used for preparation of **7**: yield 94%; MS, m/z (relative intensity) 314 (M^+, 3), 286 (8), 270 (17), 269 (37), 242 (20), 241 (18), 161 (54), 160 (37), 145 (14), 126 (27), 117 (37), 89 (55), 81 (100), 779 (35); ^1H NMR (acetone-d_6) δ 1.59, 1.99, 2.95 (m, 10 H, cyclohexyl), 7.47 (d, H, J = 1.5 Hz, Ar H-3), 7.50 (dd, H, J = 7.9, 2.0 Hz, Ar H-5), 7.84 (d, H, J = 7.9 Hz, Ar H-6).

1-(4-Azidophthalimido)cyclohexanecarboxamide (18). Amide formation was by the same method used for preparation of **8**. Purification was by prepatative TLC using benzene–acetone (2:1 v/v) as developing solvent: R_f 0.63; yield 83%; MS, m/z (relative intensity) 313 (M^+, 1), 269 (94), 241 (37), 161 (82), 145 (11), 125 (14), 117 (19), 89 (29), 81 (100), 79 (30); ^1H NMR (acetone-d_6) δ 1.59, 1.95, 3.02 (m, 10 H, cyclohexyl), 6.40 (s, H, NH), 7.00 (s, H, NH), 7.44 (d, H, J = 1.7 Hz, Ar H-3), 7.48 (dd, H, J = 8.0, 2.0 Hz, Ar H-5), 7.81 (d, H, J = 8.0 Hz, Ar H-6).

4-Methyl-3-nitrophthalic Acid (19). Cleavage of the unsubstituted ring of 1-nitro-2-methylnaphthalene was accomplished by the method of Spitzer and Lee (1974). Oxidation of ruthenium dioxide to ruthenium tetraoxide was carried out with aqueous sodium hypochlorite (household bleach, 5.25% concentration). Into a 3-L, three-necked flask fitted with mechanical stirrer and air condenser were added 300 mL of carbon tetrachloride, 0.07 g of $RuO_2 \cdot 2H_2O$, and 500 mL of bleach. The reaction was stirred until all the insoluble black ruthenium dioxide was converted into soluble yellow ruthenium tetraoxide. As the two-phase mixture was being stirred, 20 g (107 mmol) of 1-nitro-2-methylnaphthalene was added. The substituted naphthalene readily dissolved into the carbon tetrachloride layer. The reaction was allowed to stir until the black color of the ruthenium dioxide began to persist (about 1 h). Then approximately 500 mL of bleach was added and stirring was continued. Bleach was added at approximately 1-h intervals until a total of 2.4 L of bleach had been added. The reaction was then allowed to stir for about 20 h.

The aqueous phase was separated from the organic layer, concentrated, and extracted (5 × 150 mL) with diethyl ether. The ether was removed from the combined extracts, and the sample was thoroughly dried. The dried sample was redissolved in ether and filtered, which left behind a large amount of extracted salts. The product in ether solution was again taken to complete dryness and redissolved in acetone. The acetone solution was concentrated and water was added to retain any remaining undesired salts. The product (6.6 g) was allowed to precipitate from the acetone–water solution: yield 28.7%; MS, m/z (relative intensity) 225 (M^+, 3), 207 (17), 181 (8), 164 (29), 163 (29), 135 (21), 133 (18), 118 (66), 105 (71), 89 (76), 77 (100), 63 (97), 51 (80); ^1H NMR (acetone-d_6) δ 2.44 (s, 3 H, CH_3), 7.67 (d, H, J = 7.6 Hz, Ar H-5), 8.03 (d, H, J = 7.6 Hz, Ar H-6).

4-Methyl-3-nitrophthalic Anhydride (20). Synthesis of **20** was by the same method as for **1**: yield 93%; MS, m/z (relative intensity) 207 (M^+, 12), 190 (2), 163 (98), 117 (54), 89 (100), 63 (85), 62 (41); ^1H NMR (acetone-d_6) δ 2.55 (s, 3 H, CH_3), 8.17 (d, H, J = 7.9 Hz, Ar H-5), 8.22 (d, H, J = 7.9 Hz, Ar H-6).

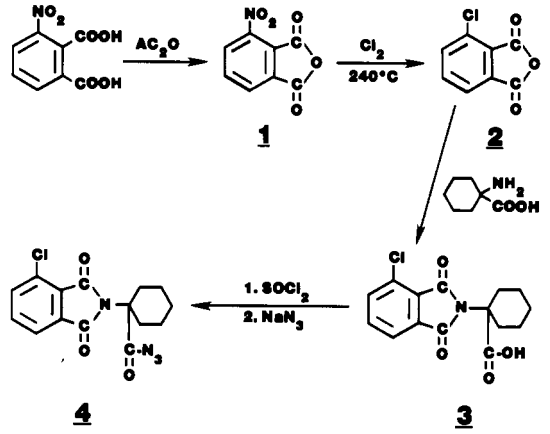
1-(4-Methyl-3-nitrophthalimido)cyclohexanecarboxylic Acid (21). Preparation of **21** was by the same procedure used for the synthesis of **3**. Reaction was heated at reflux for 24 h: yield 89%; MS, m/z (relative intensity) 332 (M^+, 13), 315 (13), 108 (76), 207 (100), 189 (23), 126 (84), 108 (29), 89 (39), 81 (88), 79 (20); ^1H NMR (acetone-d_6) δ 1.61, 2.00, 2.92 (m, 10 H, cyclohexyl), 7.97 (s, 2 H, Ar H-5,6).

1-(3-Amino-4-methylphthalimido)cyclohexanecarboxylic Acid (22). Catalytic reduction was by the same method described for preparation of **6**. Time required for reduction was 24 h: yield 95%; MS, m/z (relative intensity) 302 (M^+, 79), 287 (48), 258 (78), 257 (78), 207 (79), 189 (84), 177 (99), 176 (100), 159 (87), 126 (52), 105 (82), 81 (93), 79 (63); ^1H NMR (acetone-d_6) δ 1.62, 1.98, 2.98 (m, 10 H, cyclohexyl), 2.26 (s, 3 H, CH_3), 5.97 (s, 2 H, NH_2), 6.94 (d, H, J = 7.1 Hz, Ar H-5), 7.36 (d, H, J = 7.1 Hz, Ar H-6).

1-(3-Azido-4-methylphthalimido)cyclohexanecarboxylic Acid (23). Conversion of the amino moiety to the azido group was by the same procedure used for preparation of **8**: yield 94%; MS, m/z (relative intensity) 328 (M^+, 26), 300 (65), 287 (67), 255 (85), 207 (82), 189 (44), 177 (65), 176 (82), 175 (88), 174 (65), 159 (68), 132 (28), 131 (35), 126 (83), 104 (51), 81 (100), 79 (70); ^1H NMR (acetone-d_6) δ 1.62, 2.01, 2.95 (m, 10 H, cyclohexyl), 7.54 (d, H, J = 7.6 Hz, Ar H-5), 7.67 (d, H, J = 7.6 Hz, Ar H-6).

1-(3-Azido-4-methylphthalimido)cyclohexanecarboxamide (24). Synthesis of **24** employed the carbonyldiimidazole method that was used for preparation of **8**. Purification was by preparative TLC using benzene–acetone (2:1 v/v) as developing solvent: R_f 0.67; yield 58%; MS, m/z (relative intensity) 327 (M^+, 3), 299 (1), 283 (100), 255 (54), 177 (15), 175 (18), 131 (7), 104 (10), 81 (12), 79 (15); ^1H NMR (acetone-d_6) δ 1.54, 1.98, 3.04 (m, 10 H, cyclohexyl), 6.39, (s, H, NH), 6.99 (s, H, NH), 7.51 (d, H, J = 7.5 Hz, Ar H-5), 7.65 (d, H, J = 7.9 Hz, Ar H-6).

Scheme I

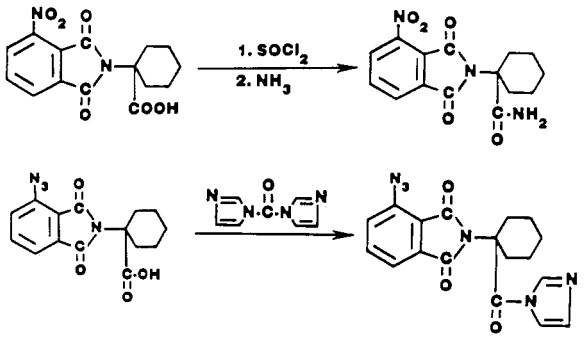


Sunflower Assay for Bioactivity. Seeds of sunflower (*Helianthus annuus* L. cv. NK265) were surface-sterilized by soaking for 10 min in a 1:4 (v/v) dilution of commercial bleach for the bioassay (Suttle and Hultstrand, 1987). The seeds were imbibed for 6 h in running deionized water. Then the imbibed seeds were placed between moist paper towels that were rolled into cylinders and placed vertically in beakers of one-third-strength Hoagland's nutrient solution. The beakers were placed in a growth chamber (14-h photoperiod) with 28 °C day and 25 °C night temperatures for 5 days. The seedlings were transferred into 250-mL aluminum foil wrapped glass jars (three seedlings/jar) containing the test compound (100 μM) in one-third-strength nutrient solution. The treated seedlings were returned to the growth chamber, and the length of the first internode was measured to the nearest millimeter after 7 days of treatment. All experiments were run in duplicate for each test compound.

RESULTS AND DISCUSSION

In Scheme I are given the reactions used for the synthesis of the acylazido photoaffinity label 4. In this scheme the last functional group to be attached was the labile azido moiety; therefore, 4 could be prepared by treatment of the carboxyl group with thionyl chloride followed by sodium azide.

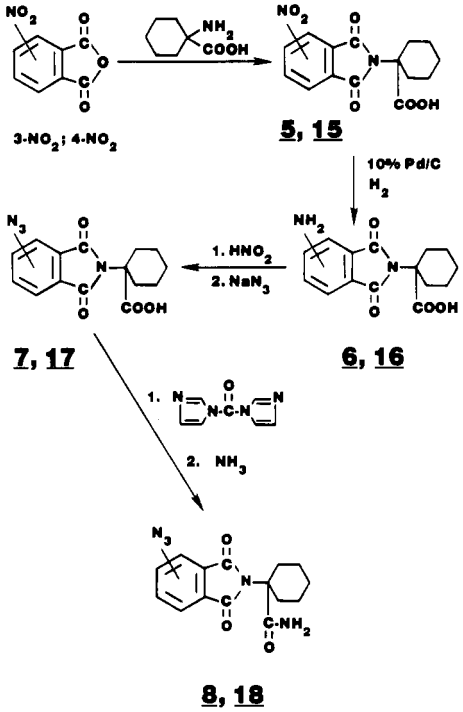
The 3-azido (8) and 4-azido (18) photoaffinity labels were prepared according to Scheme II. Initially, other approaches were attempted for the synthesis of 8, but satisfactory results could not be obtained. If the 3-azido acid (7) was treated with thionyl chloride followed by ammonium hydroxide, only an 18% yield of 8 was achieved. If anhydrous ammonia was employed for the ammonolysis of the acid chloride, the yield was increased to 41%. When the 3-azido acid was heated in thionyl chloride at approximately 60 °C, decomposition with considerable evolution of N_2 gas was observed. If the reaction temperature was reduced to 30 °C, only about 25% of the acid was chlorinated after heating for 16 h. Finally, if the amide of 5 was prepared (eq 1) and used in the reaction scheme, none of the subsequent reactions would take place with acceptable yields. Therefore, for the synthesis of 8, it was



(1)

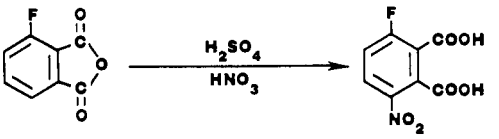
(2)

Scheme II



necessary to prepare the amide in the final step of the reaction scheme. Because of the problems just described, activation of the carboxyl group was accomplished with *N,N'*-carbonyldiimidazole to form an acylimidazole intermediate (eq 2). Then ammonolysis of the imidazole moiety was achieved to afford a satisfactory yield of the amide product.

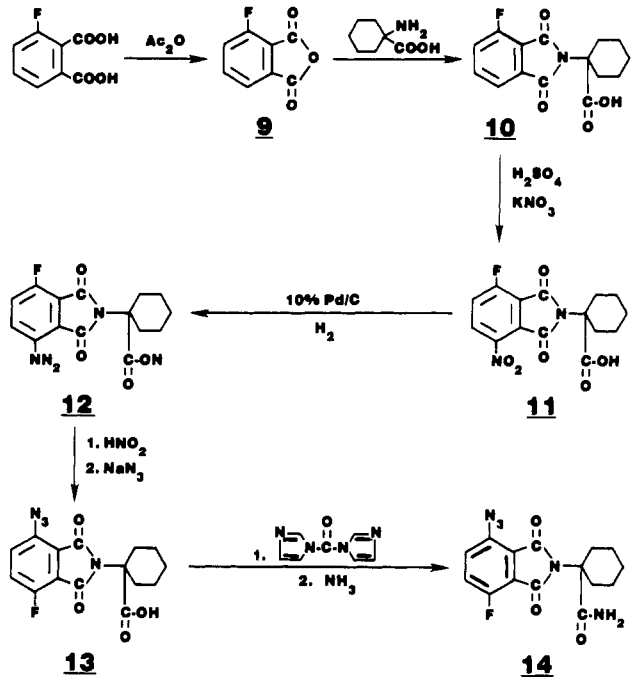
In the initial development of the reaction sequence for synthesis of the 3-azido-6-fluoro photoaffinity label (14), some problems were encountered. In the first approach, 3-fluoro-6-nitrophthalic acid was prepared according to eq 3 by using a mixture of concentrated sulfuric and nitric



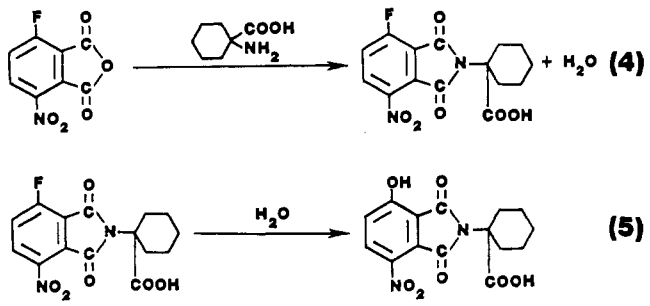
(3)

acids (Littmann, 1925). The nitration reactions went smoothly, and the yields of 3-fluoro-6-nitrophthalic acid were good. After TLC purification, NMR and mass spectral analysis verified synthesis of the product. However, the NMR spectra showed a large broad peak in the aromatic region, and the chemical shift of this peak would change according to the amount of impurity present. As the concentration of impurity was reduced, its chemical shift would move downfield. Unfortunately, it was not possible to completely remove this impurity from the product. Therefore, attempts were made to prepare 3-fluoro-6-nitrophthalic anhydride from the impure 3-fluoro-6-nitrophthalic acid by heating with excess acetic anhydride. Yields for these reactions were very low. Even if a very large excess of acetic anhydride was employed and the heating period was extended to many hours, product yield remained poor. From the acetic anhydride reactions, the chemical properties demonstrated by the impurity indicated that it was acidic material that was very strongly hydrogen bonded to the product. Because of the problems associated with this impurity, several alternative nitration procedures were attempted in the hope of obtaining 3-fluoro-6-nitrophthalic acid free from the polar impurity.

Scheme III



Nitration reactions were performed with a mixture of concentrated sulfuric and fuming nitric acids (Culhane and Woodward, 1961), with fuming nitric acid repurified by the urea method (Fieser and Fieser, 1967), and with potassium nitrate in concentrated sulfuric acid (Boyer and Buriks, 1973). All alternative methods failed to eliminate the polar impurity. As a final problem, if 3-fluoro-6-nitrophthalic anhydride was heated with 1-aminocyclohexanecarboxylic acid, a very low yield of substituted phthalimide was formed (eq 4), and a large quantity of 3-hydroxy-6-nitro byproduct was obtained owing to facile hydrolysis of the activated fluoro group (eq 5). Therefore,



this approach was abandoned and the reaction sequence given in Scheme III was employed even though the nitration reaction used in this scheme gave only fair yields. By use of Scheme III, the problem of hydrolysis of an activated fluoro group was eliminated.

The sequence of reactions given in Scheme IV was employed for the synthesis of the 3-azido-4-methyl photoaffinity label 24. Oxidative cleavage of the unsubstituted ring of 2-methyl-1-nitronaphthalene was accomplished with ruthenium tetraoxide to afford 4-methyl-3-nitrophthalic acid. The electron-withdrawing nitro group provided the deactivating effect required for selective oxidation of the unsubstituted ring of naphthalene to yield 19. Sodium hypochlorite was used for oxidation of the hydrated ruthenium dioxide to generate ruthenium tetraoxide in the reaction vessel. Inexpensive household bleach proved to be just as effective as commercial sodium hypochlorite reagent for this oxidation reaction.

Scheme IV

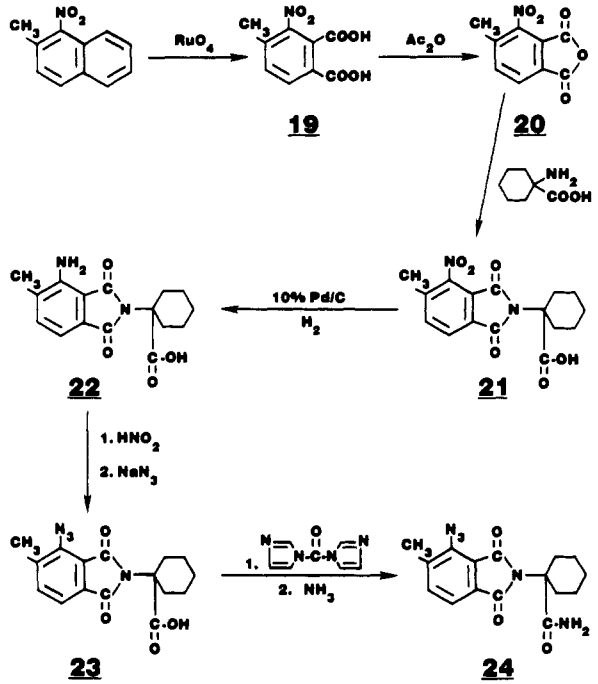


Table I. Sunflower Assay for Bioactivity of Synthetic Photoaffinity Labels

test compd	length of first internode, cm	ratio of treated to control plants
control	7.1 ± 0.2[a]	1.0
GA_3	11.1 ± 0.5	1.6
AC 94377	8.5 ± 0.3	1.2
4 (acylazido)	7.3 ± 0.1	1.0
8 (3-azido)	9.0 ± 0.2	1.3
14 (3-azido-6-fluoro)	6.8 ± 0.4	1.0
18 (4-azido)	7.5 ± 0.4	1.1
24 (3-azido-4-methyl)	9.0 ± 0.4	1.3

[a] Standard error of mean; six seedlings were used for each compound tested.

The results of the sunflower assay are given in Table I. The control showed a growth of about 7 cm for the length of the first internode. Gibberellic acid (GA_3), which is a known plant growth regulator, induced a growth of about 11 cm and AC 94377, the experimental PGR, induced a growth of greater than 8 cm.

Acyl azide (4), 3-azido-6-fluoro (14), and 4-azido (18) analogues demonstrated essentially no PGR activity. On the other hand, the 3-azido (8) and 3-azido-4-methyl (24) analogues demonstrated PGR activity greater than that shown by AC 94377 but less than that shown by GA_3. Therefore, 8 and 24 show much promise as photoaffinity labels because these compounds should compete for the active binding site that elicits the PGR activity demonstrated by AC 94377. Other assay systems are currently being tested, and 4, 14, and 18 may demonstrate PGR activity with these systems.

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